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Characterization Of A Cytolytic CD4+ T-Cell Subset In Patients With COPD

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Ich möchte allen Menschen danken, die mich bei der Verfassung dieser Arbeit durch ihren fachlichen Rat oder ihre persönlichen Worte unterstützt haben.

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1.1. ZUSAMMENFASSUNG

COPD ist eine weltweite Erkrankung, die mit einer hohen Mortalität einhergeht. Entzündliche Prozesse stehen bei der Pathogenese im Vordergrund und befallen neben der Lunge auch andere Organsysteme. Der wichtigste Risikofaktor für die Entstehung einer COPD ist Rauchen. Daneben scheinen auch Infektionen, genetisches Risiko, sowie Unweltfaktoren eine Rolle zu spielen. Die Immunantwort umfasst sowohl Elemente der angeborenen als auch der spezifischen Immunität.

CD4+CD28null Zellen stellen eine Subpopulation der CD4+ Zellen dar, die das für die Co-Stimulation wichtige CD28 an ihrer Oberfläche verloren haben und die eine eingeschränkte T-Zell Rezeptor Variabilität besitzen. Diese Zellpopulation wurde vermehrt in verschiedenen Autoimmunerkrankungen, wie z.B. der Chronischen Polyarthritis, nachgewiesen. CD4+CD28null Zellen zeigen Eigenschaften von natürlichen Killerzellen und sind in der Lage Zielzellen zu töten, als auch vermehrt Zytokine auszuschütten. Hitze Schock Proteine befinden sich unter normalen Bedingungen im Zellinneren und sorgen für die korrekte Faltung von Proteinen. Nach massivem Stress, Trauma oder Hitze können sie in die Blutstrombahn gelangen und dort Einfluss auf das Immunsystem ausüben.

Ziel dieser Studie war es, in einer gut definierten Patientengruppe mit COPD die CD4+CD28null Zellpopulation im peripheren Blut mittels Durchflusszytometrie nachzuweisen und die immunologischen Eigenschaften *in vitro* zu testen. Weiters wurden mittels ELISA Technik Hitze Schock Proteine im Serum gemessen und in Hinblick auf ihre diagnostischen Eigenschaften mittels ROC Kurven Analyse untersucht.

Es wurden 64 Patienten und Kontrollen in 4 Gruppen (Nichtraucher, Raucher, COPD Grad I-II, COPD Grad III-IV) untersucht. Dabei konnte ein vermehrtes Vorliegen von CD4+CD28null Zellen in den COPD Gruppen gezeigt werden. Diese Zellen hatten vermehrt intrazelluläres Perforin und Granzym B sowie CD94 und CD158 an ihrer Zelloberfläche. Weiters zeigten vor allem Zellen in frühen Krankheitsstadien vermehrte Zytokinproduktion. Anhand der ROC Analyse konnte gezeigt werden, dass sich der Nachweis von CD4+CD28null Zellen als

diagnostisches Instrument eignet. Die Werte von HSP27, HSP70 und HSP90alpha waren in der COPD Gruppe erhöht. HSP27 und HSP70 zeigten in der ROC Analyse hervorragende Eigenschaften als diagnostische Marker für COPD.

Sowohl zirkulierende CD4+CD28null Zellen als auch extrazelluläre HSPs deuten auf eine massive Aktivierung des Immunsystems im Rahmen einer COPD hin, die nicht auf die Lunge beschränkt ist.

1.2. ABSTRACT

COPD is a worldwide disease that causes high mortality rates. The pathogenesis is characterized by an ongoing inflammatory response that affects the lung and other organ systems. The main risk factor for the development of COPD is tobacco smoking. Infections, genetic susceptibility and environmental factors seem to be important. The immune response combines cells of the innate and the adaptive immune system.

CD4+CD28null cells are a subpopulation of CD4+ cells that have lost the co-stimulatory CD28 on the cell surface. Their T-cell receptor repertoire is usually limited. CD4+CD28null cells have been described in a number of autoimmune disorders, e.g. rheumatoid arthritis. CD4+CD28null cells exhibit features of NK-cells, are able to lyse target cells, and produce increased amounts of cytokines. Under normal conditions, heat shock proteins are intracellular proteins with chaperoning tasks. Upon massive stress, trauma or hyperthermia, HSPs enter the extracellular space and modulate the immune response.

This study was intended to evaluate CD4+CD28null cells in the blood flow of patients with COPD using flow cytometry and to test their immunological functions in *in vitro* assays. Furthermore, serum HSPs were quantified using ELISA technique and their potential to serve as diagnostic marker was analyzed using ROC curve analysis.

A total of 64 patients and controls were included in 4 groups (healthy non-smokers, healthy smokers, COPD I-II, COPD III-IV). The number of CD4+CD28null cells was increased in patients with COPD. They showed high expression of intracellular perforin and granzyme B, and surface expression of CD94 and CD158. Cytokine production was augmented at early stages of the disease. ROC curve analysis showed high sensitivity and specificity of CD4+CD28null cells as diagnostic tool. Serum levels of HSP27, HSP70 and HSP90alpha were increased in patients with COPD. HSP27 and HSP70 showed excellent potential to serve as diagnostic markers for COPD.

Circulating CD4+CD28null cells and extracellular HSPs in patients with COPD indicate an ongoing systemic immune activation exceeding the known local effects in the lung tissue.

2. BACKGROUND

2.1. EPIDEMIOLOGY AND ECONOMIC BURDEN

2.1.1. Prevalence and Mortality

By the beginning of the 21st century, mortality and loss of quality of life caused by Chronic Obstructive Pulmonary Disease (COPD) had become a globally and non-deniable health issue. As of 2001, 4.9% of the deaths in low-and-middle-income countries – equivalent to a number of 2.38 million – and 3.8% in high-income countries – 0.30 million deaths – were ascribed to direct effects of COPD. The same study ranked COPD under the ten leading causes of premature death worldwide based on years of life lost (YLL), and the ten leading causes of burden of disease in low-and-middle, and high-income countries based on disability-adjusted life years (DALY). [1] COPD is projected to be the third leading cause of death worldwide by 2020. [2] While death rates caused by heart disease, cancer, and stroke decreased in the United States of America (USA) from 1970 to 2002, a 102.8% increase was reported for mortality of COPD. [3] Despite these reported numbers, mortality rates might still be underappreciated in epidemiological studies as only a fraction of deaths in patients with COPD can be attributed to respiratory causes. In the majority of cases, death is due to cardiovascular, malign or other diseases with the chronic airway disease as an important comorbid disorder leading to the lethal illness. [4] Several reviews on the global prevalence have been published. In spite of the inhomogeneous use of diagnostic tools, the vast majority of authors estimated the prevalence of COPD in the adult population between 5 and 15% – with immense variation depending on the applied criteria. [5] [6] [7] An Austrian study published in 2007 reported airflow obstruction measured by standard spirometry – as leading diagnostic criterion for COPD – in 26.1% of the population aged 40 years and older. The authors found higher numbers with increasing age of the study participants with a peak for female and male patients in the subgroup of 70 years and above. Furthermore, the number of pack-years showed a distinct correlation with prevalence of COPD corroborating the role of smoking as a major risk factor. [8]

2.1.2. Financial Aspects

The financial burden attributable to COPD can be divided into direct (e.g. medical treatment, hospital admissions, medications) and indirect costs (e.g. reduced ability to work, social factors, morbidity, mortality). Regarding the high number of patients and the extent of medical care that is necessary to treat a patient with COPD, expenditures related to the diagnosis and treatment of COPD make up a considerable percentage of health care budgets in industrialized countries. Direct medical costs including laboratory tests, medications, oxygen therapy, procedures and health-care contacts were estimated to be as high as 10812 US dollars per year for a patient at stage III. Hospitalization costs were responsible for the largest fraction of this amount and accounted for 63% of the total expenses. Direct costs for one patient at the earlier stage I were considerably lower and were calculated to be 1681 US dollars per year. Interestingly, in this group hospitalization costs were also responsible for the major part of the total amount (40%). [9] With rising prevalence rates and the constant ageing of the world's population, health care costs arising from the treatment of COPD are expected to escalate in the future. [4] Nevertheless, detailed reports on the epidemiology of COPD and its economic impact remain scarce. Data from the USA based on evaluations from 1993 estimate the annual total costs (direct and indirect) at 23.9 billion US dollars – comparable to the costs for respiratory cancer (25.1 billion) and about twice as much as the financial expenditures for asthma (12.6 billion). [10] Taking into consideration the above mentioned development over the past decades, these figures may now be significantly higher.

2.2. RISK FACTORS

The development of the clinical symptoms of COPD and the underlying disease is a complex multifactorial process based on exogenous environmental factors and the individual predispositions of each patient. Numerous studies have aimed to identify singular risk factors and their influence on disease mechanisms linked to COPD. Table 1 summarizes possible risk factors and their corresponding level of evidence.

	Environmental Factors	**Host Factors**
Supposed	Adenovirus infection	Genetic predisposition
	Dietary deficiency of vitamin C	Blood group A
	Indoor air pollution	
Good Evidence	Outdoor air pollution	Low birth weight
	Low socioeconomic status	Childhood respiratory infection
	Alcohol intake	Atopy (high IgE)
	ETS in childhood	Bronchial hyperresponsiveness
	Other occupational exposures	Family case-history
Certain	Tobacco smoke	α1-Antitrypsin deficiency
	Some occupational exposures	

Table 1 Possible risk factors associated with increased risk to develop COPD. (adapted from *Viegi et al.* [11])

2.2.1. Tobacco Smoking

Pursuant to the available literature, tobacco smoking is considered to be the major risk factor leading to the development of COPD. [11 12 13] According to the results of *Fletcher et al.* a history of tobacco smoking is associated with virtually all patients presenting with airflow obstruction. Heavy smokers (≥15 cigarettes/day) more frequently suffered from impaired lung function than light smokers (<15 cigarettes/day). [14] *Doll et al.* showed in a prospective study over a 40-year follow-up period that former smokers and current cigarette smokers had a 5-fold and 12-fold increased annual mortality rate of COPD compared to never smokers. The increase in mortality was also dose dependent in current smokers, e.g. heavy

smokers (≥25 cigarettes/day) had a 2-fold increased rate compared to light smokers (<15 cigarettes/day). [15] In addition to its role in the progression of lung cancer and ischemic heart disease tobacco smoking was defined as a risk factor in most deaths caused by chronic bronchitis and emphysema in both men and women. [16] [17] Continuous consumption of cigarette smoke leads to chronic, progressive airflow obstruction in patients who are susceptible to the effects of tobacco smoke. Smokers who are not affected by tobacco smoking show a normal age-related loss of airflow comparable to non-smokers. [14] However, the decline of lung function parameters tends to be slower in ex-smokers who successfully refrain from smoking than in patients who continue to smoke. Capacities that have already been lost are not restored in sustained quitters, but the further progression of lung function impairment is considerably decelerated. [18] Benefits of smoking cessation on overall survival in a large study cohort were described by *Doll et al*. Smokers who successfully quit smoking before the age of 35 years had a life expectancy not different to never-smokers. However, smokers who decided to stop after the age of 35 had a pattern of survival that was better than the one of continuing smokers but did not reach the one of non-smokers. [15]

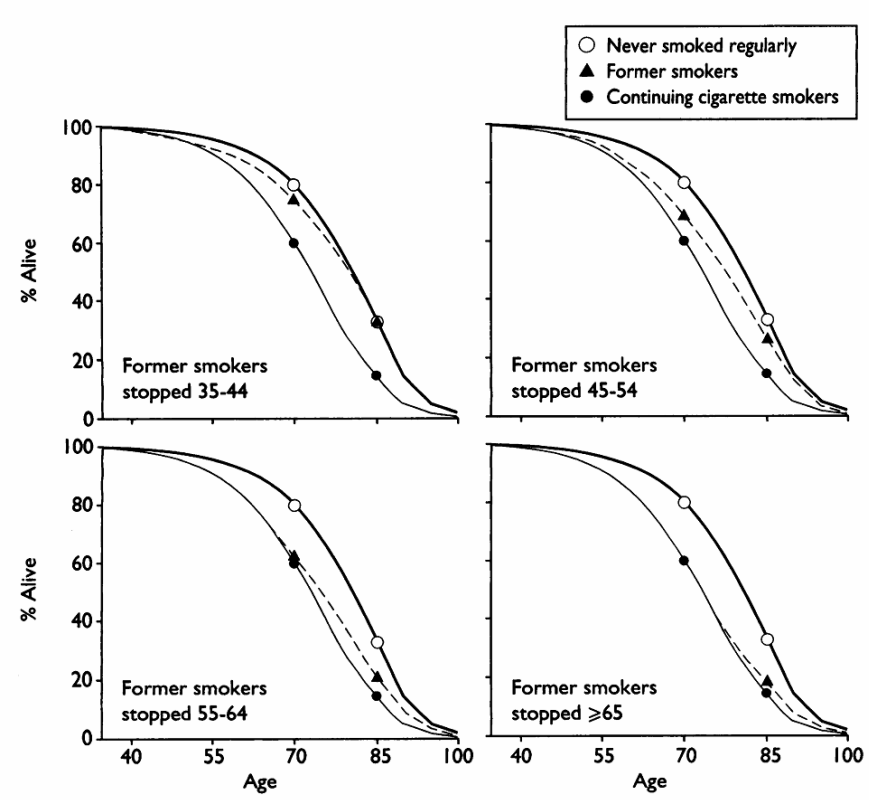

Figure 1 Survival of non-smokers, current smokers and ex-smokers. (adapted from *Doll et al.* [15])

Nevertheless, smoking alone is not responsible for airway obstruction and not every smoker ultimately suffers from COPD. A recent report showed that only up to 50% of elderly smokers developed COPD. [19] Moreover, a certain subgroup of cigarette smokers does not seem to be affected by the effects of tobacco smoking and shows no increased loss of lung function capacity over time compared to never-smokers. [14] Based on these results, several authors proposed a complex interaction between exogenous factors (e.g. tobacco smoking) and genetic susceptibility aspects that lead to the development of COPD in just a certain group of smokers. [20] [21]

2.2.2. Environmental Risk Factors

Especially in developing countries environmental factors other than cigarette smoke account for many cases of COPD. Indoor pollution due to cooking and heating with biomass fuel, e.g. wood fuel, was associated with development of obstructive airway disease in women of low socioeconomic status in Columbia. [22] A study from Mexico showed that the risk of chronic bronchitis and chronic airway obstruction increased with the amount of time of cooking with a traditional wood stove. [23] Poor indoor air quality at home – e.g. through smoking habits of other household members or elevated levels of particulate matter with a diameter of 2.5µm or less – was associated with increased prevalence of COPD and worse health conditions of patients with severe COPD, respectively. [22] [24] [25] Occupational exposure to airborne particles is an additional accepted risk factor. *Bakke et al.* calculated an odds ratio of 3.6 for the prevalence of obstructive lung disease in workers who had a job with a high degree of airborne exposure compared to workers without airborne exposure. [13] Socioeconomic aspects seem to be independently responsible for increased occurrence of COPD. Patients with low household income and lower educational levels showed reduced lung function parameters compared to patients of higher socioeconomic status. The results were adjusted for smoking habits. The risk for hospital admissions was also 3-fold increased in patients of the lowest socioeconomic level compared to the highest. [26] Similar risk assessments were found in a population based study from a developing country. [27] Based on these findings, improvement of indoor air quality and avoidance of passive smoking can be assumed to be essential measures in the management of chronic airway disease.

2.2.3. Infections

Smokers are at higher risk for acute infections of the respiratory system that can cause acute exacerbations and progression of COPD. Additionally to these effects, acute infections are suggested to play a role in the pathogenesis of chronic airway diseases. [28] Epidemiological studies provided proof for this hypothesis by showing that a history of acute lower respiratory infections during childhood was associated with an increased risk for chronic airway disease in adulthood. [29] [30] [31] Different pathogens have been associated with the occurrence of COPD.

Specifically viral infections have been widely studied. Adenovirus infections are a common cause of bronchiolitis in children and can lead to a latent infection that persists in the lung and other tissues. Adenovirus infections usually target lung epithelial cells. Pulmonary epithelial cells expressing adenoviral genetic material showed an elevated production of intercellular adhesion molecule (ICAM)-1 and interleukin (IL)-8 by lipopolysaccharide (LPS) stimulation. The production of inflammatory markers indicates that a latent adenoviral infection contributes to the inflammatory airway remodeling described in the lung tissue of patients with COPD. [32] [33] Other viral pathogens involved in the pathogenesis and exacerbations of COPD include Epstein-Barr virus (EBV), Picornaviruses (e.g. Rhinovirus), Influenza, and Respiratory syncytial virus (RSV). [34] [35] [36] A history of acute infections alone does not lead to the degree of impaired lung function as seen in COPD, but the combination with other risk factors (e.g. smoking, air pollution) may lead to an accelerated decline in lung function parameters. Effects on the bronchial system due to infections during infancy occur at early ages. Lower mean levels of performance for relevant parameters acquired through spirometry analysis have been shown in young adolescent males between the age of 11 and 22 years. [37] According to these findings, children with a significant number of infections during the first years of their life suffer from a restricted lung function capacity and are therefore more susceptible to the negative effects of other risk factors leading to chronic airway disease.

Bacterial infections and their role in the pathogenesis of COPD have been a reason for controversial reports. Several possible pathomechanisms of bacterial infections have been identified. [38]

- Lower respiratory tract infections during childhood (e.g. bronchitis, pneumonia) impair lung growth resulting in lower lung function in adulthood. [39] [40]
- If no pathology of the lung tissue is present, the tracheobronchial tree is sterile. Chronic colonization of the lower respiratory tract by bacterial pathogens induces a chronic inflammatory response with lung damage and progression of COPD. [41]
- Chronic infection of respiratory tissues by bacterial pathogens contributes to the pathogenesis of COPD by altering the host response. Infection with bacterial pathogens leads to a reduction of the mucociliary clearance and an increased mucus secretion thus facilitating bacterial infections.
- Bacteria can cause acute exacerbations of an underlying chronic bronchitis, significantly raising the morbidity and mortality of COPD.
- Bacterial antigens in the lower airway induce hypersensitivity that enhances airway hyperreactivity.

Interestingly, at stable stages of the disease, a higher bacterial load in the airways was associated with increased inflammatory markers in sputum analysis indicating a "dose-dependent" effect of bacterial colonization on the progression of chronic airway disease. [42] Bacterial pathogenic agents associated with inflammatory effects in the airways leading to COPD are Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis, Pseudomonas aeruginosa, and Chlamydia pneumoniae. [38] [43] In addition, bacterial infections of the airways are also a common cause of acute exacerbations of chronic bronchitis. [43] [44] Bacteria were found in 69.6% of patients with moderate to severe COPD at times of exacerbation, compared to 48.2% at a stable stage. [45] Exacerbations caused by bacteria are usually associated with increased volumes of purulent sputum. Based on the underlying chronic inflammatory state of the pulmonary tissue, acute bacterial infections lead to an excessive immune response resulting in an acute worsening of symptoms. The combined infection with viral and bacterial pathogens results in an even more severe exacerbation. [45]

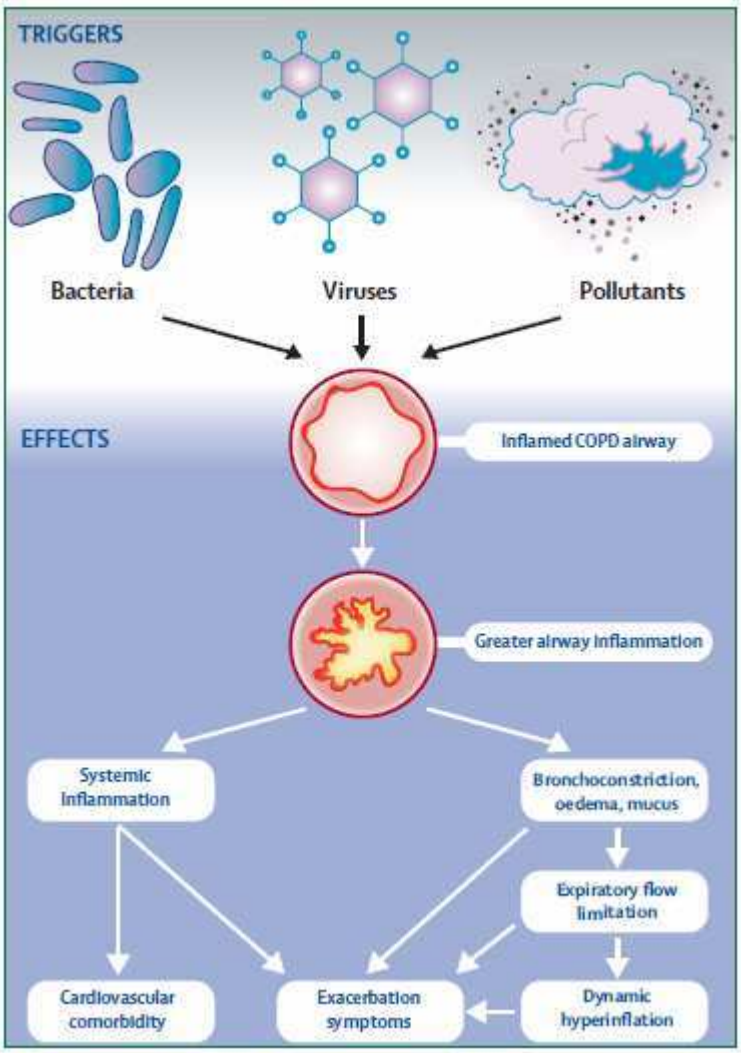

Figure 2 Exacerbations of COPD are caused by bacteria, viruses or pollutants and lead to an increased inflammatory response in the lung. (adapted from *Wedzicha et al.* [44])

2.2.4. Genetic Factors

In addition to the described exogenous risk factors relevant to the development of chronic airway disease, individual susceptibility factors of each patient contribute to the increased loss of lung function capacity. The currently accepted hypothesis that COPD is a complex disease influenced by genetic variables is corroborated by the fact that only a small amount of smokers suffers from obstructive airway disease. Based on this observation, some smokers may be more vulnerable to tobacco smoke than others – an effect most likely caused by their genotype. [46] Further evidence for a genetic background of COPD was given

by *Silverman*, who identified a significant odds ratio of 4.5 for impaired lung function measurements in first-degree relatives of COPD patients who smoked. [47]

The close association between pulmonary emphysema and α1-antitrypsin deficiency (AATD) was described by *Eriksson* in 1964. [48] Airflow obstruction and chronic bronchitis tend to be more frequent and severe in patients with AATD and usually occur at an earlier age. [21] Moreover, the extent of symptoms is often out of proportion to the smoking history of the patient. [49] α1-antitrypsin (AAT) is an important protease inhibitor of leukocyte elastase, cathepsin G, and proteinase-3. As an acute phase protein it is predominantly produced in the liver and by alveolar macrophages. The protein is transcribed from the SERPINA1 gene on chromosome 14 at the protease inhibitor (PI) locus. The 4 major allele forms are labeled based on their mobility characteristics in gel electrophoresis (F=fast, M=medium, S=slow, Z=very slow). [20] The most common alleles are the normal M allele (95%) and the deficient variants S (2–3%) and Z (1–2%), which both result from point mutations. [50] [51] The mutation accompanying the Z allele results in significantly decreased plasma levels of AAT (about 10–15% of normal values). [52] Severe AATD is an accepted risk factor for COPD and is most commonly associated with ZZ homozygosity (PI ZZ) or a PI Znull genotype. However, it only accounts for a minority (1–3%) of all cases of COPD. [50] Even in homozygote patients, the clinical course of COPD is highly variable and the ascribed role of AATD in the pathogenesis of COPD may partly be due to a selection bias in lack of other gene mutations. [53] While the association between the PI ZZ genotype and emphysema was described in several studies, the consequence of heterozygote mutations remains unclear. Patients with a PI MZ genotype have AAT levels of 60% of the normal population. [53] According to a meta-analysis by *Hersh et al.,* the increase in risk of COPD in all PI MZ heterozygotes is rather small and uncertain. [50] Loss of lung function in relation to total amount of tobacco smoking seems to be augmented in patients with PI Z genotype, indicating a significant genotype–environment interaction. [21]

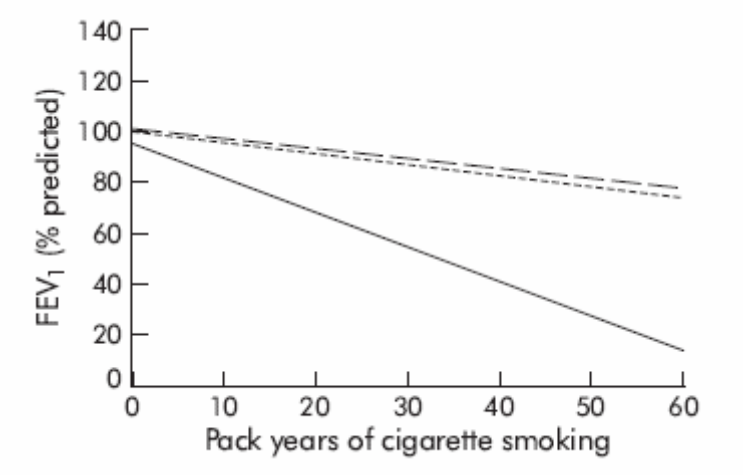

Figure 3 Forced expiratory volume in 1 second (FEV1) in PI Z subjects (solid line), heterozygote PI MZ subjects (dotted line), and PI M subjects (dashed line). (adapted from *Sandford et al.* [21])

Without the protective effects of AAT, the lung tissue is exposed to degradation by various enzymes (e.g. elastase), thus leading to the known pathological findings consistent with COPD. The imbalance between protease and protease inhibitor proteins leads to destruction of the functional lung parenchyma and is thought to be one of the major pathogenic mechanisms leading to the remodeling processes seen in patients with COPD. [46]

Numerous other gene mutations leading to increased tissue remodeling in the lung have been described, but unlike AATD, evidence for their association with COPD remains weak. Polymorphisms of α1-antichymotrypsin (AACT), α2-macroglobulin (A2M), matrix metalloproteinases (MMPs) and their inhibitors (tissue inhibitor of metalloproteinases; TIMP), and many other candidate genes and their relation to COPD are controversially discussed. [20 21 46 53]

2.3. PATHOGENESIS

According to the definition of the "Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Pulmonary 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. [54] The term "Chronic obstructive pulmonary disease" does not clearly describe a single disease entity. It rather comprises a number of different distinct processes that have been summarized in one clinical complex. The main pathological findings that are part of the concept of "COPD" include chronic bronchitis, lung emphysema and remodeling of the small airways. [55]

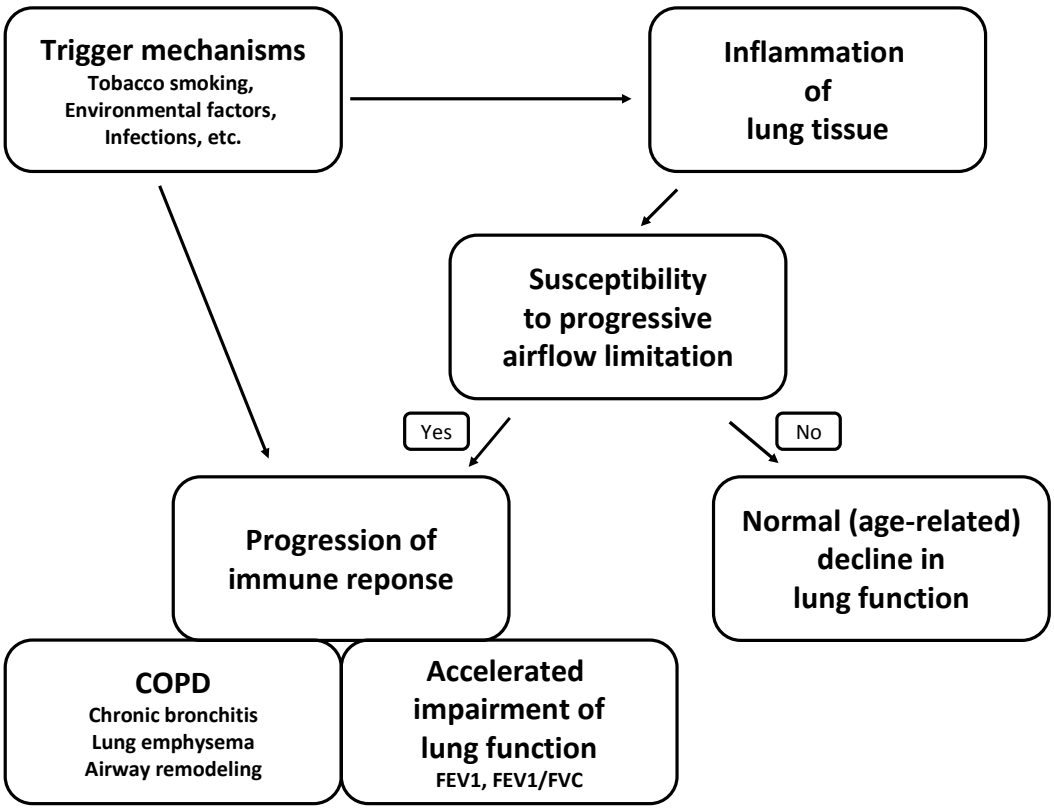

Figure 4 Based on the initial immune response in the airways, subjects who are susceptible to pathological changes of the airways may develop the clinical features of chronic obstructive airway disease.

All of these changes lead to obstruction of expiratory airflow – measurable as reduction in FEV1 and FEV1 to forced vital capacity ratio (FVC) (FEV1/FVC) – which is currently the main criterion for diagnosing COPD. As a result of this heterogeneous definition of COPD, the patient group diagnosed with the disease shows a considerable variation of symptoms.

Descriptions of pathways resulting in chronic airway disease are numerous. Yet, the underlying mechanisms appear to be intricate and are only partly specified in detail. An excessive inflammation of the lung tissue seems to be the key element for progression of airflow limitation and the associated pathological changes. [56] Though the lung of every smoker is characterized by an accumulation of inflammatory cells, only some show a more rapid decline in expiratory airflow, due to currently unknown susceptibility factors. [57] [58] Whereas in normal smokers the inflammatory reaction ceases after smoking cessation, patients with COPD continue to exhibit an ongoing immune response even years after successful quitting. [59] [60] Particularly the small airways are the predominant site of tissue remodeling and constitute the major source of the reduction of lung function parameters. [61] [62] [63]

2.3.1. Innate Immunity

Together with the mucociliary clearance system, alveolar macrophages and neutrophilic granulocytes constitute the innate immune system of the lung. [64] This initial response is able to act fast but lacks specificity and memory functions. Chronic activation may ultimately lead to the symptoms seen in patients with airway obstruction due to tissue remodeling and development of emphysema. The alveolar membrane is the largest surface of the body in direct contact with the surrounding air. Under normal conditions, alveolar macrophages account for approximately 95% of airspace leukocytes. [65] These immune cells are the first in line to counteract airborne pathogens and particles. [66] Their numbers increase significantly in patients with COPD and the amount of alveolar macrophages is associated with the severity of airflow obstruction. [56] [67] The enhanced presence of alveolar macrophages in the airways of smokers and their enhanced lifespan may partly be caused by induction of anti-apoptotic $p21^{CIP1/WAF1}$ and Bcl-x_L. [68] Alveolar macrophages exhibit multiple functions in the defense system of the lung. They are able to act as phagocytes and clear the airways of small

(smoke) particles and debris. Furthermore, as antigen-presenting cells (APCs) they are an important link to the adaptive immune system. Alveolar macrophages are also able to produce cytokines, growth factors, and MMPs. Production of MMPs by alveolar macrophages seems to be essential in the development of tissue destruction. [69] The proteolytic effect of macrophage elastase (MME; MMP12) induces degradation of extracellular matrix proteins and ultimately leads to the pathological changes of emphysema. [70] [71] Expression of MMP1 (interstitial collagenase 1) and MMP9 (gelatinase B) was also elevated in alveolar macrophages separated from bronchoalveolar lavage (BAL) fluids of patients with emphysema. [72] [73] *Hubbard et al.* were able to demonstrate that cigarette smokers' alveolar macrophages spontaneously release sufficient amounts of oxidants to inhibit the function of AAT thus leading to increased activity of proteolytic enzymes. [74] Alveolar macrophages isolated from smokers produced greater amounts of pro-inflammatory tumor necrosis factor (TNF)-α and anti-inflammatory IL-10 indicating a higher level of activation through exposure to tobacco smoke. [75] In summary, alveolar macrophages appear to be highly active in patients with COPD and contribute to the observed alterations of tissue composition.

Neutrophils are widely accepted as key inflammatory cells in COPD. [66] They are present in the lung parenchyma and are also found in the BAL fluid acquired from patients with chronic bronchitis together with an increased total number of inflammatory cells. [76] [77] [78] Of particular interest are results showing a close relation between neutrophil count in the sputum analyses and the degree of airflow limitation and decline of lung function, respectively. [79] [80] The question whether these cellular infiltrates are responsible for the chronic bronchitis or if their presence can only be regarded as surrogate marker for a general activation of the immune system has yet to be addressed. Tissue damage through activated neutrophils is caused by the release of proteins such as neutrophil elastase, MMPs, and oxygen radicals such as superoxide anion, hydrogen peroxide and hypohalides. [66] [81] Obstruction of the small airways may be a direct result of enhanced fibroblast contraction due to neutrophil elastase. [82] Increased production of mucin in a human pulmonary mucoepidermoid carcinoma cell line was observed by *Kohri et al.* after stimulation with neutrophil elastase. [83] The overproduction of mucus is an important feature of chronic airway disease.

Migration of neutrophilic granulocytes into the lung tissue requires the activity of chemotactic factors. [84] The induction of chemotaxis leads to the accumulation of leukocytes at the site of inflammation. Exposure to tobacco smoke was shown to provoke the production of chemotactic IL-8 by bronchial epithelial cells and fibroblasts under *in vitro* conditions and generally increased their chemotactic activity. [85] [86] According to these results, active smokers seem to constantly perpetuate the chronic inflammation in their airways simply by continuing to smoke. Increased levels of essential neutrophil chemotactic factors have been identified in specimens, BAL, sputum, and serum of COPD patients. [58] [87] [88] [89] Among this multitude of signaling factors, the most relevant chemokines associated with neutrophilia in COPD are IL-8, leukotriene B4 (LTB_4) growth-related oncogene (GRO)-α, epithelial cell-derived neutrophil-activating peptide (ENA)-78, monocyte chemoattractant protein (MCP)-1, and macrophage inflammatory protein (MIP)-1α. [66] [90] [91] IL-8 stimulates the transmigration of neutrophils across endothelium, pulmonary epithelium, and fibroblasts. It is produced by leukocytes [monocytes, T-cells, neutrophils, natural killer (NK) cells] as well as other cells (endothelial cells, fibroblasts, and epithelial cells). Furthermore, IL-8 leads to the activation of various functions of neutrophils including degranulation and respiratory burst. [92] Pretreatment with a neutralizing monoclonal antibody (mAb) against IL-8 in an *in vivo* rabbit smoke model showed decreased injury of alveolar epithelial cells providing evidence for the important role of IL-8 in smoke mediated lung injury. [93] Besides exposure to tobacco smoke, bacterial and viral infections – as common complications in the airways of smokers – can induce increased production of IL-8, thus leading to accumulation of neutrophils in the airways. [92] [94] Lung epithelial cells produced higher amounts of IL-8 upon infection with adenovirus in an *in vitro* model. [95] Interestingly, alveolar macrophages from patients with COPD had a five-fold higher basal secretion of IL-8 than the same cells from smokers without airway obstruction. Moreover, the addition of Dexamethasone in the COPD group showed no inhibitory effect. [96]

2.3.2. Adaptive Immunity

Activation of T-lymphocytes (CD3+) – as part of the adaptive immune system – is reliant on the presentation of an antigen via the major histocompatibility complex (MHC) on APCs or

target cells. Ultimately, CD8+ lymphocytes act as cytotoxic cells that are able to lyse target cells upon activation. CD4+ lymphocytes usually function as "helper cells" capable of promoting either a cellular T_H1 or a humoral T_H2 immune response. Generally, the immune response in COPD appears to be mostly T_H1 mediated. [97] The total number of lymphocytes in the lung parenchyma as well as in the central and peripheral airways of patients with COPD was shown to be elevated in comparison to healthy controls. [98] The total count of CD3+ cells in the alveolar wall showed a remarkable relation with the total amount of exposure to tobacco smoke. The number of CD3+ cells evidenced a steep increment after an inflection point at about 30 pack years. The same phenomenon was observed in the analyses of CD4+ and CD8+ cells. The dose effect of tobacco smoke on the inflammation in the lung tissue therefore seems evident. Apparently, a long period of smoking – until finally reaching a threshold dose – is necessary to initiate the immune response involving antigen specific T-lymphocytes. [99] Also, the degree of emphysematous destruction of the lung tissue showed a strong correlation with the number of T-lymphocytes identified by immunohistochemistry in lung tissue samples. [67]

In particular, CD8+ T-lymphocytes were predominantly found in patients with COPD and their numbers increased with severity of airflow obstruction. [100] [101] [102] Some authors described a shift of the CD4+/CD8+ ratio towards a more dominant CD8+ cell effect in smokers and patients with COPD. [103] [104] Interestingly, HIV-seropositive smokers (with a lower total CD4+ cell count) had a higher prevalence of emphysema. [105] Traditionally, cytolytic CD8+ cells are essential in the clearing of airways in the case of viral infections. Since the occurrence of obstructive airway disease is frequently associated with virus persistence in the airways, accumulation of CD8+ cells in susceptible patients may thus lead to airway destruction. [106] The mechanism of action of cytolytic lymphocytes in the progression of COPD is partly based on the induction of apoptosis in lung cells. [99] Killing of the target cells can be caused by the release of potentially lytic proteins like perforin and granzymes. Sputum CD8+ cells acquired from patients with COPD showed elevated levels of intracellular perforin expression and cytotoxic activity compared to both asymptomatic smokers and non-smokers. [104] Besides direct destructive effects, CD8+ cells are an essential part of the inflammatory network that leads to the remodeling of the airways as seen in COPD. [107]

Some authors also pointed out the role of CD4+ T-cells in the pathogenesis of chronic airway disease. [108] However, studies describing the role of these cells in COPD remain scarce. CD4+ cells were detected in the tissue surrounding the small airways where they were organized in follicles together with B-lymphocytes and CD8+ cells. [109]

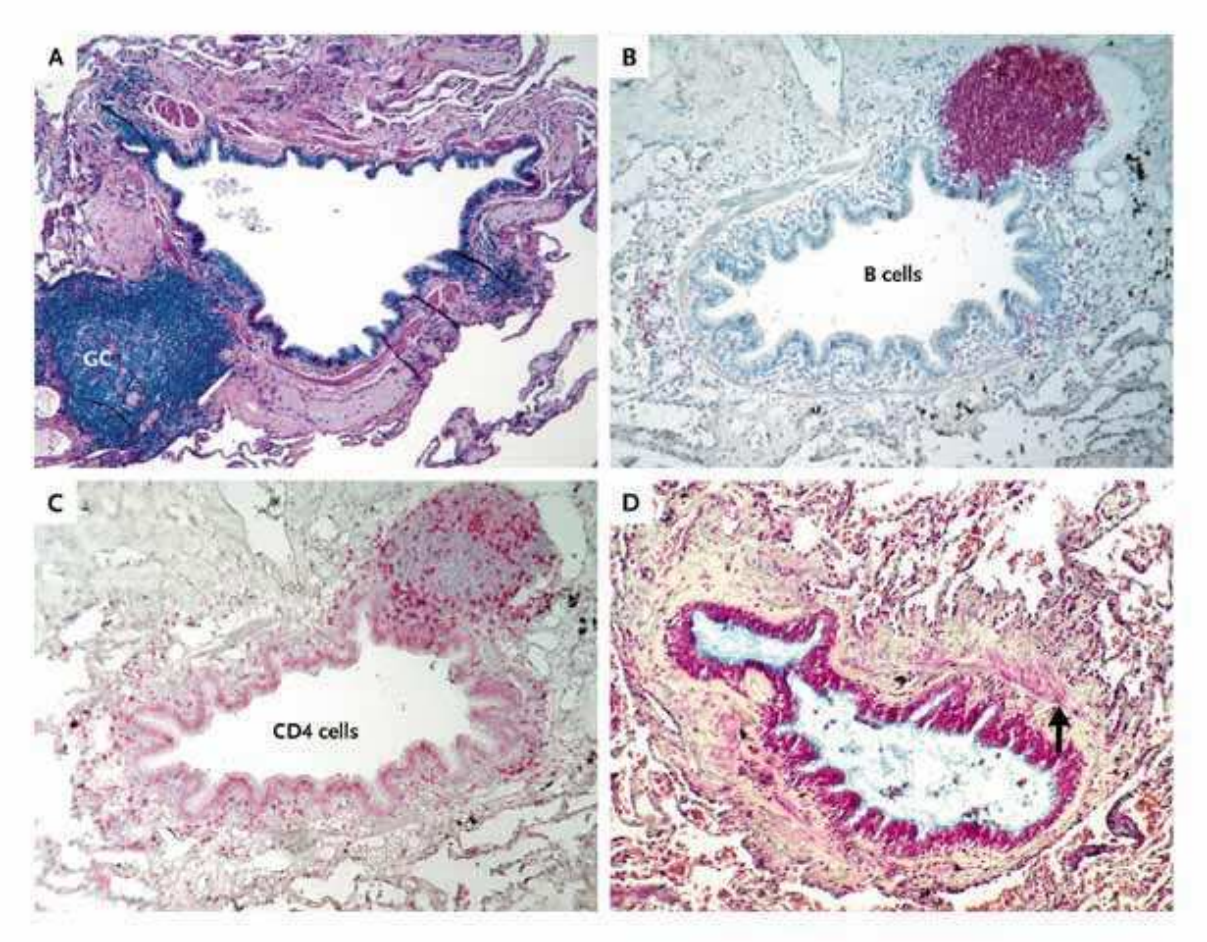

Figure 5 (A) shows bronchial lymphoid tissue with a lymphoid follicle containing a germinal center (GC). (B) (C) are sections of the same airway. The lymphoid follicle surrounding the airway stained positive for B-cells and CD4+ cells. (D) shows a remodeled airway. (adapted from *Hogg et al.* [109]*)*

An immunohistological study performed by *Aoshiba et al.* found different distribution patterns of CD8+ and CD4+ cells in the airway walls of patients with emphysema. Whereas CD8+ cells were predominantly found in regions with mild lesions, CD4+ cells were increased at sites that were more severely damaged. The finding of CD4+ cells was closely related with the extent of emphysema. [110] *Sullivan et al.* showed that CD4+ cells isolated from the lung tissue of patients with emphysema at time of transplantation had a higher expression of the activation marker CD45RO compared to cells in the peripheral blood. Furthermore, these cells had lost receptors necessary for lymph node trafficking (CD62L, CCR7) and displayed

the phenotype of differentiated memory T-cells. T-lymphocytes of patients with emphysema showed increased proliferation rates after stimulation with IL-2 indicating that they had been activated previously. Of particular interest are their results indicating the presence of a subset of oligoclonal CD4+ cells in the emphysematous lungs. [111] Yet, the exact role of CD4+ cells in the development of COPD remains unclear. Suggested pathways include the stimulation of fibroblast-mediated extracellular matrix degradation, and the increased expression of interferon-gamma (IFN-γ) and signal transducer and activator of transcription 4 (STAT4) in CD4+ cells. [112] [113] Despite the lack of functional studies on CD4+ cells in the development of COPD, the role of activated and differentiated CD4+ cells with a restricted T-cell receptor (TCR) repertoire in the process of tissue remodeling of the lung seems evident.

2.3.3. Autoimmune Aspects

Some years ago, the idea of COPD as autoimmune disease emerged. [114] Several observations during the initiation and the natural course of the disease corroborate this hypothesis. First, only a small percentage of smokers reach the later stages of chronic airway disease. The majority of tobacco smokers only suffer from the regularly observed and age-related decline in lung function. Thus, a certain genetic background seems necessary to bring smokers on the pathway to COPD. Clustering of a certain genetic variant was also described in known autoimmune disorders like ankylosing spondylitis, rheumatoid arthritis, and systemic lupus erythematosus. [115] [116] [117] Yet, the exact susceptibility factors leading to an increase in risk for development of COPD have not been identified. A second finding that suggests an autoimmune aspect in COPD is the persistence of airway inflammation despite successful smoking cessation. With smoking being the most important factor leading to the disease, its discontinuation should also be associated with a return of the immunologic response in the lung to normal conditions. However, several authors found an ongoing infiltration of the lung with activated immune cells in COPD patients who had successfully quit smoking. [60] [118] [119] *Motz et al.* evidenced in a mouse model of smoke induced emphysema, that smoke exposure alone leads to the expansion of oligoclonal, antigen-specific T-cells that were found in the lung up to 6 months after smoking cessation. [120] The possible (auto-)antigens responsible for this continuous specific response of the adaptive immune response remain unknown. However, the persistence of the ongoing inflammatory response despite the lack

of an exogenous trigger mechanism is a strong argument towards the autoimmune hypothesis of COPD.

The search for the specific antigens involved in the immune response of chronic airway inflammation has generated different hypotheses over the years. Some authors argued that exposure to tobacco smoke leads to an increased liberation of self-antigen that is subsequently recognized and processed by antigen-presenting cells. Since most proteins are also expressed in organs other than the lung, the immune response can lead to systemic effects of the initially local disease of the lung. [114] [121] An additional factor in this proposed pathway is the defective clearance of apoptotic cells in COPD. Clearance of apoptotic material was shown to be impaired in patients versus control subjects and thus leads to the accumulation of cell material in the lung and further promotes the exposure of self-antigen. [122] Interestingly, smoking leads to increased levels of antigen-presenting cells in the lung – yet another element of the self-antigen hypothesis. [123] As viral infections are common in the COPD population, other groups found an association of increased immune activation with expression of a specific antigen due to latent adenoviral infections. [124] Reactivity of CD4+ and CD8+ cells to a viral neo-antigen exclusively expressed in the lung was also observed in an animal model which corroborates this hypothesis. [125] [126] *Taraseviciene-Stewart et al.* showed that injection of rats with human umbilical vein endothelial cells (HUVECs) lead to the development of emphysema. This effect was CD4+ dependent as transfer of CD4+ cells from immunized rats caused airway alterations in naïve animals. [127]

In summary, these results suggest an autoimmune component in the pathogenesis of COPD. The necessity of a neo-antigen – either through loss of tolerance for a self-antigen or exogenous factors – seems evident. Smoking has been identified as major risk factor in multiple autoimmune diseases like Graves' disease and autoimmune hypothyroidism, rheumatoid arthritis, primary biliary cirrhosis, and accelerated atherosclerosis in patients with autoimmune rheumatoid arthritis. [128] Years of tobacco smoking cause airway inflammation, damage cells through oxidants, damage DNA, and cause apoptosis. Smoking can therefore be considered the exogenous trigger mechanism that eventually results in the occurrence of an auto-antigen. [99] This "point of no return" can be reached after a considerably long period of time. It is at the late stages of the disease, when the adaptive

immune response in form of lymphoid follicles becomes more evident in the airways of affected smokers. [129] From this point on, the inflammation represents a self-perpetuating process in response to the auto-antigen and cessation of smoking has little to no effect on the course of chronic airway disease.

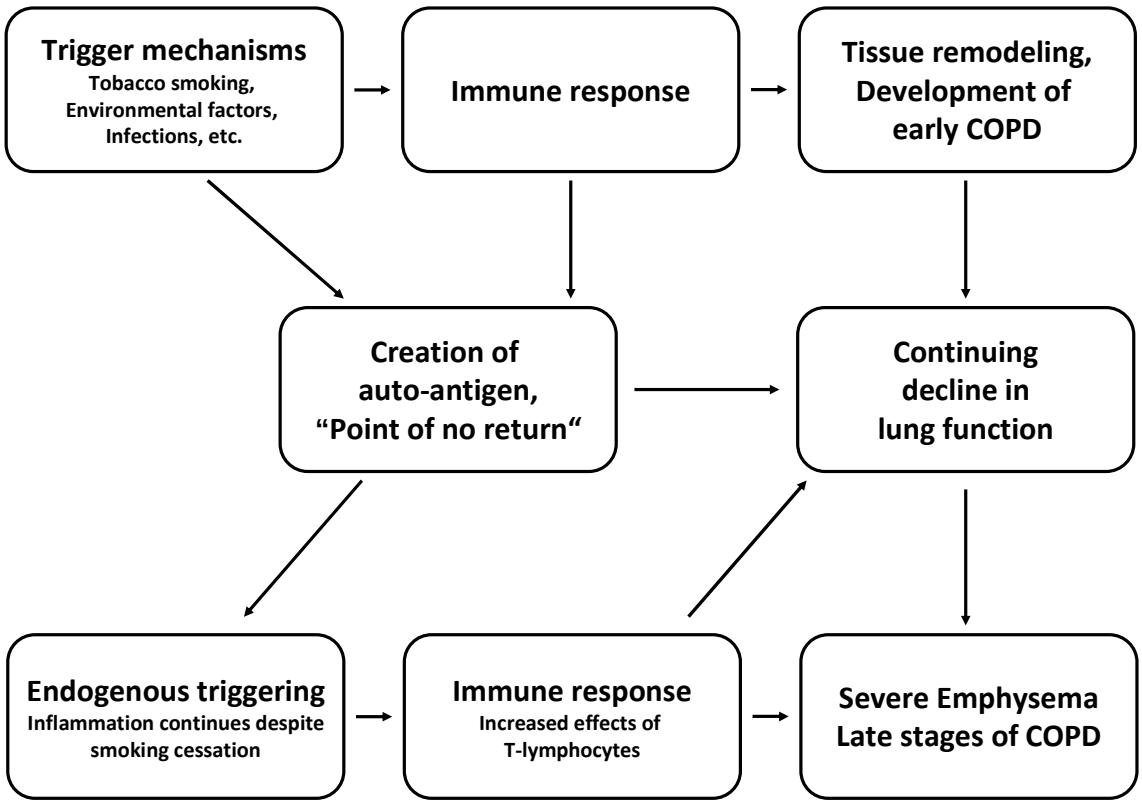

Figure 6 Possible autoimmune pathways leading to COPD are shown. Alterations of self-antigen or latent viral infections lead to the forming of auto-antigens in susceptible subjects. After this, the immune response is ongoing – mainly involving parts of the adaptive immune system – and leads to the development of severe COPD.

2.3.4. COPD is a Systemic Disease

Most patients with chronic airway disease die of non-respiratory causes with cardiovascular disease, diabetes mellitus, and cancer being the most prevalent and crucial factors for mortality. [130 131] Current reports highlight the role of systemic inflammation with heightened levels of inflammatory cytokines and acute phase proteins in the systemic circulation of

patients with COPD. [132] This indicates that the systemic immune activation caused by the local effects in the lung may lead to the extrapulmonary co-morbidities frequently seen in COPD.

Systemic effects of COPD related to systemic inflammation

Involuntary weight loss	Impaired bone metabolism
Muscle wasting	Normocytic anemia
Reduced functional capacity	Cancer
and health status	Depression
Increased cardiovascular	Diabetes
morbidity and mortality	Peptic ulceration

Table 2 Systemic effects observed in patients with COPD. (adapted from *Cazzola et al.* [132]*)*

2.4. CD4+CD28NULL CELLS

CD28 is a co-stimulatory, membrane bound molecule regularly found on human T-cells. Together with the interaction of TCR and peptide/MHC complexes, the binding of CD28 to its ligands CD80 and CD86 is required as the "second signal" for the response and proliferation of T-cells. CD80 and CD86 are expressed on APCs, are upregulated upon activation of these cells, and therefore regulate the number of clonogenic T-cells. In T-cells that express CD28, the induction of a T-cell response lacking the co-stimulatory mechanism via the CD28 pathway leads to anergy to the specific antigen. [133] Upon activation via the TCR and CD28, T-cells challenged with a specific antigen undergo an oligoclonal expansion and differentiate into effector and memory cells. After completion of the antigen-specific function, this expansion is usually counterbalanced by apoptotic cell death. Interestingly, a certain subset of CD4+ cells lacking the co-stimulatory CD28 was found to have prolonged survival and was less susceptible to undergo apoptosis. This effect is partly due to a defect in downregulating B-cell lymphoma protein 2 (Bcl-2) when deprived of T-cell growth factors. This specific cell compartment consists of a limited number of expanded T-cell clones. [134] Loss of the co-stimulatory molecule CD28 occurs after replicative stress and indicates premature lymphocyte senescence. [135] CD4+CD28null cells are rare in healthy individuals. However, elevated numbers of circulating CD4+CD28null cells were found in patients with autoimmune disorders including rheumatoid arthritis, Wegener's granulomatosis, inflammatory bowel disease, ankylosing spondylitis, cardiovascular disease, and multiple sclerosis. [136] [137] [138] [139] [140] [141] Moreover, increased numbers of CD4+CD28null cells were found in patients with chronic viral infections. [142] [143] CD4+CD28null cells are able to produce large amounts of IFN-γ. [141] They also contain intracellular perforin and granzyme B, providing them with the ability to lyse target cells. [144] As an alternative to the regular co-stimulatory pathway, CD4+CD28null cells express receptors characteristic of natural killer cells including CD94, CD158, and CD161, and therefore combine relevant features of the adaptive and innate immunity. [145]

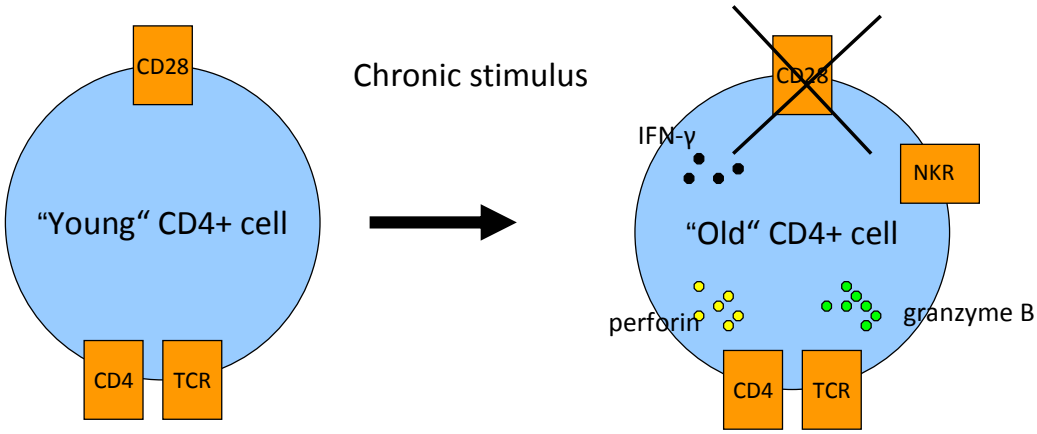

Figure 7 Chronic stimulus of naïve T-cells leads to phenotypic changes including loss of CD28 and expression of natural killer cell receptors (NKR).

2.5. HEAT SHOCK PROTEINS

Heat shock proteins (HSPs) are a group of highly conserved proteins that under normal conditions play a critical role in the folding and transport of intracellular proteins. [146] Upon exposure of the cell to external stressors like heat, infections, or ischemia-reperfusion, the expression of intracellular HSPs is increased as they are able to prevent apoptosis and protein aggregation and preserve the integrity of the cellular compartment. [147] [148] Besides the known intracellular effects, the response to stress also results in a release of HSPs in the extracellular space. Further mechanisms that lead to increased levels of HSPs in the bloodstream are necrotic cell death due to extreme cell damage with adjacent release of the intracellular proteins and the active secretion of HSPs by certain cell lines. [149] [150]

Extracellular HSPs have the ability to actively modulate the innate and adaptive immune response. HSP70 was shown to bind specifically to human monocytes and to induce the production of pro-inflammatory cytokines. [151] Heat shock proteins may serve as immunological "danger signals" because they are upregulated after exposure of cells to stressors. [146] In the adaptive immune response, HSPs exhibit high immunogenic potential by leading to cross-presentation of antigens on APCs by formation of HSP-antigen complexes that are subsequently processed and presented by APCs. These mechanisms result in the activation of antigen specific T-cells via the MHC pathway. [152]

	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 HSPs have different effects in the intracellular compartment and after release in the extracellular space. (adapted from *Calderwood et al.* [150])

Interestingly, HSP60 and other HSPs have been identified as possible auto-antigens in the pathogenesis of atherosclerosis. [153] The constant inflammatory process together with inadequate clearance of tissue debris leads to increased contact of normally intracellular HSPs with immune cells and triggers an autoimmune response. CD4+ cells reactive to HSP60 were found in the atherosclerotic plaques of patients. [154] Other studies demonstrate the role of HSP90 as auto-antigen for humoral and cellular immunity in the pathogenesis of carotid atherosclerosis. [155] Whether the autoimmune effects to HSPs are a result of molecular mimicry – based on the close structural homology with bacterial HSPs – or a consequence of increased liberation of HSPs in response to cell stress remains a point of discussion.

3. AIMS OF THE STUDY

The current study was performed in order to

1. identify CD4+CD28null cells in the peripheral blood of patients with diagnosed COPD at different stages of the disease.
2. further describe the phenotype of these cells as link between the innate and adaptive immune system.
3. evaluate the serum levels of circulating HSPs in serum samples of patients with COPD.
4. characterize the potential of serum HSPs to serve as biomarker and a new easy-to-use diagnostic tool in the management of COPD.

4. ORIGINAL RESEARCH

T-cell senescence and contraction of T-cell repertoire diversity in patients with chronic obstructive pulmonary disease

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4.1. INTRODUCTION

Chronic obstructive pulmonary disease is a leading cause of death worldwide: [156] By 2020, only ischemic heart disease and cerebrovascular disease will account for a higher mortality among the world's population. [2] Prevalence and hospitalization rates have increased significantly over the past years. [157] [158] Several studies were able to show a strong correlation between tobacco abuse and the development of COPD. However, not every smoker develops clinical features of COPD. [159] [160] Pathogenesis of the disease is characterized by irreversible airflow obstruction because of constant remodeling of the airways and chronic inflammatory responses. [161] The impairment of the immune system is not restricted to the lungs, as COPD patients are also at higher risk for systemic failures including cardiovascular diseases. [162] Diagnosis of airway obstruction according to the guidelines of the Global Initiative for Chronic Obstructive Lung Diseases (GOLD) requires the use of spirometry. A post-bronchodilator FEV_1/forced vital capacity ratio of less than 70% indicates an irreversible airflow obstruction, and is therefore considered to be the main parameter for the diagnosis of COPD. [163]

Although smoking is accepted widely as the major risk factor for the development of the disease, descriptions of specific pathogenic mechanisms remain vague. For decades, neutrophils and macrophages, as part of the innate immunity, were considered pivotal in the airway remodeling process occurring in patients with COPD. Recent reports have challenged

this pathognomonic concept by demonstrating increased CD8+ and CD4+ T-cells as part of the adaptive immune system in bronchoalveolar lavage and sputum analyses of COPD patients. These T-lymphocytes contained higher levels of perforin and revealed cytotoxic activity compared with cells of healthy donors or non-COPD smokers. [164] [165] Furthermore, *Di Stefano et al.* presented two papers in which they were able to show that stable mild/moderate COPD is associated with an active T helper 1 cell/type-1 cytotoxic T-cell inflammatory process involving activation of signal transducer and activator of transcription 4 and IFN-γ production and NK-cells in COPD lung tissue and bronchoalveolar lavage. [113] [56]

Based on the data of *Hodge et al.*, who described CD8+CD28null in COPD and *Di Stefano et al.*'s data, we hypothesized that a specific chronic inflammatory reaction of the adaptive immune system is occurring in patients with COPD. Antigenic stimulation causes a rapid expansion of antigen-specific T-cells that increase to large clonal size. This physiological increment is counterbalanced by a pre-programmed clonal contraction. This process is robust and usually suffices to maintain a diverse memory T-cell compartment. [166] [167] Chronic antigen exposure because of infections with human immunodeficiency virus or cytomegalovirus and advanced age also leads to expansion of monoclonal T-cell populations. [168] [169] [167]

Replicatively stressed CD4+ T-cells undergo multiple phenotypic and functional changes. The most widely acknowledged phenotypic change is the loss of the co-stimulatory surface marker CD28. Expansion of CD4+ T-cells and loss of CD28 are presumably senescent (CD4+CD28null). This has been described in several autoimmune diseases, such as diabetes mellitus, rheumatoid arthritis, Wegener's granulomatosis, multiple sclerosis and ankylosing spondylitis. [137] [170] [171] CD4+CD28null cells are clonally expanded and are known to include autoreactive T-cells, implicating a direct role in autoimmune disease. These expanded CD4+ clonotypes are phenotypically distinct from the classic T helper cells. Because of a transcriptional block of the CD28 gene, clonally expanded CD4+ T-cells lack surface expression of the major co-stimulatory molecule CD28. CD4+CD28null T-cells release large amounts of IFN-γ and contain intracellular perforin and granzyme B, providing them with the ability to lyse target cells. Their outgrowth into large clonal populations may be attributed partially to a defect in down-regulating Bcl-2 when deprived of T-cell growth factors. In the

absence of the CD28 molecule, these unusual CD4+ T-cells use alternative co-stimulatory pathways. Several of these functional features in CD4+CD28null T-cells are reminiscent of NK-cells. Like NK-cells, CD4+CD28null T-cells are cytotoxic and can express NK-cell receptors such as CD94 and CD158. NK-cells are regulated closely by a family of polymorphic receptors that interact with major histocompatibility complex class I molecules, resulting in signals that control NK-mediated cytotoxicity and cytokine production. MHC class I-mediated triggering of the full-length NK-cell receptors transduces a dominant inhibitory signal that blocks the cytolytic activity and cytokine release of NK-cells. These receptors also contain highly homologous members that have truncated cytoplasmatic domains and transmit activating signals. [172 134 173 174]

Because chronic antigen exposure because of history of smoking can be assumed in most COPD patients, we hypothesized that chronic stimulation of the adaptive immune system leads to increased levels of systemic clonogenic CD4+CD28null T-cell populations. To prove this we included age- and sex-matched healthy non-smokers, smokers with a history of tobacco abuse and normal lung function and patients with diagnosed COPD according to GOLD classification (groups: mild, COPD I-II; severe, COPD III-IV; respectively). We determined intracellular expression of cytolytic proteins perforin and granzyme B as well as surface expression of the NK-cell receptors CD94 and CD158 on CD4+CD28null T-cells. We further designed *in vitro* experiments to explore whether peripheral blood mononuclear cells (PBMCs) obtained from each study group secrete augmented levels of IFN-γ, TNF-α and IL-12 after T-cell triggering. Because systemic inflammation is associated with systemic proinflammatory cytokines *in vivo*, we correlated serum levels of IL-1β, TNF-α, IFN-γ and IL-10 with lung function parameters. We conclude in this work that patients with COPD show increased circulating clonogenic T-cells that have diagnostic potential for detection of COPD according to the GOLD classification.

4.2. METHODS

Patients

The study protocol was approved by the ethics committee of the Medical University of Vienna (EK No. 091/2006) and was performed in accordance with the Declaration of Helsinki and current revisions of the Good Clinical Practice Guidelines of the Medical University of Vienna. A total number of 64 volunteers, at least 40 years old, participated in this trial. Healthy non-smokers (n=15), smokers (n=14) and smokers meeting the GOLD diagnostic criteria for COPD I-II (n=19) and COPD III-IV (n=16) were recruited. [175] COPD patients with acute exacerbation as defined by the guidelines from the WHO and GOLD within 14 days before study entry were excluded. [163] [176] Additional exclusion criteria were a history of asthma, autoimmune diseases or other relevant lung diseases (e.g. lung cancer, known α1-antitrypsin deficiency). Furthermore, all patients were free from known coronary artery disease, peripheral artery disease and carotid artery disease. All patients provided written, informed consent before collection of blood samples and lung function. Height and weight (Seca; Vogel and Halke, Hamburg, Germany) were measured and body mass index (BMI) was determined. Pulmonary function was measured using the same model spirometer (AutoboxV6200; SensorMedics, Vienna, Austria). Measurements were made before and – if criteria for airflow obstruction were met – 15-30 minutes after inhaling 200µg salbutamol. Arterial blood gases (PaO_2, $PaCO_2$) were obtained at rest while breathing room air in a sitting position. Measurement of arterial blood gases was performed with an ABL 510 gas analyzer (Radiometer, Copenhagen, Denmark). Results are expressed as absolute values and as percentages of predicted values for age, sex and height, according to the European Community for Steel and Coal prediction equations. [177] Predicted normal values were derived from the reference values of the Austrian Society of Pulmonary Medicine.

Flow cytometry analysis

Heparinized blood samples were incubated on ice with fluorochrome-labeled antibodies. Prior to antibody incubation, erythrocytes were lysed by addition of BD fluorescence

activated cell sorter lysing solution (Becton Dickinson, Franklin Lakes, NJ, USA). Cells were then stained with fluorescein isothiocyanate-conjugated anti-CD4 (BD Biosciences Pharmingen, San Jose, CA, USA), phycoerythrin (PE)-labeled anti-CD158 (R&D Systems, Minneapolis, MN, USA), PE-Cy5-labeled anti-CD28 (Biolegend, San Diego, CA, USA) and PE-conjugated anti-CD94 (eBioscience, San Diego, CA, USA) at various combinations. Stained cells were analyzed using a Cytomics FC 500 flow cytometer (Beckman Coulter, Fullerton, CA, USA). For intracellular staining, PE-conjugated antibodies directed against perforin and granzyme B (BD Biosciences Pharmingen; Serotec, Dusseldorf, Germany) were used and incubated with pre-stained cells after permeabilization of the cell membrane with saponin solution.

Enzyme-linked immunosorbent assays

The enzyme-linked immunosorbent assay (ELISA) technique (BenderMedSystems, Vienna, Austria) was used to quantify levels of IL-1β, TNF-α, IFN-γ and IL-10 in serum samples obtained after centrifugation of whole blood. Ninety-six-well plates were coated with a monoclonal antibody directed against the specific antigen and incubated overnight at 4°C. After a washing step, plates were blocked with assay 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. Tetramethylbenzidine (TMB) substrate solution was applied after the appropriate incubation time and another washing step. Color development was then monitored using a Wallac Multilabel counter 1420 (PerkinElmer, Boston, MA, USA). The optical density values obtained were compared with the standard curve calculated from optical density values of standards with known concentrations of antigen.

Stimulation of freshly prepared PBMCs

Freshly prepared PBMCs were separated by standard Ficoll densitiy gradient centrifugation. Cells were then washed twice in phosphate-buffered saline, counted and transferred to a 96-well flat-bottomed plate at 1×10^5 cells per well in 200µl serum-free ultra culture medium

(Cambrex Corp., East Rutherford, NJ, USA) containing 0.2% gentamycinsulphate (Sigma, St Louis, MO, USA), 0.5% β-mercapto-ethanol (Sigma), and 1% L-glutamine (Sigma). Anti-CD3 (CD3) (10µg/ml) or phytohemagglutinin (PHA) (7µg/ml) were added and plates were transferred to a humidified atmosphere (5% CO_2, 37°C) for 18h. Supernatants were harvested and stored at −20°C.

Quantification of IFN-γ, TNF-α and IL-12 in supernatants

The ELISA technique (BenderMedSystems) was used to quantify levels of IFN-γ, TNF-α and IL-12 in supernatants of stimulated cells, as described above.

Statistical methods

Comparison of the primary end-point CD4+CD28null% of CD4+ and the second end-points (IFN-γ, TNF-α and IL-12 *ex vivo* CD3 and PHA, IL-1β, TNF-α, IFN-γ, and IL-10 serum values) between healthy non-smokers, healthy smokers, COPD I-II and COPD III-IV patients was performed with the non-parametric Kruskal-Wallis test. Pairwise comparisons between groups were performed with Wilcoxon tests. For the six pairwise between-group comparisons of the primary end-point CD4+CD28null% of CD4+ additionally adjusted critical values, according to *Shaffer* (1986), were applied to control the familywise error rate in the strong sense. [178] Parametric 95% confidence intervals (CI) for the mean CD4+CD28null percentages in each group were computed. Correlations of percentage of CD4+CD28null cells and serum cytokine levels with parameters of lung function were calculated using the Spearman's correlation coefficient. These correlations were performed for all patients, the subgroup of smokers and the subgroup of COPD patients. The prevalence of perforin, granzyme B and expression of CD94 and CD158 was compared between CD4+CD28null and CD4+CD28+ cells using Wilcoxon's signed-rank tests. Additionally, parametric 95% CI for the mean percentages for each variable are given. In the subgroup of smokers a logistic regression with dependent variable COPD (yes/no) and independent variable CD4+CD28null% was performed. To account for an outlying observation, the square root of the percentages was used in this analysis. To assess the predictive capacity of the

percentage of CD4+CD28null a receiver operating characteristic (ROC) curve with its area under the curve (AUC) was computed.

4.3. RESULTS

Demographic characteristics of study patients

Demographic characteristics of patients are depicted in Table 4. Healthy non-smokers, healthy smokers, GOLD-classified COPD I-II and COPD III-IV were included. In all groups a similar number of patients were included and age and sex were distributed equally.

CD4+CD28null cells show increased occurrence in patients suffering from COPD

To test our hypothesis whether CD4+CD28null cells are increased in patients with COPD, we evaluated blood samples using multi-stain flow cytometry. Figure 8a and Table 5 illustrate percentages of CD4+CD28null cells of the total CD4+ cell population. The COPD III-IV group showed significantly increased values compared with the healthy non-smoker and smoker groups (Wilcoxon test: p=0.012, p=0.002, Kruskal-Wallis test for the overall comparison: p=0.005). Additionally, we observed a significant difference between the COPD I-II group and the healthy smoker group (Wilcoxon test: p=0.046). Applying the *Shaffer* (1986) multiplicity adjusted critical values, only the differences between the COPD III-IV and the healthy groups remained significant. [178]

Unstimulated CD4+CD28null cells contain cytolytic proteins perforin and granzyme B

To evaluate the intra-cytoplasmic content of cytolytic proteins perforin and granzyme B in CD4+ cells, flow cytometric analysis of blood samples was performed after co-incubation with saponin solution and intracellular staining. Content of perforin was more prevalent in CD4+CD28null cells compared with CD4+CD28+ cells (Figure 8b) [46.13% (39.34-52.91) versus 4.68% (3.04-6.32), p<0.001; all means (95% CI)]. Positive staining for intracellular granzyme B in CD4+CD28null cells was more frequent than in CD4+CD28+ cells (Figure 8c) [78.63% (72.65-84.61) versus 2.36% (1.63-3.11), p<0.001; all means (95% CI)].

Increased prevalence of NK-cell receptors on CD4+CD28null cells

Flow cytometry analysis was used to evaluate expression of CD94 and CD158 on the surface of CD4+ cells. Figure 8d and e show increased expression of surface antigens CD94 and CD158 on CD4+CD28null cells [CD94, 10.00% (6.04–13.97) versus 1.41% (0.85-1.97), p<0.001; CD158, 9.35% (6.22-12.47) versus 2.00% (1.61-2.39), p<0.001; all means (95% CI)].

Percentage of CD4+CD28null cells correlates negatively with routine parameters of spirometry

For verification of our flow cytometry data with routine clinical data, we correlated the percentage of CD4+CD28null with FEV1% of vital capacity, 50% maximum expiratory flow (MEF50%) of predicted value and MEF25% of predicted value. All parameters showed a statistically significant negative correlation with percentage of CD4+CD28null cells (Spearman's correlation coefficients: FEV1%, R=−0.49, p<0.001; MEF50%, R=−0.40, p=0.001; MEF25%, R=−0·38, p=0.002; Figure 9a-c). Similarly, we observed significant correlations in the subgroup of smokers (Spearman's correlation coefficients: FEV1%, R=−0.52, p<0.001; MEF50%, R=−0.48, p=0.001; MEF25%, R=−0.40, p=0.004). In the subgroup of COPD patients marginally significant correlations with FEV1% and MEF50% (Spearman's correlation coefficients: FEV1%, R=−0.32, p=0.068; MEF50%, R=−0.36, p=0.04) and no significant correlation with MEF25% (Spearman's correlation coefficient: MEF25%, R=−0.15, p=0.38) were found.

Prediction capacity of the percentage of CD4+CD28null cells for COPD in smokers

In the logistic regression analysis for the subset of smokers the independent variable percentage of CD4+CD28null cells showed a significant association with COPD (p=0.012). The corresponding ROC curve (Figure 9d) has an AUC=0.76.

Correlations of serum cytokine concentrations (IL-1β, TNF-α, IFN-γ and IL-10) with FEV1%, MEF50% and MEF25%

Table 6 embraces the results of non-parametric correlations of serum cytokines IL-1β, TNF-α, IFN-γ and IL-10 with routine lung function parameters.

Stimulated PBMCs of patients suffering from early-stage COPD produce increased levels of IFN-γ and TNF-α *ex vivo*

To verify the functional activity of PBMCs we performed blastogenesis assays using lymphocyte-specific anti-CD3 and PHA. This analysis was performed for seven patients per group (except for the COPD III-IV group, where only five patients were included). Groupwise means and 95% CI are given in Table 5. Supernatants of patients with COPD I-II showed increased levels of IFN-γ compared with healthy smokers (Wilcoxon test: CD3 p=0.026, PHA: p=0.038); however, the differences failed to reach significance after correcting for multiple testing (Kruskal-Wallis test: CD3: p=0.06; PHA: p=0.09). Concentrations of the healthy group and of patients with COPD III-IV were lower but showed no significant difference to the COPD I-II group. None of the remaining pairwise comparisons was statistically significant. Significant differences of TNF-α (PHA) levels between groups were observed (Kruskal-Wallis test: p=0.007). The COPD I-II group showed significantly elevated levels of TNF-α (PHA) compared with healthy smokers (Wilcoxon test: p=0.001) and non-smokers (Wilcoxon test: p=0.007) and marginally significant elevated levels compared with COPD III-IV patients (Wilcoxon test: p=0.03). None of the remaining pairwise comparisons was statistically significant. For TNF-α (CD3) no significant differences between groups were observed. For IL-12 (PHA) we observed marginally significant between-group differences (Kruskal-Wallis test: p=0.048). There were higher IL-12 (PHA) levels in the COPD I-II group compared with the other groups. However, only the difference to the COPD III-IV group reached statistical significance (Wilcoxon test: p=0.018). Additionally, the difference between non-smokers and COPD III-IV patients was marginally significant (Wilcoxon test: p=0.048). Concentrations of IL-12 (CD3) showed no significant between group differences.

Patients with severe COPD (GOLD III-IV) show decreased serum levels of IFN-γ

Significant differences of IFN-γ serum levels between groups have been observed (Kruskal–Wallis test: $p=0.002$). COPD III-IV patients showed lower IFN-γ serum levels than healthy smokers (Wilcoxon test: $p<0.001$) and healthy non-smokers (Wilcoxon test: $p=0.002$). Additionally, marginally significantly lower values were observed in the COPD I-II group compared with the healthy smoker group. Note that in 94% of COPD III-IV and in 74% of COPD I-II patients (compared with 40% in healthy controls and 21% in healthy smokers) no serum IFN-γ could be detected. For serum TNF-α, serum IL-10 and serum IL-1β no significant between-group differences were found.

4.4. DISCUSSION

The total number of lymphocytes circulating in the blood and their subset distribution is under strict homeostatic control. We report for the first time that patients with COPD show a profound change in the representation of functionally and phenotypically distinct subsets of CD4+ T-cells. We propose that clonogenic CD4+ T-cells with characterized loss of co-stimulatory CD28 and intracellular storage of the cytolytic proteins granzyme B and perforin might be causal for continuing systemic inflammatory state in COPD patients. The basic mechanisms causing replacement of other CD4+ T-cells by CD4+CD28null clonotypes are incompletely understood. However, phenotypic and functional analyses of CD4+CD28null T-cells have suggested that they are related to NK-cells and represent a population of NK-like T-cells. [179] In support of this hypothesis, we found that CD4+CD28null T-cells express MHC class I-recognizing receptors of the immunoglobulin superfamily (CD94, CD158). [145] [180] Our data corroborate the concept that CD4+CD28null T-cells share multiple features with NK-cells and may combine functional properties of innate and adaptive immunity in COPD patients.

To prove relevant immune functions we separated PBMCs of the study groups and activated them via specific and unspecific T-cell stimulation *in vitro*. We were able to show that systemic white blood cells derived from COPD GOLD I-II secreted augmented levels of IFN-γ and TNF-α – cytokines that are known to increase macrophage and dendritic cell activity – compared with controls and severe COPD (GOLD III-IV). This observation is particularly interesting, as this *in vitro* phenomenon was observed only in patients at the initial stage of COPD progression (GOLD stages I-II), indicating a specific role of NK-like T-cells in triggering initial lung tissue destruction. Our data confirm and corroborate the pathophysiological speculation by *Hodge et al.* and *Di Stefano et al.*, who argued that T-cell activation is leading to enhanced secretion of IFN-γ, a cytokine that activates macrophages and enhances innate immunity, and is thus causing tissue destruction in COPD-susceptible patients. [165] [113] [56] [181] [182] This *in vitro* finding led us to explore whether systemic serum levels of IL-1β, TNF-α, IFN-γ and IL-10 were elevated in COPD patients without recent exacerbation of COPD disease. Contrary to our assumption, the level of inflammatory cytokine IFN-γ correlated negatively with spirometric parameters. This finding underlines the importance of a local interaction of

cell-based immune system and lung tissue interphase in the presence of T-cell-triggering noxious substances (e.g. inhaled smoke). In a final attempt we investigated whether systemic presence of clonogenic CD4+CD28null T-cells is relevant for diagnosing COPD by means of flow cytometry analysis *ex vivo*. We performed a logistic regression analysis and were able to show that presence of systemic CD4+CD28null T-cells was highly predictive for diagnosing COPD. Because of these data we are currently designing a clinical trial to evaluate whether systemic determination of CD4+CD28null by means of flow cytometry analysis is an appropriate tool to identify COPD patients at risk.

Clinical perspective in comparison with other etiologies

Whatever competing mechanism is causative for COPD, the presence of systemic chronic inflammation in COPD has been associated with a variety of co-morbidities including cachexia, osteoporosis, and cardiovascular diseases. [183] [184] The relationship between COPD and cardiovascular diseases is especially germane, as more than half of patients with COPD die of cardiovascular causes. [185] [186] [187] *Nakajima et al.* demonstrated that patients with acute ischemic heart disease are characterized by a perturbation of functional T-cell repertoire (CD4+CD28null) with a bias towards increased IFN-γ production compared with controls. [188] [189] Of particular importance is a study by *Pingiotti et al.* They were able to show that patients with rheumatoid arthritis show increased circulating CD4+CD28null T-cells that are related directly to pre-clinical atherosclerotic changes, such as arterial endothelial dysfunction and carotid artery wall thickening. [190] Our observation of T-cell pool perturbation in COPD might be relevant in explaining the previously observed long-term cardiovascular risk in this disease entity. However, it remains unclear whether the higher percentage of CD4+CD28null T-cells is the result of the inflammatory process, i.e. prematurely senescent CD4+ cells that are unable to go into cell death but still secrete cytokines, or if it represents a subset of COPD subjects whose pathogenic process includes generation of this T-cell subset at an early stage of the disease.

If we interpret our data correctly, a detailed picture is emerging. Chronic antigen exposure, e.g. through contents of tobacco smoke, leads to loss of CD28 and up-regulation of NK-cell receptors expression on T-cells in potentially genetically susceptible patients. This induced

immunological “senescence” is accompanied by a dysregulation of apoptosis-inducing signals, e.g. Bcl-2, fostering longevity of cytotoxic T-cells and increased secretion of IFN-γ and TNF-α upon T-cell triggering. [167] In conclusion, we believe that the appearance of clonogenic T-cells in COPD patients is partially causative for the progressive cell-based inflammatory process in lung tissue irrespective of smoking status.

4.5. ACKNOWLEDGEMENTS

Drs Lambers and Hacker contributed equally to this manuscript. Dr Lambers was responsible for clincial data evaluation. Drs Hacker, Hoetzenecker, Pollreisz and Lichtenauer performed laboratory work. Dr Hacker and Dr Lichtenauer helped to edit the paper. Professor Klepetko provided infrastructure support. Professor Posch was responsible for statistical analysis. This study was supported by FOLAB Chirurgie, private funding (HJ.A.) and the Medical University of Vienna. Dr Hacker was awarded the poster award of the Austrian Society of Pulmonary Medicine (ÖGP) 2008. Dr Ankersmit edited the manuscript and designed and coordinated the study. We are thankful to all participants who supported our investigation voluntarily.

4.6. DISCLOSURES

The Medical University of Vienna claims financial interest.

4.7. TABLES

	Healthy	Healthy Smoker	COPD I-IV	COPD I-II	COPD III-IV
n	15	14	35	19	16
Male/Female	10/5	7/7	20/15	10/9	10/6
Age	57.20 (12.50)	56.64 (9.17)	59.60 (8.01)	60.68 (7.39)	58.31 (8.75)
Lung Function		-			
FVC (L)	4.55 (0.94)	3.84 (0.66)	2.80 (1.08)	3.33 (1.06)	2.14 (0.70)
FEV1 (%)	105.37 (17.11)	94.40 (11.96)	52.76 (23.71)	70.21 (13.33)	30.67 (12.66)
FEV1/VC (%)	76.80 (7.85)	75.95 (3.99)	51.18 (16.83)	61.74 (8.36)	37.80 (15.33)
MEF50 (%)	100.67 (28.92)	87.64 (21.45)	27.29 (18.68)	39.42 (15.93)	11.93 (6.60)
MEF25 (%)	103.53 (33.89)	75.71 (31.33)	29.71 (15.31)	37.37 (16.19)	20.00 (5.94)
Smoking History					
Never-smoker	15	0	0	0	0
Ex-smoker	0	3	7	4	3
Current-smoker	0	11	28	15	13
Pack Years	0	34 (25.2)	45.8 (30.6)	47.3 (29.7)	44.0 (32.6)
Body Weight (kg)	71.6 (13.9)	76.4 (8.6)	80.4 (21.6)	79.7 (16.7)	81.1 (27.2)
Body Height (cm)	172.7 (10.9)	168.7 (8.1)	169.2 (10.5)	167.7 (12.1)	171.2 (7.9)

Table 4 Clinical characteristics. Severity of airflow obstruction was determined using lung function test in all subjects; COPD patients meeting the Global Initiative for Chronic Obstructive Lung Diseases diagnostic criteria for COPD. Data are given as mean (+/- standard deviation) if not otherwise stated.

	Healthy	Healthy Smoker	COPD I-II	COPD III-IV
CD4+CD28null% of CD4+	1.96 (1.07-2.84)	1.5 (0.41-2.59)	3·22 (1.83-4.62)	7.53 (2.67-12.39)
IFN-γ CD3 (pg/ml)	272 (188-356)	240 (178-301)	440 (286-594)	328 (214-442)
IFN-γ PHA (pg/ml)	116 (83-149)	91 (53-129)	375 (135-615)	134 (1-266)
TNF-α CD3 (pg/ml)	922 (368-1476)	731 (333-1128)	1234 (793-1674)	1508 (860-2157)
TNF-α PHA (pg/ml)	1096 (551-1641)	777 (411-1143)	2465 (1532-3398)	1144 (387-1901)
IL-12 CD3 (pg/ml)	93 (46-139)	63 (34-92)	72 (36-108)	42 (13-71)
IL-12 PHA (pg/ml)	44 (8-80)	33 (19-47)	78 (31-125)	17 (8-25)

Table 5 The table shows the percentage of CD4+CD28null cells in the peripheral blood flow. Furthermore, cytokine expression in supernatants of PBMCs stimulated with either anti-CD3 or PHA is described. All data are given as mean (95% confidence interval).

	All patients			**All smokers**			**All COPD**		
	n	**Coeff.**	**p-value**	**n**	**Coeff.**	**p-value**	**n**	**Coeff.**	**p-value**
CD4+CD28null - FEV1%	62	-0.485	<0.001	48	-0.517	<0.001	34	-0.317	0.068
CD4+CD28null - MEF50%	62	-0.404	0.001	48	-0.479	<0.001	34	-0.355	0.040
CD4+CD28null - MEF25%	62	-0.380	0.002	48	-0.403	0.004	34	-0.154	0.384
IFN-γ - FEV1%	62	0.461	<0.001	48	0.613	<0.001	34	0.491	0.003
IFN-γ - MEF50%	62	0.556	<0.001	48	0.645	<0.001	34	0.541	<0.001
IFN-γ - MEF25%	62	0.489	<0.001	48	0.618	<0.001	34	0.492	0.003
TNF-α -FEV1%	62	0.374	0.003	48	0.336	0.019	34	0.226	0.198
TNF-α -MEF50%	62	0.337	0.007	48	0.275	0.058	34	0.123	0.489
TNF-α -MEF25%	62	0.309	0.014	48	0.249	0·087	34	0.039	0.828
IL-1ß - FEV1%	62	0.344	0.006	48	0.287	0.048	34	0.066	0.709
IL-1ß - MEF50%	62	0.282	0.026	48	0.256	0.079	34	0.078	0.663
IL-1ß - MEF25%	62	0.266	0.037	48	0.220	0.133	34	0.002	0.993
IL-10 - FEV1%	62	0.256	0.044	48	0.178	0.226	34	0.096	0.587
IL-10 - MEF50%	62	0.328	0.009	48	0.278	0.055	34	0.329	0.058
IL-10 - MEF25%	62	0.300	0.018	48	0.226	0.122	34	0.214	0.223

Table 6 Correlations of serum cytokine levels with parameters of lung function test.

4.8. FIGURES

Figure 8a

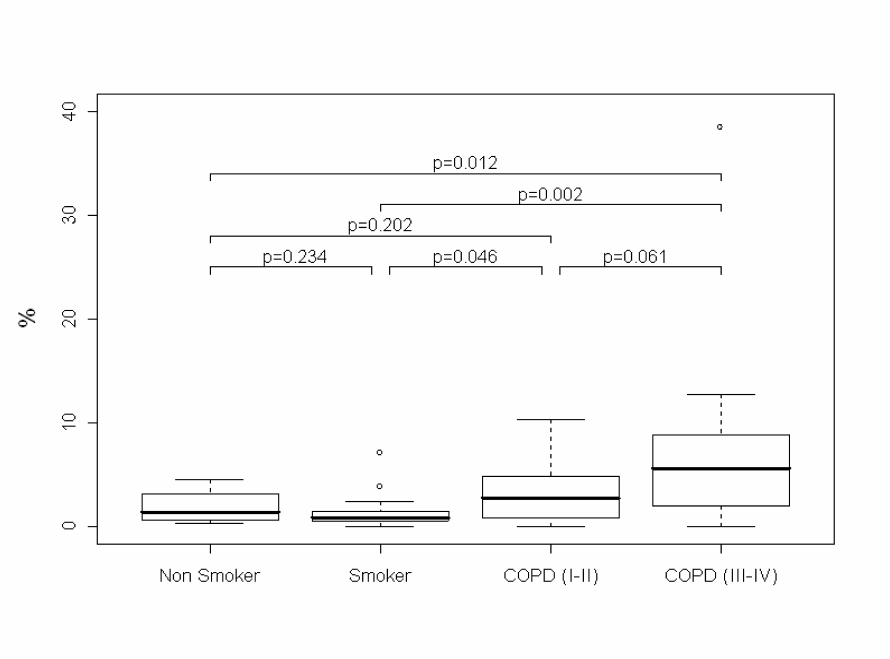

Figure 8b

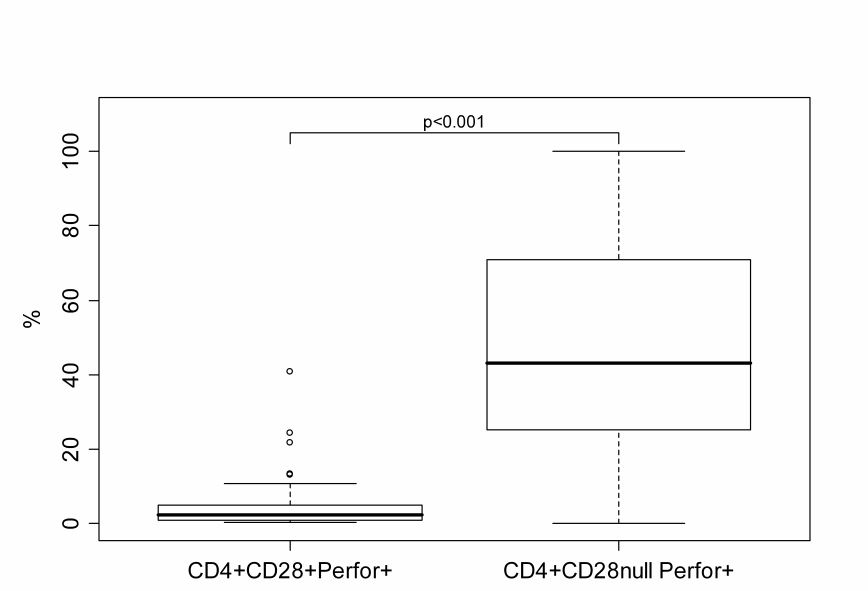

Figure 8c

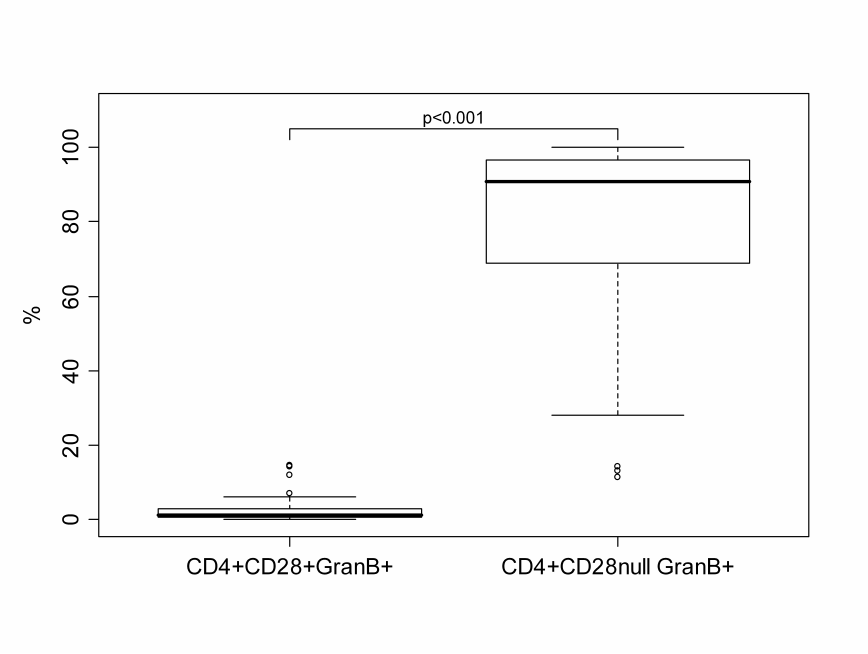

Figure 8d

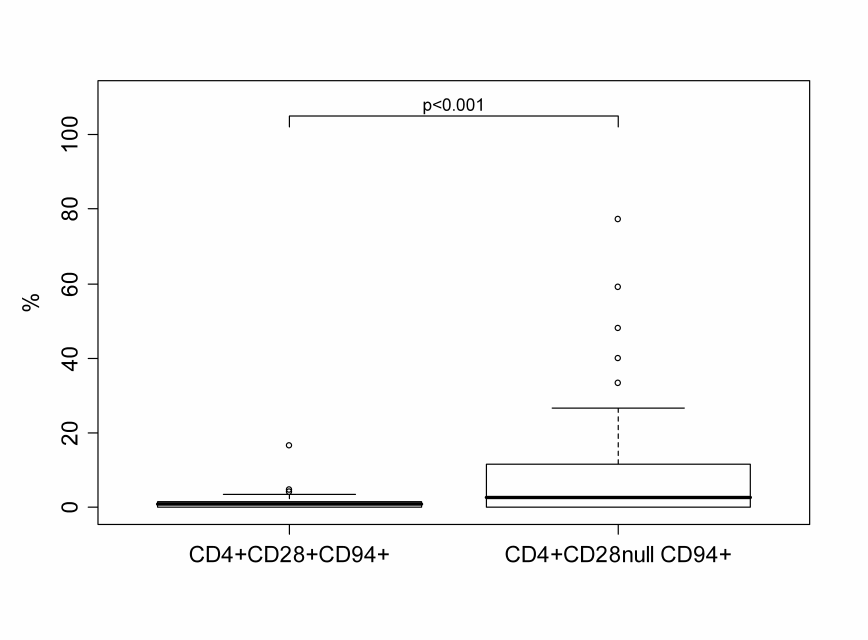

Figure 8e

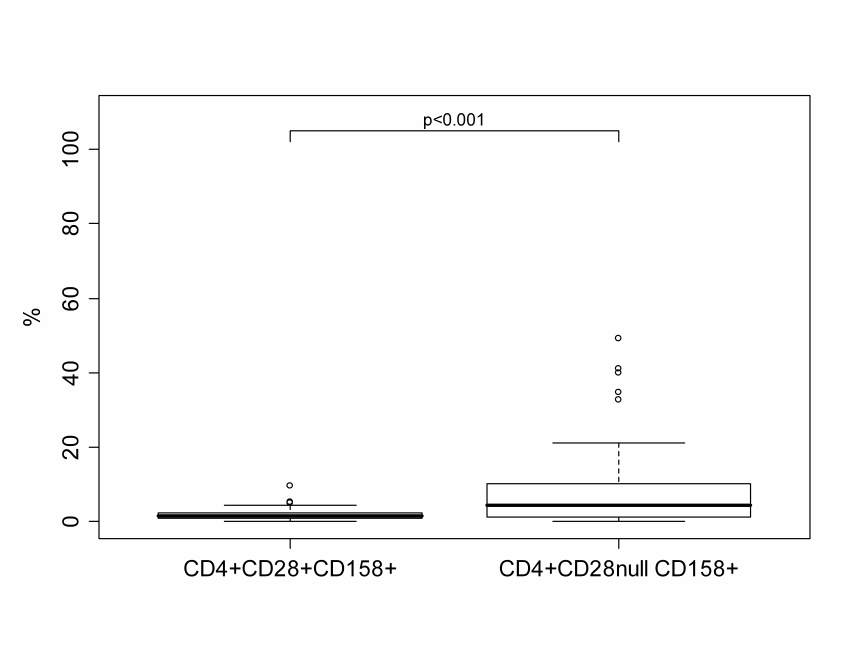

Figure 8 (a) Boxplot showing percentage of CD4+CD28null cells in the peripheral blood flow. (b, c) Subset of CD4+ T-cells lacking co-stimulatory CD28 contained intracellular cytolytic proteins perforin and granzyme B. (d, e) CD4+CD28null cells showed significantly increased surface expression of NK-cell receptors CD94 and CD158. Bars indicate medians; solid boxes show span between 25th and 75th percentiles; whiskers illustrate lowest and highest values. Outliers are marked as open circles.

Figure 9a

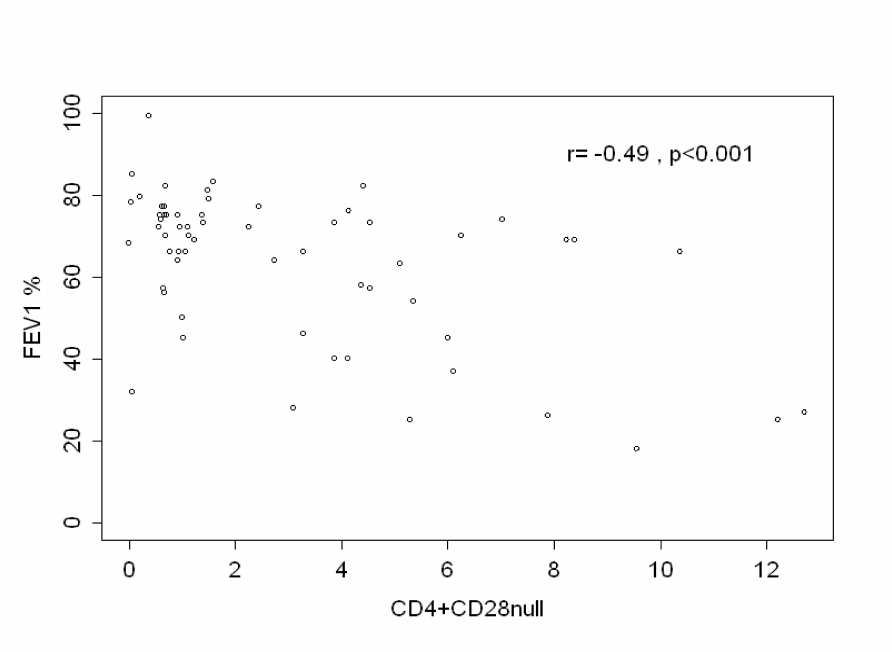

Figure 9b

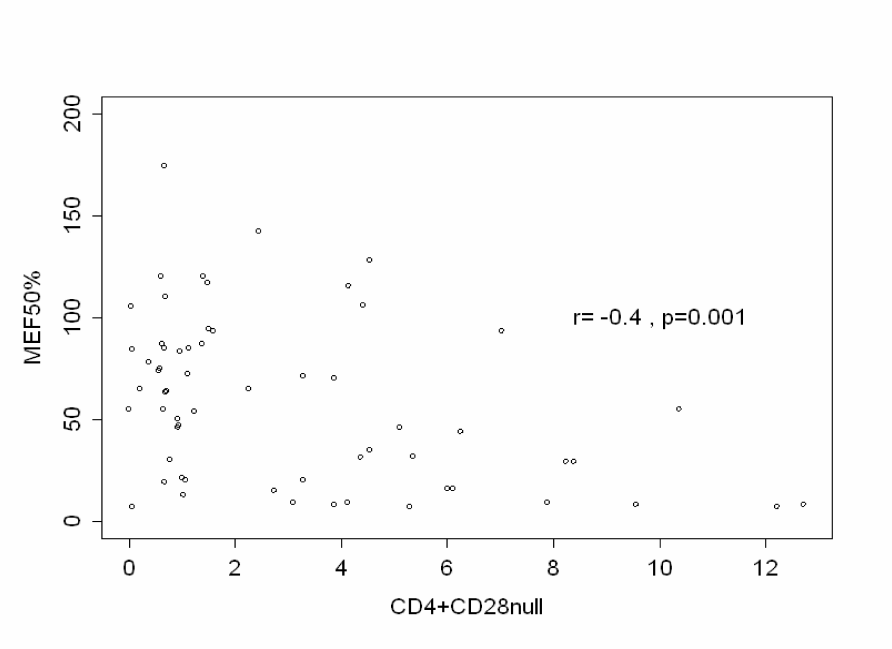

Figure 9c

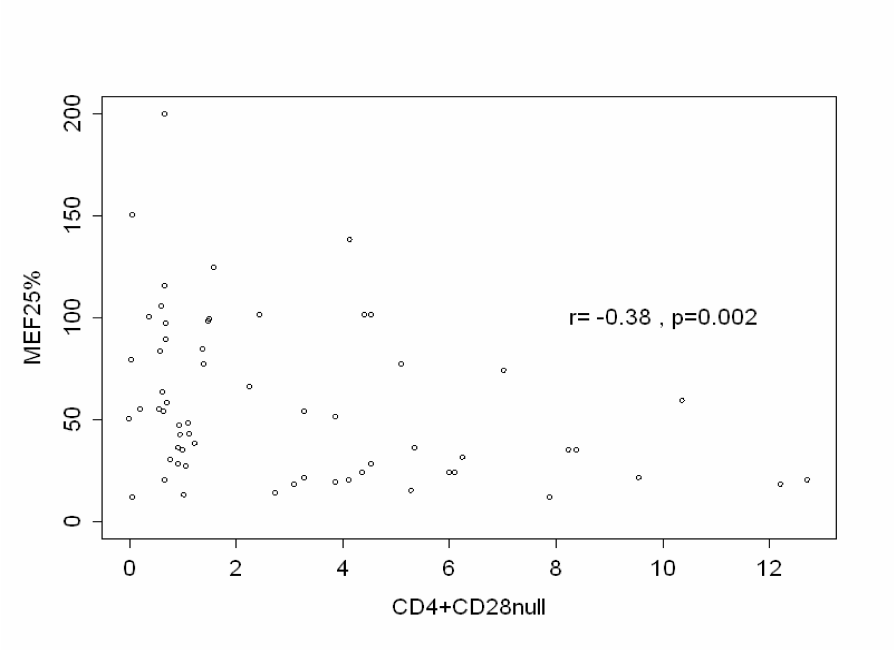

Figure 9d

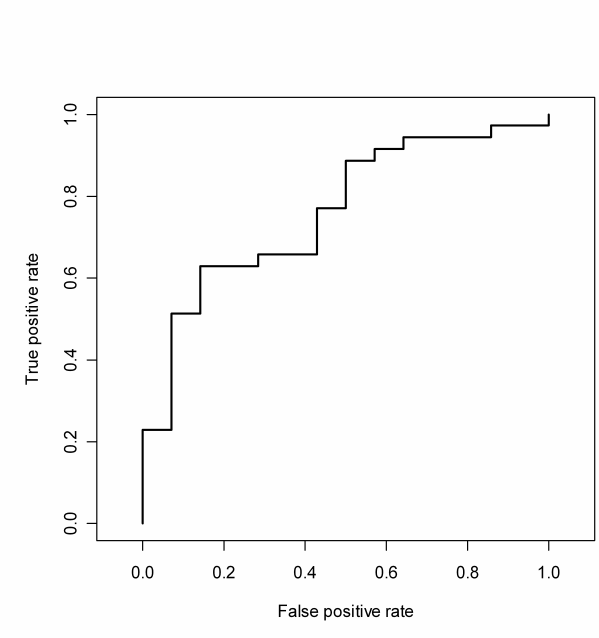

Figure 9 (a, b, c) Scatterplots showing correlations of CD4+CD28null% of CD4+ and forced expiratory volume in 1 second, maximum expiratory flow (MEF50%), and MEF25%, Spearman's correlation coefficients and p-values

are given. (d) Receiver operating characteristic curve for the prediction of chronic obstructive pulmonary disease in the subgroup of smokers based on the CD4+CD28null% measurement.

5. ORIGINAL RESEARCH

Elevated HSP27, HSP70 and HSP90alpha in chronic obstructive pulmonary disease: markers for immune activation and tissue destruction

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5.1. INTRODUCTION

Chronic obstructive pulmonary disease is a significant burden to health care systems worldwide. Despite COPD being one of the leading causes of death and disability, well designed epidemiological studies remain vague leading to the impression of COPD as a rare disease. [1 7] Contrary, prevalence has inclined dramatically over the past years. [158] Although active tobacco smoking is thought to be the predominant risk factor only a fraction of smokers develop the clinical features of COPD. [160 191] Currently, the diagnostic process requires the assessment of lung function parameters. In several recent studies genetic factors were considered to contribute to the susceptibility to develop COPD. [192] Results of these evaluations varied and did not reveal a definite answer. The only accepted genetic cause of emphysema to date is severe AATD. Like the many possible etiologies triggering the development of COPD, the underlying pathogenic pathways remain poorly understood. The disease is characterized by irreversible airflow obstruction – the current diagnostic criterion – due to remodeling and an aberrant inflammatory response. [193] Chronic bronchitis and lung emphysema are pathologic characteristics of COPD and both conditions result from progressive inflammatory destruction of the lung parenchyma. Airflow limitation is slowly progressive, leading to dyspnoea and limitations of physical exercise capacities. [194] However, immune activation is not restricted to the lungs, as patients suffering from COPD are also at higher risk for cardiovascular diseases and autoimmune diseases such as Ulcerative Colitis

and Crohn's Disease. [162] [195] Interestingly, some authors presented data of persisting inflammatory reactions in COPD patients despite cessation of smoking and without other forms of exogenous triggering of an inflammatory response. These findings are suggesting an autoimmune aspect contributing to the disease progression. [196]

Heat shock proteins are a group of highly preserved stress proteins that are ubiquitously expressed in all cells. The main functions of these proteins comprise a process referred to as chaperoning – the conservation of the correct protein structure under stress conditions – as well as the regulation of death pathways. [149] The classification of these stress proteins follows their molecular weight, e.g. the HSP70 has an approximate molecular weight of 70kDa. Under normal physiological conditions HSPs usually account for up to 5% of the total protein content of a cell. Expression of HSPs is upregulated under stressful events like heat, bacterial or viral infections. [197] [147] [198] Newly synthesized HSPs are thought to fold denatured proteins and prevent activation of caspases otherwise leading to active cell death, e.g. apoptosis. Besides known intracellular chaperoning, HSPs may also be released into the extracellular space following massive trauma or stress. [199] [200] This spillage of proteins serves as "danger signal" leading to cytokine transcription and release. [151] Furthermore, extracellular stress proteins are able to induce the adaptive immune system through binding to antigenic peptides. These HSP-peptide complexes are then processed by antigen presenting cells via MHC class I molecules and lead to activation of cytotoxic T-lymphocytes. [201] Extracellular and intravascular HSPs seem to play a key role in the activation of the immune response following stressors like trauma, heat or infection.

The 20S proteasome is a multicatalytic protease complex, localized in the cytosol as well as in the nucleus of all eukaryotic cells. It is crucially involved in the enzymatic degradation of ubiquitinated proteins. The proteasome consists of a cylindrical-shaped core particle, the 20S proteasome, which itself contains two sets of seven different α and ß subunits assembled in four heptameric rings. Spillage of intracellular 20S proteasome was recently shown to occur during degradative processes of cells, e.g. sepsis, trauma, acute liver failure, and on-pump coronary artery bypass grafting. [202] [203] [204]

The aim of the present study was to evaluate in a well-defined study cohort whether the serum levels of various heat shock proteins and 20S proteasome as markers for immune activation and cellular destruction are elevated in patients with COPD. Furthermore, we tested the ability of serum HSPs and 20S proteasome to serve as diagnostic markers for the detection of COPD. The diagnostic use of serum proteins prior to lung function testing may support the diagnostic process and lead to an earlier treatment of patients with COPD.

5.2. METHODS

Patients

A total number of 64 patients and controls were included in this case control study. The study protocol was approved by the ethics committee of the Medical University of Vienna (EK No. 091/2006). All clinical and laboratory tests were performed in accordance with the Declaration of Helsinki and the guidelines for Good Clinical Practice of the Medical University of Vienna. All patients and controls provided written, informed consent before entering the study. Healthy non-smoking volunteers (n=15), smokers without COPD (n=14), patients with mild to moderate COPD (n=19) and patients with severe or very severe COPD (n=16) were evaluated in four study groups. Patient characteristics are depicted in Table 7. Exclusion criteria were acute exacerbation as defined by the guidelines of the WHO and the Global Initiative for Chronic Obstructive Lung Disease or use of immunomodulatory drugs – including steroids – within the past 14 days, history of asthma, autoimmune diseases, other relevant lung diseases (e.g. lung cancer, known α1-antitrypsin deficiency), or any known cardiopulmonary co-morbidity. Height and weight (Seca; Vogel and Halke, Hamburg, Germany) were measured and the body mass index was determined. Pulmonary function parameters (FEV1, FVC, FEV1/FVC ratio) were measured using the same model spirometer (AutoboxV6200, SensorMedics, Vienna, Austria). Measurements were made before and – if criteria for airflow obstruction were met – 15-30 minutes after inhaling of 200µg salbutamol. Arterial blood gases (PaO_2, $PaCO_2$) were obtained at rest while breathing room air in a sitting position. Measurement of arterial blood gases was performed with an ABL 510 gas analyzer (Radiometer, Copenhagen, Denmark). Results are expressed as absolute values and as percentages of predicted values for age, sex and height, according to the European Community for Steel and Coal prediction equations. Predicted normal values were derived from the reference values of the Austrian Society of Pulmonary Medicine. Blood samples were collected at the time of pulmonary evaluation. Serum was acquired after centrifugation and aliquots were kept frozen at -20° Celsius until further testing.

Heat Shock Proteins 27, 60, and 70

Levels of HSP27, HSP60, and HSP70 were determined using adapted ELISA kits for the quantification of intracellular HSP (Duoset IC; R&D Systems, Minneapolis, MN, USA). Ninety-six–well microtiter plates were coated overnight at 4°C with the capture antibody at a concentration of 1μg/ml. After blocking of plates, serum samples and standard protein in different concentrations were added to the wells. After a washing step, a biotin-labeled antibody was added to each well and incubated for 1 hour. Plates were washed and Streptavidin-HRP was added. Color reaction was achieved using TMB (Sigma, St. Louis, MO, USA) and was stopped by an acid stop solution. Optical density was measured at 450nm on an ELISA reader.

Heat Shock Protein 90alpha

Serum levels of HSP90α were measured with a commercially available ready-to-use ELISA kit (Stressgen, Ann Arbor, MI, USA). In brief, serum samples and standards were incubated in 96-well microtiter plates, precoated with anti-human HSP90α antibody. After a washing step, anti-HSP90α:HRP-conjugated antibody was added, and plates were incubated for 24 hours. Plates were washed and TMB substrate was added. Color development was stopped by an acid stop solution, and optical density was determined at 450nm. The amount of protein in each sample was calculated according to a standard curve of optical density values constructed for known levels of HSP90α. The sensitivity of the ELISA was determined to be 50pg/ml; the intra-assay variability was stated to be less than 10% by the manufacturer.

20S Proteasome

Microtiter plates were incubated overnight at 4°C with a monoclonal antibody against the C6-subunit of the 20S proteasome (Biomol, Plymouth Meeting, PA, USA). Plates were washed and blocked for 1 hour with 1% BSA in phosphate-buffered saline. Serum samples and different concentrations of a standard protein (Biomol) were added, then plates were sealed and incubated for 24 hours at 4°C. A rabbit polyclonal antibody to 20S proteasome

α/β subunits (Biomol) – serving as the detection antibody – was added; and after a washing step, plates were incubated with a peroxidase-labeled donkey anti-rabbit IgG (Jackson ImmunoResearch, Soham, United Kingdom) for another 2 hours. TMB served as color substrate. The reaction was stopped by adding 1N sulphuric acid. Plates were read at 450nm using a Wallac Multilabel counter 1420 (PerkinElmer, Boston, MA, USA).

Interleukin-6

Serum levels of IL-6 were determined by a commercially available ELISA kit (BenderMedSystems, Vienna, Austria). Assays were performed according to the manufacturer's instructions. Plates were read at 450nm on an ELISA reader, and IL-6 contents were calculated comparing optical density values of samples with optical density values of known IL-6 concentrations.

C-reactive Protein

Serum levels of C-reactive protein (CRP) were routinely analyzed by the Department of Laboratory Medicine at the Medical University of Vienna.

Statistical Methods

SPSS Software (SPSS Inc., Chicago, IL, USA) was used to calculate all results. A p-value <0.05 was considered statistically significant. Pair-wise comparisons between groups were performed using the Mann-Whitney-U-Test. Correlations were calculated using the Spearman-Correlation-Coefficient. Univariate logistic regression models in a subgroup excluding healthy non-smokers were calculated for HSPs and 20S proteasome. ROC curves were plotted to demonstrate sensitivity and specificity of the evaluated serum proteins. Results were not corrected for multiple testing.

5.3. RESULTS

HSP27

Serum levels of HSP27 were 2042.57pg/ml [1599.58-2485.57] (mean [95% confidence interval]) in healthy controls, 2199.64 [1641.52-2757.75] in healthy smokers, 2862.62 [2280.49-3444.74] in COPD GOLD I-II, and 3717.58 [3079.35-4355.81] in COPD GOLD III-IV. Statistically significant differences were found between healthy controls and COPD I-II ($p=0.025$), healthy controls and COPD III-IV ($p<0.001$), healthy smokers and COPD III-IV ($p=0.001$), and COPD I-II and COPD III-IV ($p=0.026$). Serum levels of HSP27 did not correlate with body weight. Figure 10a.

HSP60

Serum levels of HSP60 were 1836.69pg/ml [153.30-3520.08] in healthy controls, 4378.40 [-3851.48-12608.27] in healthy smokers, 3497.42 [-1561.38-8556.23] in COPD GOLD I-II, and 531.81 [132.57-931.05] in COPD GOLD III-IV. No statistically significant differences were found between the groups. Serum levels of HSP60 showed a weak correlation with body weight ($R=-0.269$; $p= 0.034$). Figure 10b.

HSP70

Serum levels of HSP70 were 140.50pg/ml [67.97-213.04] in healthy controls, 108.50 [43.30-173.69] in healthy smokers, 454.29 [327.05-581.52] in COPD GOLD I-II, and 437.92 [143.41-732.42] in COPD GOLD III-IV.

Statistically significant differences were found between healthy controls and COPD I-II ($p<0.001$), healthy controls and COPD III-IV ($p=0.009$), healthy smokers and COPD I-II ($p<0.001$), and healthy smokers and COPD III-IV ($p<0.001$). Serum levels of HSP70 did not correlate with body weight. Figure 10c.

HSP90alpha

Serum levels of HSP90alpha were 13133.78pg/ml [9791.40-16476.15] in healthy controls, 12827.91 [10838.21-14817.62] in healthy smokers, 17884.50 [13307.14-22461.85] in COPD GOLD I-II, and 17273.02 [12573.96-21972.08] in COPD GOLD III-IV. Statistically significant differences were found between healthy controls and COPD I-II ($p=0.025$), and healthy controls and COPD III-IV ($p=0.049$). Serum levels of HSP90alpha showed a weak correlation with body weight ($R=0.257$; $p=0.044$). Figure 10d.

20S Proteasome

Serum levels of 20S proteasome were 194.78ng/ml [164.94-224.62] in healthy controls, 188.25 [159.26-217.25] in healthy smokers, 172.33 [133.78-210.88] in COPD GOLD I-II, and 187.50 [145.54-229.46] in COPD GOLD III-IV. No statistically significant differences were found between the groups. Serum levels of 20S proteasome did not correlate with body weight. Figure 10e.

Interleukin-6

Serum levels of IL-6 were 5.18pg/ml [0.17-10.19] in healthy controls, 1.65 [-0.11-3.42] in healthy smokers, 7.14 [1.19-13.09] in COPD GOLD I-II, and 2.99 [0.99-5.00] in COPD GOLD III-IV. A statistically significant difference was found between healthy smokers and COPD I-II ($p=0.017$). Serum levels of IL-6 did not correlate with body weight.

C-reactive Protein

Serum levels of CRP were 0.19mg/dl [0.00–0.37] in healthy controls, 0.20 [0.11-0.29] in healthy smokers, 1.10 [0.54-1.66] in COPD GOLD I-II, and 0.71 [0.37-1.05] in COPD GOLD III-IV. Statistically significant differences were found between healthy controls and COPD I-II ($p<0.001$), healthy controls and COPD III-IV ($p=0.001$), healthy smokers and COPD I-II

(p<0.001), and healthy smokers and COPD III-IV (p=0.004). Serum levels of CRP did not correlate with body weight.

Regression Models

In univariate logistic regression models including only healthy smokers and patients with COPD, HSP27 had an AUC in the ROC curve of 0.763 (0.624-0.902; 95% CI; p=0.004), and HSP70 showed an AUC of 0.885 (0.786-0.983: 95% CI; p<0.001). All other variables showed no significant result in the univariate logistic regression analysis. Figure 11.

5.4. DISCUSSION

Patients suffering from chronic obstructive pulmonary disease present progressive inflammation of the bronchial airways, small airways, and lung parenchyma. Lung biopsies revealed massive infiltration of the peribronchial tissue with neutrophils, macrophages, and lymphocytes as part of the innate and adaptive immune system. [193] [109] Activation of these cells is believed to lead to remodeling of the lung tissue. [112] In COPD, both endogenous factors including neutrophils and cytotoxic T-cells as well as exogenous stimuli like tobacco smoke are thought to contribute towards tissue destruction. [99] [205] However, cessation of smoking does not alter impairment of the inflammatory response. The constant induction of inflammatory signals and increased cellular turnover result in upregulation of intracellular heat shock proteins and augmented release into the extracellular environment. We were able to show a significant increase of HSP27 in serum samples taken from the peripheral blood flow of patients suffering from COPD as compared to healthy smokers. HSP27 functions as repair mechanism aiming at the stability and correct posttranslational folding of intracellular proteins as well as the prevention of apoptotic cell death. Elevated serum levels of HSP27 were reported in inflammatory disorders including acute coronary syndrome and chronic allograft nephropathy. [206] [207] Expression of HSP27 is transiently induced as a response to stress events. Termination of the acute triggering results in an immediate downregulation of HSP27 concentrations to normal levels. Thus, HSP27 is only upregulated when its cytoprotective properties are required. [204] Interestingly, our results demonstrate a continuous increase of serum HSP27 concentrations with disease severity. This effect may be due to increased tissue devastation especially in late stages of COPD and spreading of the inflammatory disease to other organ systems resulting in a systemic spillage of HSP27 into the vascular bed. HSP27 generally acts as anti-apoptotic mediator and can be seen as an endogenous immunosuppressive attempt to control excessive inflammation in COPD. [208] Serum contents of HSP27 showed diagnostic potential to determine the occurrence of COPD in a logistic regression model and may serve as marker for diagnosis and prediction of disease severity. Further explorations are needed to determine optimal cut-off values and improve the proposed sensitivity and specificity of serum HSP27 in a clinical setting. The role of extracellular HSP60 has not been well defined. Some authors have described pro-inflammatory features, primarily in atherosclerosis. [149] [209] Our data did not provide any

support for HSP60 being a key element in the pathogenesis of COPD. Serum concentrations of HSP60 did not correlate with levels of other HSPs. However, as we did not include any patients with known coronary artery disease in our study, further investigations are needed to define the role of soluble HSP60 in patients with COPD at increased risk for coronary events.

Serum levels of HSP70 were elevated in patients at early and late stages of COPD. We evidenced a four-fold increase in the GOLD I-II group compared to non-symptomatic smokers. HSP70 is an intracellular chaperone that is released into the extracellular space upon cell death or by means of various secreting pathways. [210] [211] Extracellular HSP70 has been reported to activate cells of the innate and adaptive immune system and to stimulate cytokine production. [151] [211] We found increased concentrations of soluble HSP70 in COPD samples. Values peaked in the COPD I-II group indicating a state of vast immune activation primarily at the early stages of the disease. Furthermore, serum contents of HSP70 showed high sensitivity and specificity to determine the occurrence of COPD in a logistic regression model and could serve as diagnostic marker. Because there was no significant difference between the COPD groups, HSP70 – unlike HSP27 – might not be suitable as marker for disease progression or response to therapy. Soluble HSP90α was significantly upregulated in the peripheral blood flow in the COPD groups as compared to healthy non-smokers. Elevated levels of HSP90α have previously been described in on-pump coronary artery bypass grafting and wound healing after hypoxia. [204] [212] *Rajagopal et al.* characterized HSP90 as central factor in antigen presentation to T-lymphocytes via MHC II. [213] In synopsis with our data, we hypothesize that elevated levels of extracellular HSP90α in COPD are an essential elicitor of the adaptive immune system, triggering a possible autoreactive response to self-antigen. This function of HSP90α may also change the immunogenicity of the associated antigen. Therefore, HSP90α has immunomodulatory effects through cross-presentation of associated peptides in the context of MHC molecules. The exact immunological role of increased serum HSP90α in COPD has to be addressed in further studies. The extracellular content of 20S proteasome was not statistically increased or decreased in the investigated study cohorts. Levels remained under 200ng/ml in all groups and appear to be of subordinate importance in the progression of COPD.

In conclusion, we were able to demonstrate elevated serum concentrations of soluble heat shock proteins 27, 70 and 90α in patients with COPD. This spillage into the vascular bed may be caused by continuous activation of the immune system in the deterioration of COPD through endogenous and exogenous trigger mechanisms. This is the first study to demonstrate elevated serum levels of the described HSPs in patients with COPD at stable stages of the disease. Furthermore, HSP27 and HSP70 showed statistical trends to serve as diagnostic markers or markers for disease progression. Further investigations employing higher numbers of patients are needed to establish diagnostic algorithms using serum levels of HSPs.

5.5. TABLES

	Healthy	Healthy Smoker	COPD I-II	COPD III-IV
n	15	14	19	16
Male/Female	10/5	7/7	10/9	10/6
Age	57.20 (12.50)	56.64 (9.17)	60.68 (7.39)	58.31 (8.75)
Body Weight (kg)	71.6 (13.9)	76.4 (8.6)	79.7 (16.7)	81.1 (27.2)
Body Height (cm)	172.7 (10.9)	168.7 (8.1)	167.7 (12.1)	171.2 (7.9)
Lung Function		-		
FVC (L)	4.55 (0.94)	3.84 (0.66)	3.33 (1.06)	2.14 (0.70)
FEV1 (%)	105.37 (17.11)	94.40 (11.96)	70.21 (13.33)	30.67 (12.66)
FEV1/VC (%)	76.80 (7.85)	75.95 (3.99)	61.74 (8.36)	37.80 (15.33)
MEF50 (%)	100.67 (28.92)	87.64 (21.45)	39.42 (15.93)	11.93 (6.60)
MEF25 (%)	103.53 (33.89)	75.71 (31.33)	37.37 (16.19)	20.00 (5.94)
Smoking History				
Never-smoker	15	0	0	0
Ex-smoker	0	3	4	3
Current-smoker	0	11	15	13
Pack Years	0	34 (25.2)	47.3 (29.7)	44.0 (32.6)

Table 7 Clinical characteristics (severity of airflow obstruction was determined using lung function test in all subjects; COPD patients meeting the GOLD diagnostic criteria for COPD). Data are given as mean (+/- standard deviation) if not otherwise stated.

5.6. FIGURES

Figure 10a

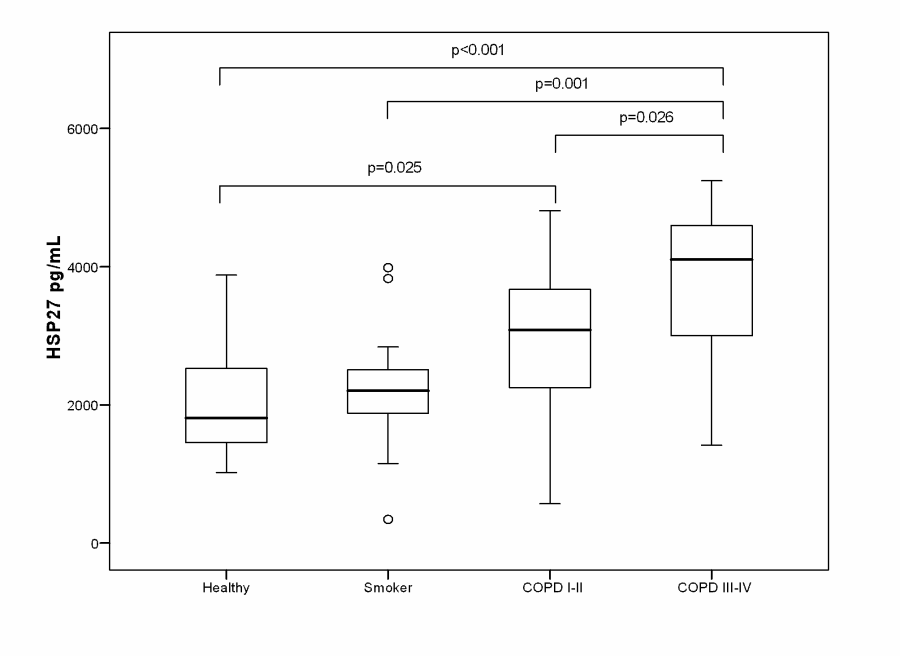

Figure 10b

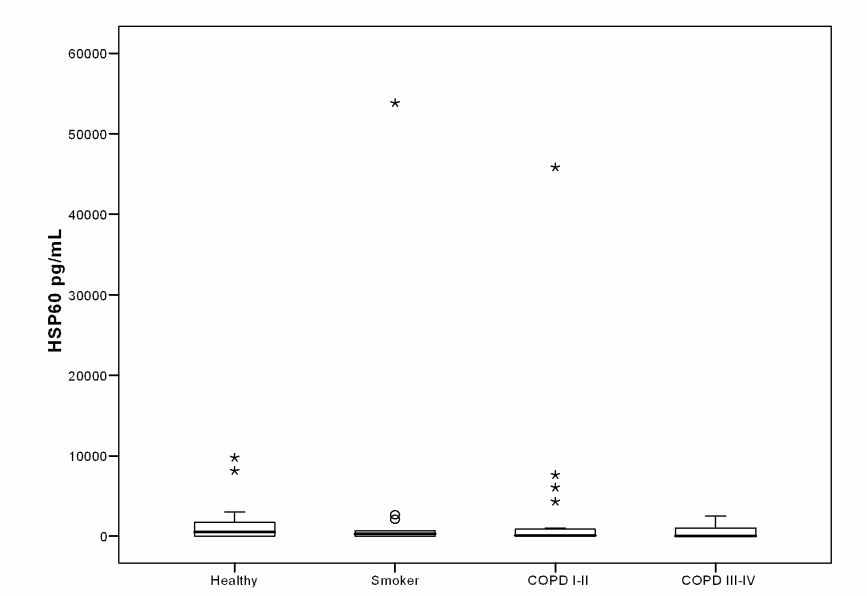

Figure 10c

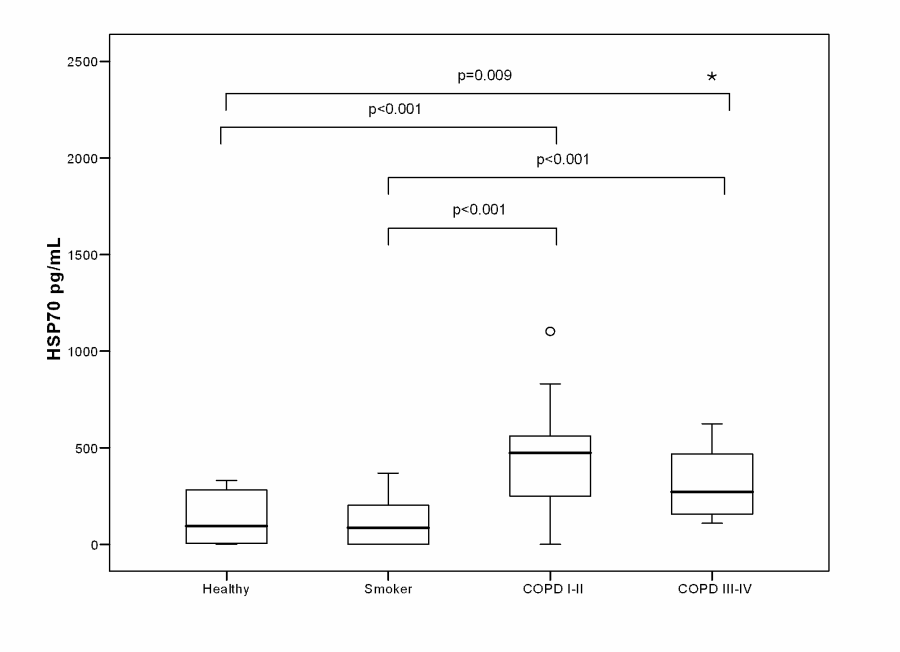

Figure 10d

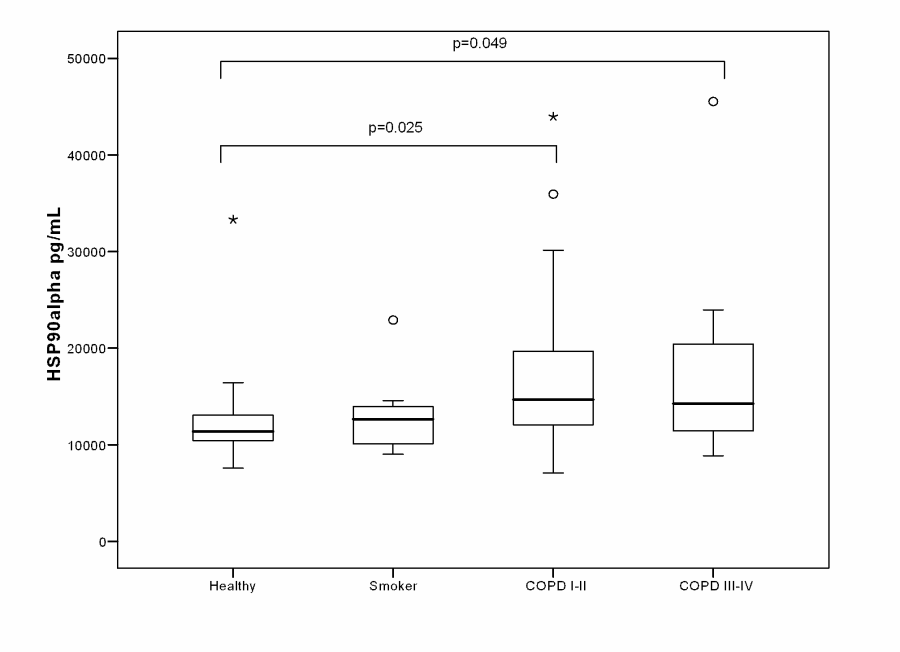

Figure 10e

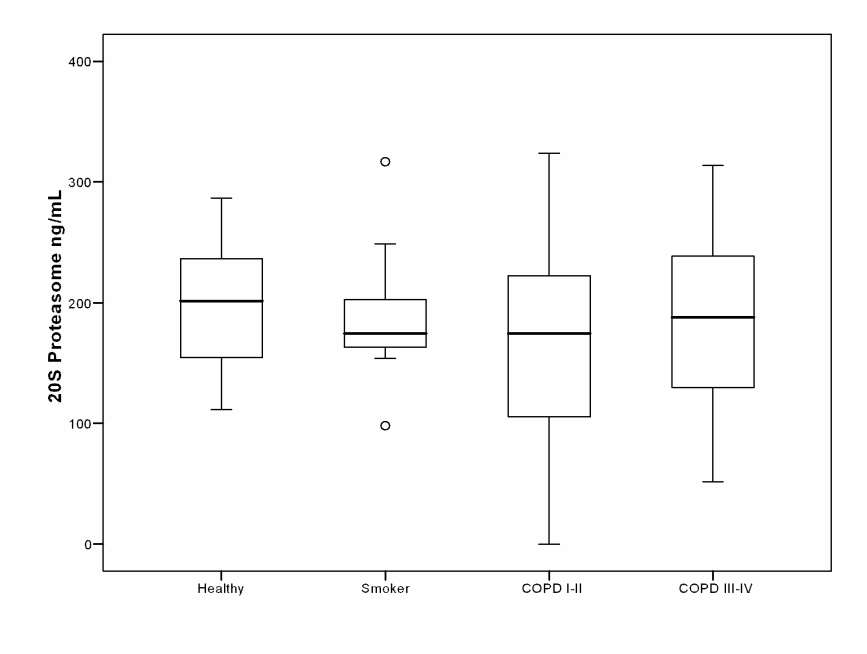

Figure 10 (a, b, c, d, e) Serum levels of heat shock proteins and 20S proteasome were determined in the systemic blood flow of patients and controls. Bars indicate medians; solid boxes show span between 25th and 75th percentile; whiskers illustrate lowest and highest values; outliers are marked. P-values indicate significant differences.

Figure 11

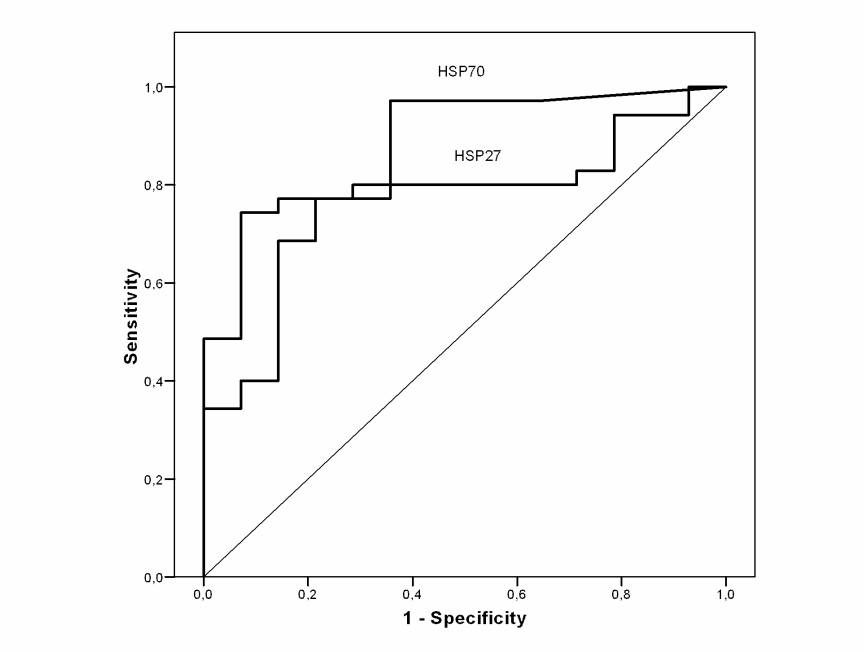

Figure 11 ROC curve indicating sensitivity and specificity of HSP27 and HSP70 to diagnose COPD in the smoking study population.

6. DISCUSSION

The progression of COPD is characterized by excessive inflammation of the lung tissue that may also affect other organ systems. We were able to show increased levels of potentially autoreactive CD4+CD28null cells in the peripheral blood flow of a patient group with COPD due to tobacco smoking. These cells combine features of the innate and adaptive immune system and showed expression of NKR. CD4+CD28null cells have been described in various autoimmune diseases, e.g. rheumatoid arthritis. They are the result of continuous stimulation of the immune system and represent a senescent effector T-cell population that has a limited TCR repertoire. Further investigations are needed to find the target antigens of CD4+CD28null cells in COPD as they are the source of the ongoing immune response. These cells might represent the link between local tissue destruction of the lung and systemic effects seen in COPD. In conclusion, CD4+CD28null cells seem to play a central role in the pathogenesis of COPD – either by their known ability to act as cytotoxic cells or by the production of large amounts of cytokines. Furthermore, quantification of CD4+CD28null cells in the systemic blood can be used in the diagnostic process.

In the second part of the study, we evaluated serum levels of known heat shock proteins. Extracellular HSPs have known effects on the immune response and support the antigen presentation via APCs. Elevated levels of HSP27, HSP70 and HSP90alpha were found in patients with COPD. The exact role of serum HSPs in COPD has to be addressed in further studies. However, the measurement of HSPs has excellent qualities to serve as diagnostic tool in the management of COPD.

7. REFERENCES

1. Lopez AD, Mathers CD, Ezzati M, Jamison DT, Murray CJ. Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. *Lancet* 2006;**367**(9524)**:**1747-57.
2. Murray CJ, Lopez AD. Alternative projections of mortality and disability by cause 1990-2020: Global Burden of Disease Study. *Lancet* 1997;**349**(9064)**:**1498-504.
3. Jemal A, Ward E, Hao Y, Thun M. Trends in the leading causes of death in the United States, 1970-2002. *Jama* 2005;**294**(10)**:**1255-9.
4. Mannino DM, Buist AS. Global burden of COPD: risk factors, prevalence, and future trends. *Lancet* 2007;**370**(9589)**:**765-73.
5. Mannino DM. Epidemiology and global impact of chronic obstructive pulmonary disease. *Semin Respir Crit Care Med* 2005;**26**(2)**:**204-10.
6. Chapman KR, Mannino DM, Soriano JB, et al. Epidemiology and costs of chronic obstructive pulmonary disease. *Eur Respir J* 2006;**27**(1)**:**188-207.
7. Halbert RJ, Isonaka S, George D, Iqbal A. Interpreting COPD prevalence estimates: what is the true burden of disease? *Chest* 2003;**123**(5)**:**1684-92.
8. Schirnhofer L, Lamprecht B, Vollmer WM, et al. COPD prevalence in Salzburg, Austria: results from the Burden of Obstructive Lung Disease (BOLD) Study. *Chest* 2007;**131**(1)**:**29-36.
9. Hilleman DE, Dewan N, Malesker M, Friedman M. Pharmacoeconomic evaluation of COPD. *Chest* 2000;**118**(5)**:**1278-85.
10. Sullivan SD, Ramsey SD, Lee TA. The economic burden of COPD. *Chest* 2000;**117**(2 Suppl)**:**5S-9S.
11. Viegi G, Scognamiglio A, Baldacci S, Pistelli F, Carrozzi L. Epidemiology of chronic obstructive pulmonary disease (COPD). *Respiration* 2001;**68**(1)**:**4-19.
12. Lindberg A, Jonsson AC, Ronmark E, Lundgren R, Larsson LG, Lundback B. Ten-year cumulative incidence of COPD and risk factors for incident disease in a symptomatic cohort. *Chest* 2005;**127**(5)**:**1544-52.

13. Bakke PS, Baste V, Hanoa R, Gulsvik A. Prevalence of obstructive lung disease in a general population: relation to occupational title and exposure to some airborne agents. *Thorax* 1991;**46**(12)**:**863-70.
14. Fletcher C, Peto R. The natural history of chronic airflow obstruction. *Br Med J* 1977;**1**(6077)**:**1645-8.
15. Doll R, Peto R, Wheatley K, Gray R, Sutherland I. Mortality in relation to smoking: 40 years' observations on male British doctors. *Bmj* 1994;**309**(6959)**:**901-11.
16. Doll R, Peto R. Mortality in relation to smoking: 20 years' observations on male British doctors. *Br Med J* 1976;**2**(6051)**:**1525-36.
17. Doll R, Gray R, Hafner B, Peto R. Mortality in relation to smoking: 22 years' observations on female British doctors. *Br Med J* 1980;**280**(6219)**:**967-71.
18. Anthonisen NR, Connett JE, Murray RP. Smoking and lung function of Lung Health Study participants after 11 years. *Am J Respir Crit Care Med* 2002;**166**(5)**:**675-9.
19. Lundback B, Lindberg A, Lindstrom M, et al. Not 15 but 50% of smokers develop COPD?--Report from the Obstructive Lung Disease in Northern Sweden Studies. *Respir Med* 2003;**97**(2)**:**115-22.
20. Arinir U, Hoffjan S, Knoop H, Schultze-Werninghaus G, Epplen JT, Rohde G. [The genetics of chronic obstructive pulmonary disease]. *Pneumologie* 2009;**63**(1)**:**41-8.
21. Sandford AJ, Silverman EK. Chronic obstructive pulmonary disease. 1: Susceptibility factors for COPD the genotype-environment interaction. *Thorax* 2002;**57**(8)**:**736-41.
22. Dennis RJ, Maldonado D, Norman S, Baena E, Martinez G. Woodsmoke exposure and risk for obstructive airways disease among women. *Chest* 1996;**109**(1)**:**115-9.
23. Perez-Padilla R, Regalado J, Vedal S, et al. Exposure to biomass smoke and chronic airway disease in Mexican women. A case-control study. *Am J Respir Crit Care Med* 1996;**154**(3 Pt 1)**:**701-6.
24. Yin P, Jiang CQ, Cheng KK, et al. Passive smoking exposure and risk of COPD among adults in China: the Guangzhou Biobank Cohort Study. *Lancet* 2007;**370**(9589)**:**751-7.
25. Osman LM, Douglas JG, Garden C, et al. Indoor air quality in homes of patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2007;**176**(5)**:**465-72.

26. Prescott E, Lange P, Vestbo J. Socioeconomic status, lung function and admission to hospital for COPD: results from the Copenhagen City Heart Study. *Eur Respir J* 1999;**13**(5)**:**1109-14.
27. Menezes AM, Victora CG, Rigatto M. Prevalence and risk factors for chronic bronchitis in Pelotas, RS, Brazil: a population-based study. *Thorax* 1994;**49**(12)**:**1217-21.
28. Lebowitz MD. Multivariate analysis of smoking and other risk factors for obstructive lung diseases and related symptoms. *J Chronic Dis* 1982;**35**(9)**:**751-8.
29. Burrows B, Knudson RJ, Lebowitz MD. The relationship of childhood respiratory illness to adult obstructive airway disease. *Am Rev Respir Dis* 1977;**115**(5)**:**751-60.
30. Gold DR, Tager IB, Weiss ST, Tosteson TD, Speizer FE. Acute lower respiratory illness in childhood as a predictor of lung function and chronic respiratory symptoms. *Am Rev Respir Dis* 1989;**140**(4)**:**877-84.
31. Barker DJ, Godfrey KM, Fall C, Osmond C, Winter PD, Shaheen SO. Relation of birth weight and childhood respiratory infection to adult lung function and death from chronic obstructive airways disease. *Bmj* 1991;**303**(6804)**:**671-5.
32. Hayashi S. Latent adenovirus infection in COPD. *Chest* 2002;**121**(5 Suppl)**:**183S-187S.
33. Ogawa E, Elliott WM, Hughes F, Eichholtz TJ, Hogg JC, Hayashi S. Latent adenoviral infection induces production of growth factors relevant to airway remodeling in COPD. *Am J Physiol Lung Cell Mol Physiol* 2004;**286**(1)**:**L189-97.
34. McManus TE, Marley AM, Baxter N, et al. High levels of Epstein-Barr virus in COPD. *Eur Respir J* 2008;**31**(6)**:**1221-6.
35. Rohde G, Wiethege A, Borg I, et al. Respiratory viruses in exacerbations of chronic obstructive pulmonary disease requiring hospitalisation: a case-control study. *Thorax* 2003;**58**(1)**:**37-42.
36. McManus TE, Marley AM, Baxter N, et al. Respiratory viral infection in exacerbations of COPD. *Respir Med* 2008;**102**(11)**:**1575-80.
37. Voter KZ, Henry MM, Stewart PW, Henderson FW. Lower respiratory illness in early childhood and lung function and bronchial reactivity in adolescent males. *Am Rev Respir Dis* 1988;**137**(2)**:**302-7.
38. Sethi S. Bacterial infection and the pathogenesis of COPD. *Chest* 2000;**117**(5 Suppl 1)**:**286S-91S.

39. Shaheen SO, Barker DJ, Shiell AW, Crocker FJ, Wield GA, Holgate ST. The relationship between pneumonia in early childhood and impaired lung function in late adult life. *Am J Respir Crit Care Med* 1994;**149**(3 Pt 1)**:**616-9.

40. Shaheen SO, Sterne JA, Tucker JS, Florey CD. Birth weight, childhood lower respiratory tract infection, and adult lung function. *Thorax* 1998;**53**(7)**:**549-53.

41. Wedzicha JA. Airway infection accelerates decline of lung function in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2001;**164**(10 Pt 1)**:**1757-8.

42. Hill AT, Campbell EJ, Hill SL, Bayley DL, Stockley RA. Association between airway bacterial load and markers of airway inflammation in patients with stable chronic bronchitis. *Am J Med* 2000;**109**(4)**:**288-95.

43. Wedzicha JA, Donaldson GC. Exacerbations of chronic obstructive pulmonary disease. *Respir Care* 2003;**48**(12)**:**1204-13; discussion 1213-5.

44. Wedzicha JA, Seemungal TA. COPD exacerbations: defining their cause and prevention. *Lancet* 2007;**370**(9589)**:**786-96.

45. Wilkinson TM, Hurst JR, Perera WR, Wilks M, Donaldson GC, Wedzicha JA. Effect of interactions between lower airway bacterial and rhinoviral infection in exacerbations of COPD. *Chest* 2006;**129**(2)**:**317-24.

46. Teramoto S. 1. COPD pathogenesis from the viewpoint of risk factors. *Intern Med* 2007;**46**(2)**:**77-9.

47. Silverman EK. Genetic Epidemiology of COPD. *Chest* 2002;**121**(3 Suppl)**:**1S-6S.

48. Eriksson S. Pulmonary Emphysema and Alpha1-Antitrypsin Deficiency. *Acta Med Scand* 1964;**175:**197-205.

49. Needham M, Stockley RA. Alpha 1-antitrypsin deficiency. 3: Clinical manifestations and natural history. *Thorax* 2004;**59**(5)**:**441-5.

50. Hersh CP, Dahl M, Ly NP, Berkey CS, Nordestgaard BG, Silverman EK. Chronic obstructive pulmonary disease in alpha1-antitrypsin PI MZ heterozygotes: a meta-analysis. *Thorax* 2004;**59**(10)**:**843-9.

51. Blanco I, Bustillo EF, Rodriguez MC. Distribution of alpha1-antitrypsin PI S and PI Z frequencies in countries outside Europe: a meta-analysis. *Clin Genet* 2001;**60**(6)**:**431-41.

52. Lomas DA, Silverman EK. The genetics of chronic obstructive pulmonary disease. *Respir Res* 2001;**2**(1)**:**20-6.

53. Joos L, Pare PD, Sandford AJ. Genetic risk factors of chronic obstructive pulmonary disease. *Swiss Med Wkly* 2002;**132**(3-4)**:**27-37.

54. Rabe KF, Hurd S, Anzueto A, et al. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *Am J Respir Crit Care Med* 2007;**176**(6)**:**532-55.

55. Chung KF, Adcock IM. Multifaceted mechanisms in COPD: inflammation, immunity, and tissue repair and destruction. *Eur Respir J* 2008;**31**(6)**:**1334-56.

56. Di Stefano A, Capelli A, Lusuardi M, et al. Severity of airflow limitation is associated with severity of airway inflammation in smokers. *Am J Respir Crit Care Med* 1998;**158**(4)**:**1277-85.

57. Niewoehner DE, Kleinerman J, Rice DB. Pathologic changes in the peripheral airways of young cigarette smokers. *N Engl J Med* 1974;**291**(15)**:**755-8.

58. Spurzem JR, Rennard SI. Pathogenesis of COPD. *Semin Respir Crit Care Med* 2005;**26**(2)**:**142-53.

59. Skold CM, Hed J, Eklund A. Smoking cessation rapidly reduces cell recovery in bronchoalveolar lavage fluid, while alveolar macrophage fluorescence remains high. *Chest* 1992;**101**(4)**:**989-95.

60. Rutgers SR, Postma DS, ten Hacken NH, et al. Ongoing airway inflammation in patients with COPD who do not currently smoke. *Thorax* 2000;**55**(1)**:**12-8.

61. Hogg JC, Macklem PT, Thurlbeck WM. Site and nature of airway obstruction in chronic obstructive lung disease. *N Engl J Med* 1968;**278**(25)**:**1355-60.

62. Yanai M, Sekizawa K, Ohrui T, Sasaki H, Takishima T. Site of airway obstruction in pulmonary disease: direct measurement of intrabronchial pressure. *J Appl Physiol* 1992;**72**(3)**:**1016-23.

63. Hogg JC. State of the art. Bronchiolitis in chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2006;**3**(6)**:**489-93.

64. Knowles MR, Boucher RC. Mucus clearance as a primary innate defense mechanism for mammalian airways. *J Clin Invest* 2002;**109**(5)**:**571-7.

65. Martin TR, Frevert CW. Innate immunity in the lungs. *Proc Am Thorac Soc* 2005;**2**(5)**:**403-11.

66. Sarir H, Henricks PA, van Houwelingen AH, Nijkamp FP, Folkerts G. Cells, mediators and Toll-like receptors in COPD. *Eur J Pharmacol* 2008;**585**(2-3)**:**346-53.

67. Finkelstein R, Fraser RS, Ghezzo H, Cosio MG. Alveolar inflammation and its relation to emphysema in smokers. *Am J Respir Crit Care Med* 1995;**152**(5 Pt 1)**:**1666-72.

68. Tomita K, Caramori G, Lim S, et al. Increased p21(CIP1/WAF1) and B cell lymphoma leukemia-x(L) expression and reduced apoptosis in alveolar macrophages from smokers. *Am J Respir Crit Care Med* 2002;**166**(5)**:**724-31.

69. Tetley TD. Macrophages and the pathogenesis of COPD. *Chest* 2002;**121**(5 Suppl)**:**156S-159S.

70. Hautamaki RD, Kobayashi DK, Senior RM, Shapiro SD. Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. *Science* 1997;**277**(5334)**:**2002-4.

71. Demedts IK, Morel-Montero A, Lebecque S, et al. Elevated MMP-12 protein levels in induced sputum from patients with COPD. *Thorax* 2006;**61**(3)**:**196-201.

72. Finlay GA, O'Driscoll LR, Russell KJ, et al. Matrix metalloproteinase expression and production by alveolar macrophages in emphysema. *Am J Respir Crit Care Med* 1997;**156**(1)**:**240-7.

73. Russell RE, Culpitt SV, DeMatos C, et al. Release and activity of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 by alveolar macrophages from patients with chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol* 2002;**26**(5)**:**602-9.

74. Hubbard RC, Ogushi F, Fells GA, et al. Oxidants spontaneously released by alveolar macrophages of cigarette smokers can inactivate the active site of alpha 1-antitrypsin, rendering it ineffective as an inhibitor of neutrophil elastase. *J Clin Invest* 1987;**80**(5)**:**1289-95.

75. Lim S, Roche N, Oliver BG, Mattos W, Barnes PJ, Chung KF. Balance of matrix metalloprotease-9 and tissue inhibitor of metalloprotease-1 from alveolar macrophages in cigarette smokers. Regulation by interleukin-10. *Am J Respir Crit Care Med* 2000;**162**(4 Pt 1)**:**1355-60.

76. Baraldo S, Turato G, Badin C, et al. Neutrophilic infiltration within the airway smooth muscle in patients with COPD. *Thorax* 2004;**59**(4)**:**308-12.

77. Saetta M, Turato G, Facchini FM, et al. Inflammatory cells in the bronchial glands of smokers with chronic bronchitis. *Am J Respir Crit Care Med* 1997;**156**(5)**:**1633-9.

78. Thompson AB, Daughton D, Robbins RA, Ghafouri MA, Oehlerking M, Rennard SI. Intraluminal airway inflammation in chronic bronchitis. Characterization and correlation with clinical parameters. *Am Rev Respir Dis* 1989;**140**(6)**:**1527-37.

79. O'Donnell RA, Peebles C, Ward JA, et al. Relationship between peripheral airway dysfunction, airway obstruction, and neutrophilic inflammation in COPD. *Thorax* 2004;**59**(10)**:**837-42.

80. Stanescu D, Sanna A, Veriter C, et al. Airways obstruction, chronic expectoration, and rapid decline of FEV1 in smokers are associated with increased levels of sputum neutrophils. *Thorax* 1996;**51**(3)**:**267-71.

81. Henricks PA, Nijkamp FP. Reactive oxygen species as mediators in asthma. *Pulm Pharmacol Ther* 2001;**14**(6)**:**409-20.

82. Skold CM, Liu X, Umino T, et al. Human neutrophil elastase augments fibroblast-mediated contraction of released collagen gels. *Am J Respir Crit Care Med* 1999;**159**(4 Pt 1)**:**1138-46.

83. Kohri K, Ueki IF, Nadel JA. Neutrophil elastase induces mucin production by ligand-dependent epidermal growth factor receptor activation. *Am J Physiol Lung Cell Mol Physiol* 2002;**283**(3)**:**L531-40.

84. Balbi B. COPD: is chemotaxis the key? *Chest* 2003;**123**(4)**:**983-6.

85. Mio T, Romberger DJ, Thompson AB, Robbins RA, Heires A, Rennard SI. Cigarette smoke induces interleukin-8 release from human bronchial epithelial cells. *Am J Respir Crit Care Med* 1997;**155**(5)**:**1770-6.

86. Sato E, Koyama S, Takamizawa A, et al. Smoke extract stimulates lung fibroblasts to release neutrophil and monocyte chemotactic activities. *Am J Physiol* 1999;**277**(6 Pt 1)**:**L1149-57.

87. Pesci A, Balbi B, Majori M, et al. Inflammatory cells and mediators in bronchial lavage of patients with chronic obstructive pulmonary disease. *Eur Respir J* 1998;**12**(2)**:**380-6.

88. Beeh KM, Beier J, Kornmann O, Mander A, Buhl R. Long-term repeatability of induced sputum cells and inflammatory markers in stable, moderately severe COPD. *Chest* 2003;**123**(3)**:**778-83.

89. Seggev JS, Thornton WH, Jr., Edes TE. Serum leukotriene B4 levels in patients with obstructive pulmonary disease. *Chest* 1991;**99**(2)**:**289-91.

90. Beeh KM, Kornmann O, Buhl R, Culpitt SV, Giembycz MA, Barnes PJ. Neutrophil chemotactic activity of sputum from patients with COPD: role of interleukin 8 and leukotriene B4. *Chest* 2003;**123**(4)**:**1240-7.

91. Traves SL, Culpitt SV, Russell RE, Barnes PJ, Donnelly LE. Increased levels of the chemokines GROalpha and MCP-1 in sputum samples from patients with COPD. *Thorax* 2002;**57**(7)**:**590-5.

92. Mukaida N. Pathophysiological roles of interleukin-8/CXCL8 in pulmonary diseases. *Am J Physiol Lung Cell Mol Physiol* 2003;**284**(4)**:**L566-77.

93. Laffon M, Pittet JF, Modelska K, Matthay MA, Young DM. Interleukin-8 mediates injury from smoke inhalation to both the lung endothelial and the alveolar epithelial barriers in rabbits. *Am J Respir Crit Care Med* 1999;**160**(5 Pt 1)**:**1443-9.

94. Mukaida N. Interleukin-8: an expanding universe beyond neutrophil chemotaxis and activation. *Int J Hematol* 2000;**72**(4)**:**391-8.

95. Alcorn MJ, Booth JL, Coggeshall KM, Metcalf JP. Adenovirus type 7 induces interleukin-8 production via activation of extracellular regulated kinase 1/2. *J Virol* 2001;**75**(14)**:**6450-9.

96. Culpitt SV, Rogers DF, Shah P, et al. Impaired inhibition by dexamethasone of cytokine release by alveolar macrophages from patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2003;**167**(1)**:**24-31.

97. Grumelli S, Corry DB, Song LZ, et al. An immune basis for lung parenchymal destruction in chronic obstructive pulmonary disease and emphysema. *PLoS Med* 2004;**1**(1)**:**e8.

98. O'Shaughnessy TC, Ansari TW, Barnes NC, Jeffery PK. Inflammation in bronchial biopsies of subjects with chronic bronchitis: inverse relationship of CD8+ T lymphocytes with FEV1. *Am J Respir Crit Care Med* 1997;**155**(3)**:**852-7.

99. Majo J, Ghezzo H, Cosio MG. Lymphocyte population and apoptosis in the lungs of smokers and their relation to emphysema. *Eur Respir J* 2001;**17**(5)**:**946-53.

100. Saetta M, Baraldo S, Corbino L, et al. CD8+ve cells in the lungs of smokers with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1999;**160**(2)**:**711-7.

101. Saetta M, Di Stefano A, Turato G, et al. CD8+ T-lymphocytes in peripheral airways of smokers with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1998;**157**(3 Pt 1)**:**822-6.

102. Lams BE, Sousa AR, Rees PJ, Lee TH. Subepithelial immunopathology of the large airways in smokers with and without chronic obstructive pulmonary disease. *Eur Respir J* 2000;**15**(3)**:**512-6.

103. Lams BE, Sousa AR, Rees PJ, Lee TH. Immunopathology of the small-airway submucosa in smokers with and without chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1998;**158**(5 Pt 1)**:**1518-23.

104. Chrysofakis G, Tzanakis N, Kyriakoy D, et al. Perforin expression and cytotoxic activity of sputum CD8+ lymphocytes in patients with COPD. *Chest* 2004;**125**(1)**:**71-6.

105. Diaz PT, King MA, Pacht ER, et al. Increased susceptibility to pulmonary emphysema among HIV-seropositive smokers. *Ann Intern Med* 2000;**132**(5)**:**369-72.

106. Turato G, Zuin R, Saetta M. Pathogenesis and pathology of COPD. *Respiration* 2001;**68**(2)**:**117-28.

107. Maeno T, Houghton AM, Quintero PA, Grumelli S, Owen CA, Shapiro SD. CD8+ T Cells are required for inflammation and destruction in cigarette smoke-induced emphysema in mice. *J Immunol* 2007;**178**(12)**:**8090-6.

108. Lofdahl MJ, Roos-Engstrand E, Pourazar J, et al. Increased intraepithelial T-cells in stable COPD. *Respir Med* 2008;**102**(12)**:**1812-8.

109. Hogg JC, Chu F, Utokaparch S, et al. The nature of small-airway obstruction in chronic obstructive pulmonary disease. *N Engl J Med* 2004;**350**(26)**:**2645-53.

110. Aoshiba K, Koinuma M, Yokohori N, Nagai A. Differences in the distribution of CD4+ and CD8+ T cells in emphysematous lungs. *Respiration* 2004;**71**(2)**:**184-90.

111. Sullivan AK, Simonian PL, Falta MT, et al. Oligoclonal CD4+ T cells in the lungs of patients with severe emphysema. *Am J Respir Crit Care Med* 2005;**172**(5)**:**590-6.

112. Mikko M, Fredriksson K, Wahlstrom J, Eriksson P, Grunewald J, Skold CM. Human T cells stimulate fibroblast-mediated degradation of extracellular matrix in vitro. *Clin Exp Immunol* 2008;**151**(2)**:**317-25.

113. Di Stefano A, Caramori G, Capelli A, et al. STAT4 activation in smokers and patients with chronic obstructive pulmonary disease. *Eur Respir J* 2004;**24**(1)**:**78-85.

114. Agusti A, MacNee W, Donaldson K, Cosio M. Hypothesis: does COPD have an autoimmune component? *Thorax* 2003;**58**(10)**:**832-4.

115. Brewerton DA, Hart FD, Nicholls A, Caffrey M, James DC, Sturrock RD. Ankylosing spondylitis and HL-A 27. *Lancet* 1973;**1**(7809)**:**904-7.

116. Silman AJ, Pearson JE. Epidemiology and genetics of rheumatoid arthritis. *Arthritis Res* 2002;**4 Suppl 3:**S265-72.

117. Criswell LA. The genetic contribution to systemic lupus erythematosus. *Bull NYU Hosp Jt Dis* 2008;**66**(3)**:**176-83.

118. Babusyte A, Stravinskaite K, Jeroch J, Lotvall J, Sakalauskas R, Sitkauskiene B. Patterns of airway inflammation and MMP-12 expression in smokers and ex-smokers with COPD. *Respir Res* 2007;**8:**81.

119. Gamble E, Grootendorst DC, Hattotuwa K, et al. Airway mucosal inflammation in COPD is similar in smokers and ex-smokers: a pooled analysis. *Eur Respir J* 2007;**30**(3)**:**467-71.

120. Motz GT, Eppert BL, Sun G, et al. Persistence of lung CD8 T cell oligoclonal expansions upon smoking cessation in a mouse model of cigarette smoke-induced emphysema. *J Immunol* 2008;**181**(11)**:**8036-43.

121. Lee SH, Goswami S, Grudo A, et al. Antielastin autoimmunity in tobacco smoking-induced emphysema. *Nat Med* 2007;**13**(5)**:**567-9.

122. Hodge S, Hodge G, Scicchitano R, Reynolds PN, Holmes M. Alveolar macrophages from subjects with chronic obstructive pulmonary disease are deficient in their ability to phagocytose apoptotic airway epithelial cells. *Immunol Cell Biol* 2003;**81**(4)**:**289-96.

123. Casolaro MA, Bernaudin JF, Saltini C, Ferrans VJ, Crystal RG. Accumulation of Langerhans' cells on the epithelial surface of the lower respiratory tract in normal subjects in association with cigarette smoking. *Am Rev Respir Dis* 1988;**137**(2)**:**406-11.

124. Retamales I, Elliott WM, Meshi B, et al. Amplification of inflammation in emphysema and its association with latent adenoviral infection. *Am J Respir Crit Care Med* 2001;**164**(3)**:**469-73.

125. Enelow RI, Stoler MH, Srikiatkhachorn A, et al. A lung-specific neo-antigen elicits specific CD8+ T cell tolerance with preserved CD4+ T cell reactivity. Implications for immune-mediated lung disease. *J Clin Invest* 1996;**98**(4)**:**914-22.

126. Enelow RI, Mohammed AZ, Stoler MH, et al. Structural and functional consequences of alveolar cell recognition by CD8(+) T lymphocytes in experimental lung disease. *J Clin Invest* 1998;**102**(9)**:**1653-61.

127. Taraseviciene-Stewart L, Scerbavicius R, Choe KH, et al. An animal model of autoimmune emphysema. *Am J Respir Crit Care Med* 2005;**171**(7)**:**734-42.

128. Taraseviciene-Stewart L, Douglas IS, Nana-Sinkam PS, et al. Is alveolar destruction and emphysema in chronic obstructive pulmonary disease an immune disease? *Proc Am Thorac Soc* 2006;**3**(8)**:**687-90.

129. Curtis JL, Freeman CM, Hogg JC. The immunopathogenesis of chronic obstructive pulmonary disease: insights from recent research. *Proc Am Thorac Soc* 2007;**4**(7)**:**512-21.

130. Fabbri LM, Luppi F, Beghe B, Rabe KF. Complex chronic comorbidities of COPD. *Eur Respir J* 2008;**31**(1)**:**204-12.

131. Mannino DM, Thorn D, Swensen A, Holguin F. Prevalence and outcomes of diabetes, hypertension and cardiovascular disease in COPD. *Eur Respir J* 2008;**32**(4)**:**962-9.

132. Cazzola M, Matera MG, Rogliani P, Page C. Treating systemic effects of COPD. *Trends Pharmacol Sci* 2007;**28**(10)**:**544-50.

133. Acuto O, Michel F. CD28-mediated co-stimulation: a quantitative support for TCR signalling. *Nat Rev Immunol* 2003;**3**(12)**:**939-51.

134. Schirmer M, Vallejo AN, Weyand CM, Goronzy JJ. Resistance to apoptosis and elevated expression of Bcl-2 in clonally expanded CD4+CD28- T cells from rheumatoid arthritis patients. *J Immunol* 1998;**161**(2)**:**1018-25.

135. Vallejo AN, Weyand CM, Goronzy JJ. T-cell senescence: a culprit of immune abnormalities in chronic inflammation and persistent infection. *Trends Mol Med* 2004;**10**(3)**:**119-24.

136. Schmidt D, Goronzy JJ, Weyand CM. CD4+ CD7- CD28- T cells are expanded in rheumatoid arthritis and are characterized by autoreactivity. *J Clin Invest* 1996;**97**(9)**:**2027-37.

137. Moosig F, Csernok E, Wang G, Gross WL. Costimulatory molecules in Wegener's granulomatosis (WG): lack of expression of CD28 and preferential up-regulation of its ligands B7-1 (CD80) and B7-2 (CD86) on T cells. *Clin Exp Immunol* 1998;**114**(1)**:**113-8.

138. Garcia de Tena J, Manzano L, Leal JC, San Antonio E, Sualdea V, Alvarez-Mon M. Active Crohn's disease patients show a distinctive expansion of circulating memory CD4+CD45RO+CD28null T cells. *J Clin Immunol* 2004;**24**(2)**:**185-96.

139. Duftner C, Goldberger C, Falkenbach A, et al. Prevalence, clinical relevance and characterization of circulating cytotoxic CD4+CD28- T cells in ankylosing spondylitis. *Arthritis Res Ther* 2003;**5**(5)**:**R292-300.

140. Zal B, Kaski JC, Arno G, et al. Heat-shock protein 60-reactive CD4+CD28null T cells in patients with acute coronary syndromes. *Circulation* 2004;**109**(10)**:**1230-5.

141. Miyazaki Y, Iwabuchi K, Kikuchi S, et al. Expansion of CD4+CD28- T cells producing high levels of interferon-{gamma} in peripheral blood of patients with multiple sclerosis. *Mult Scler* 2008;**14**(8)**:**1044-55.

142. van Leeuwen EM, Remmerswaal EB, Vossen MT, et al. Emergence of a CD4+CD28- granzyme B+, cytomegalovirus-specific T cell subset after recovery of primary cytomegalovirus infection. *J Immunol* 2004;**173**(3)**:**1834-41.

143. Haffar OK, Smithgall MD, Wong JG, Bradshaw J, Linsley PS. Human immunodeficiency virus type 1 infection of CD4+ T cells down-regulates the expression of CD28: effect on T cell activation and cytokine production. *Clin Immunol Immunopathol* 1995;**77**(3)**:**262-70.

144. Namekawa T, Wagner UG, Goronzy JJ, Weyand CM. Functional subsets of CD4 T cells in rheumatoid synovitis. *Arthritis Rheum* 1998;**41**(12)**:**2108-16.

145. Warrington KJ, Takemura S, Goronzy JJ, Weyand CM. CD4+,CD28- T cells in rheumatoid arthritis patients combine features of the innate and adaptive immune systems. *Arthritis Rheum* 2001;**44**(1)**:**13-20.

146. Osterloh A, Breloer M. Heat shock proteins: linking danger and pathogen recognition. *Med Microbiol Immunol* 2008;**197**(1)**:**1-8.

147. Sedger L, Ruby J. Heat shock response to vaccinia virus infection. *J Virol* 1994;**68**(7)**:**4685-9.

148. Kregel KC. Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. *J Appl Physiol* 2002;**92**(5)**:**2177-86.

149. Calderwood SK, Mambula SS, Gray PJ, Jr., Theriault JR. Extracellular heat shock proteins in cell signaling. *FEBS Lett* 2007;**581**(19)**:**3689-94.

150. Calderwood SK, Mambula SS, Gray PJ, Jr. Extracellular heat shock proteins in cell signaling and immunity. *Ann N Y Acad Sci* 2007;**1113:**28-39.

151. Asea A, Kraeft SK, Kurt-Jones EA, et al. HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. *Nat Med* 2000;**6**(4)**:**435-42.

152. Noessner E, Gastpar R, Milani V, et al. Tumor-derived heat shock protein 70 peptide complexes are cross-presented by human dendritic cells. *J Immunol* 2002;**169**(10)**:**5424-32.

153. Blasi C. The autoimmune origin of atherosclerosis. *Atherosclerosis* 2008.

154. Benagiano M, D'Elios MM, Amedei A, et al. Human 60-kDa heat shock protein is a target autoantigen of T cells derived from atherosclerotic plaques. *J Immunol* 2005;**174**(10)**:**6509-17.

155. Rigano R, Profumo E, Buttari B, et al. Heat shock proteins and autoimmunity in patients with carotid atherosclerosis. *Ann N Y Acad Sci* 2007;**1107:**1-10.

156. Murray CJ, Lopez AD. Mortality by cause for eight regions of the world: Global Burden of Disease Study. *Lancet* 1997;**349**(9061)**:**1269-76.

157. Hurd S. The impact of COPD on lung health worldwide: epidemiology and incidence. *Chest* 2000;**117**(2 Suppl)**:**1S-4S.

158. Mannino DM. COPD: epidemiology, prevalence, morbidity and mortality, and disease heterogeneity. *Chest* 2002;**121**(5 Suppl)**:**121S-126S.

159. Lange P, Groth S, Nyboe GJ, et al. Effects of smoking and changes in smoking habits on the decline of FEV1. *Eur Respir J* 1989;**2**(9)**:**811-6.

160. Higenbottam T, Clark TJ, Shipley MJ, Rose G. Lung function and symptoms of cigarette smokers related to tar yield and number of cigarettes smoked. *Lancet* 1980;**1**(8165)**:**409-11.

161. Mercer PF, Shute JK, Bhowmik A, Donaldson GC, Wedzicha JA, Warner JA. MMP-9, TIMP-1 and inflammatory cells in sputum from COPD patients during exacerbation. *Respir Res* 2005;**6:**151.

162. Sin DD, Man SF. Why are patients with chronic obstructive pulmonary disease at increased risk of cardiovascular diseases? The potential role of systemic inflammation in chronic obstructive pulmonary disease. *Circulation* 2003;**107**(11)**:**1514-9.

163. Global Strategy for Diagnosis, Management, and Prevention of COPD: Global Initiative For Chronic Obstructive Lung Disease, 2007.

164. Hodge S, Hodge G, Nairn J, Holmes M, Reynolds PN. Increased airway granzyme b and perforin in current and ex-smoking COPD subjects. *Copd* 2006;**3**(4)**:**179-87.

165. Hodge G, Nairn J, Holmes M, Reynolds PN, Hodge S. Increased intracellular T helper 1 proinflammatory cytokine production in peripheral blood, bronchoalveolar lavage and intraepithelial T cells of COPD subjects. *Clin Exp Immunol* 2007;**150**(1)**:**22-9.

166. Campisi J. Replicative senescence: an old lives' tale? *Cell* 1996;**84**(4)**:**497-500.

167. Goronzy JJ, Weyand CM. Aging, autoimmunity and arthritis: T-cell senescence and contraction of T-cell repertoire diversity - catalysts of autoimmunity and chronic inflammation. *Arthritis Res Ther* 2003;**5**(5)**:**225-34.

168. Choremi-Papadopoulou H, Viglis V, Gargalianos P, Kordossis T, Iniotaki-Theodoraki A, Kosmidis J. Downregulation of CD28 surface antigen on CD4+ and CD8+ T lymphocytes during HIV-1 infection. *J Acquir Immune Defic Syndr* 1994;**7**(3)**:**245-53.

169. Hooper M, Kallas EG, Coffin D, Campbell D, Evans TG, Looney RJ. Cytomegalovirus seropositivity is associated with the expansion of CD4+CD28- and CD8+CD28- T cells in rheumatoid arthritis. *J Rheumatol* 1999;**26**(7)**:**1452-7.

170. Markovic-Plese S, Cortese I, Wandinger KP, McFarland HF, Martin R. CD4+CD28-costimulation-independent T cells in multiple sclerosis. *J Clin Invest* 2001;**108**(8)**:**1185-94.

171. Schirmer M, Goldberger C, Wurzner R, et al. Circulating cytotoxic CD8(+) CD28(-) T cells in ankylosing spondylitis. *Arthritis Res* 2002;**4**(1)**:**71-6.

172. Griffiths GM, Alpert S, Lambert E, McGuire J, Weissman IL. Perforin and granzyme A expression identifying cytolytic lymphocytes in rheumatoid arthritis. *Proc Natl Acad Sci U S A* 1992;**89**(2)**:**549-53.

173. Lopez-Botet M, Bellon T. Natural killer cell activation and inhibition by receptors for MHC class I. *Curr Opin Immunol* 1999;**11**(3)**:**301-7.

174. Lanier LL. NK cell receptors. *Annu Rev Immunol* 1998;**16:**359-93.

175. Global Strategy For the Diagnosis, Management And Prevention of Chronic Pulmonary Disease (GOLD): National Heart, Lung, and Blood Institute/World Health Organization, 2005: NIH Publication No. 2701.

176. Rabe KF, Beghe B, Luppi F, Fabbri LM. Update in chronic obstructive pulmonary disease 2006. *Am J Respir Crit Care Med* 2007;**175**(12)**:**1222-32.

177. Quanjer PH, Tammeling GJ, Cotes JE, Pedersen OF, Peslin R, Yernault JC. Lung volumes and forced ventilatory flows. Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. *Eur Respir J Suppl* 1993;**16:**5-40.

178. Shaffer JP. Modified Sequentially Rejective Multiple Test Procedures. *J Am Stat Assoc* 1986;**81**(395)**:**826-831.

179. Lanier LL. Back to the future--defining NK cells and T cells. *Eur J Immunol* 2007;**37**(6)**:**1424-6.

180. Speiser DE, Valmori D, Rimoldi D, et al. CD28-negative cytolytic effector T cells frequently express NK receptors and are present at variable proportions in circulating lymphocytes from healthy donors and melanoma patients. *Eur J Immunol* 1999;**29**(6)**:**1990-9.

181. Gallin JI, Farber JM, Holland SM, Nutman TB. Interferon-gamma in the management of infectious diseases. *Ann Intern Med* 1995;**123**(3)**:**216-24.

182. Antoniou KM, Ferdoutsis E, Bouros D. Interferons and their application in the diseases of the lung. *Chest* 2003;**123**(1)**:**209-16.

183. Agusti AG. Systemic effects of chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2005;**2**(4)**:**367-70; discussion 371-2.

184. Ionescu AA, Schoon E. Osteoporosis in chronic obstructive pulmonary disease. *Eur Respir J Suppl* 2003;**46:**64s-75s.

185. Calverley PM, Scott S. Is airway inflammation in chronic obstructive pulmonary disease (COPD) a risk factor for cardiovascular events? *Copd* 2006;**3**(4)**:**233-42.

186. Friedman GD, Klatsky AL, Siegelaub AB. Lung function and risk of myocardial infarction and sudden cardiac death. *N Engl J Med* 1976;**294**(20)**:**1071-5.

187. Camilli AE, Robbins DR, Lebowitz MD. Death certificate reporting of confirmed airways obstructive disease. *Am J Epidemiol* 1991;**133**(8)**:**795-800.

188. Nakajima T, Schulte S, Warrington KJ, et al. T-cell-mediated lysis of endothelial cells in acute coronary syndromes. *Circulation* 2002;**105**(5)**:**570-5.

189. Nakajima T, Goek O, Zhang X, et al. De novo expression of killer immunoglobulin-like receptors and signaling proteins regulates the cytotoxic function of CD4 T cells in acute coronary syndromes. *Circ Res* 2003;**93**(2)**:**106-13.

190. Pingiotti E, Cipriani P, Marrelli A, et al. Surface expression of fractalkine receptor (CX3CR1) on CD4+/CD28 T cells in RA patients and correlation with atherosclerotic damage. *Ann N Y Acad Sci* 2007;**1107:**32-41.

191. Fairclough L, Urbanowicz RA, Corne J, Lamb JR. Killer cells in chronic obstructive pulmonary disease. *Clin Sci (Lond)* 2008;**114**(8)**:**533-41.

192. DeMeo DL, Hersh CP, Hoffman EA, et al. Genetic determinants of emphysema distribution in the national emphysema treatment trial. *Am J Respir Crit Care Med* 2007;**176**(1)**:**42-8.

193. Barnes PJ. Immunology of asthma and chronic obstructive pulmonary disease. *Nat Rev Immunol* 2008;**8**(3)**:**183-92.

194. Celli BR, Cote CG, Marin JM, et al. The body-mass index, airflow obstruction, dyspnea, and exercise capacity index in chronic obstructive pulmonary disease. *N Engl J Med* 2004;**350**(10)**:**1005-12.

195. Ekbom A, Brandt L, Granath F, Lofdahl CG, Egesten A. Increased risk of both ulcerative colitis and Crohn's disease in a population suffering from COPD. *Lung* 2008;**186**(3)**:**167-72.

196. Willemse BW, ten Hacken NH, Rutgers B, Lesman-Leegte IG, Postma DS, Timens W. Effect of 1-year smoking cessation on airway inflammation in COPD and asymptomatic smokers. *Eur Respir J* 2005;**26**(5)**:**835-45.

197. Feder ME, Hofmann GE. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu Rev Physiol* 1999;**61:**243-82.

198. Zheng L, He M, Long M, Blomgran R, Stendahl O. Pathogen-induced apoptotic neutrophils express heat shock proteins and elicit activation of human macrophages. *J Immunol* 2004;**173**(10)**:**6319-26.

199. Basu S, Binder RJ, Suto R, Anderson KM, Srivastava PK. Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kappa B pathway. *Int Immunol* 2000;**12**(11)**:**1539-46.

200. Hightower LE, Guidon PT, Jr. Selective release from cultured mammalian cells of heat-shock (stress) proteins that resemble glia-axon transfer proteins. *J Cell Physiol* 1989;**138**(2)**:**257-66.

201. Arnold-Schild D, Hanau D, Spehner D, et al. Cutting edge: receptor-mediated endocytosis of heat shock proteins by professional antigen-presenting cells. *J Immunol* 1999;**162**(7)**:**3757-60.

202. Roth GA, Moser B, Krenn C, et al. Heightened levels of circulating 20S proteasome in critically ill patients. *Eur J Clin Invest* 2005;**35**(6)**:**399-403.

203. Hetz H, Hoetzenecker K, Hacker S, et al. Caspase-cleaved cytokeratin 18 and 20 S proteasome in liver degeneration. *J Clin Lab Anal* 2007;**21**(5)**:**277-81.

204. Szerafin T, Hoetzenecker K, Hacker S, et al. Heat shock proteins 27, 60, 70, 90alpha, and 20S proteasome in on-pump versus off-pump coronary artery bypass graft patients. *Ann Thorac Surg* 2008;**85**(1)**:**80-7.

205. Kang MJ, Homer RJ, Gallo A, et al. IL-18 is induced and IL-18 receptor alpha plays a critical role in the pathogenesis of cigarette smoke-induced pulmonary emphysema and inflammation. *J Immunol* 2007;**178**(3)**:**1948-59.

206. Park HK, Park EC, Bae SW, et al. Expression of heat shock protein 27 in human atherosclerotic plaques and increased plasma level of heat shock protein 27 in patients with acute coronary syndrome. *Circulation* 2006;**114**(9)**:**886-93.

207. Djamali A, Reese S, Oberley T, Hullett D, Becker B. Heat shock protein 27 in chronic allograft nephropathy: a local stress response. *Transplantation* 2005;**79**(12)**:**1645-57.

208. Vander Heide RS. Increased expression of HSP27 protects canine myocytes from simulated ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol* 2002;**282**(3)**:**H935-41.

209. Xu Q. Role of heat shock proteins in atherosclerosis. *Arterioscler Thromb Vasc Biol* 2002;**22**(10)**:**1547-59.

210. Madach K, Molvarec A, Rigo J, Jr., et al. Elevated serum 70 kDa heat shock protein level reflects tissue damage and disease severity in the syndrome of hemolysis, elevated liver enzymes, and low platelet count. *Eur J Obstet Gynecol Reprod Biol* 2008;**139**(2)**:**133-8.

211. Vega VL, Rodriguez-Silva M, Frey T, et al. Hsp70 Translocates into the Plasma Membrane after Stress and Is Released into the Extracellular Environment in a Membrane-Associated Form that Activates Macrophages. *J Immunol* 2008;**180**(6)**:**4299-307.

212. Li W, Li Y, Guan S, et al. Extracellular heat shock protein-90alpha: linking hypoxia to skin cell motility and wound healing. *Embo J* 2007;**26**(5)**:**1221-33.

213. Rajagopal D, Bal V, Mayor S, George A, Rath S. A role for the Hsp90 molecular chaperone family in antigen presentation to T lymphocytes via major histocompatibility complex class II molecules. *Eur J Immunol* 2006;**36**(4)**:**828-41.

ABBREVIATIONS

AACT	α1-antichymotrypsin
AAT	α1-antitrypsin
AATD	α1-antitrypsin deficiency
APC	Antigen-presenting cell
AUC	Area under the curve
A2M	α2-macroglobulin
BAL	Bronchoalveolar lavage
Bcl-2	B-cell lymphoma protein 2
BMI	Body mass index
C	Celsius
CCR7	Chemokine (C-C motif) receptor 7
CD	Cluster of differentiation
CI	Confidence interval
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
DALY	Disability-adjusted life years
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
ENA	Epithelial cell-derived neutrophil-activating peptide
ETS	Environmental tobacco smoke
FEV1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
GC	Germinal center
GOLD	Global initiative for chronic obstructive lung disease
GRO	Growth-related oncogene
HIV	Human immunodeficiency virus
HSP	Heat shock protein
HUVEC	Human umbilical vein endothelial cell
ICAM	Intercellular adhesion molecule

IFN	Interferon
IL	Interleukin
kDa	Kilodalton
LPS	Lipopolysaccharide
LTB_4	Leukotriene B4
mAb	Monoclonal antibody
MCP	Monocyte chemoattractant protein
MEF	Maximum expiratory flow
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MME	Macrophage elastase
MMP	Matrix metalloproteinase
NK	Natural killer
NKR	Natural killer cell receptor
PBMC	Peripheral blood mononuclear cell
PE	Phycoerythrin
PHA	Phytohemagglutinin
PI	Protease inhibitor
ROC	Receiver operating characteristic
RSV	Respiratory syncytial virus
STAT	Signal transducer and activator of transcription
TCR	T-cell receptor
TIMP	Tissue inhibitor of metalloproteinase
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor
USA	United States of America
YLL	Years of life lost due to premature mortality

APPENDIX

Parts of this work have been published in peer-reviewed journals. The complete publications are attached.

Clinical & Experimental Immunology
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T cell senescence and contraction of T cell repertoire diversity in patients with chronic obstructive pulmonary disease

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Summary

Pathogenetic mechanisms leading to chronic obstructive pulmonary disease (COPD) remain poorly understood. Because clonogenic T cells ($CD4^+CD28^{null}$) were shown to be increased in autoimmune diseases we hypothesized that $CD4^+CD28^{null}$ T cells play a role in COPD. Here we describe that enhanced presence of $CD4^+CD28^{null}$ cells is associated with impaired lung function. Sixty-four patients and controls were included. T cell phenotype was analysed using flow cytometry. Enzyme-linked immunosorbent assays were utilized to determine cytokines. Statistical evaluations were performed using non-parametric group comparisons and correlations. A logistic regression model was used to determine predictive values of $CD4^+CD28^{null}$ in the diagnosis of COPD. Populations of $CD4^+$ T cells lacking surface co-stimulatory CD28 were enlarged significantly in evaluated patients when compared with controls. Natural killer (NK)-like T cell receptors (CD94, 158) and intracellular perforin, granzyme B were increased in $CD4^+CD28^{null}$ cells. Cytokine production after triggering of peripheral blood mononuclear cells (PBMCs) was elevated in patients at early disease stages. Receiver operating characteristic curve plotting revealed that presence of $CD4^+CD28^{null}$ T cells has a diagnostic value. These $CD4^+CD28^{null}$ T cells show increased expression of NK-like T cell receptors (CD94, 158) and intracellular perforin and granzyme B. Furthermore, triggering of PBMCs obtained from patients with mild COPD led to increased interferon-γ and tumour necrosis factor-α production *in vitro* compared with controls. Our finding of increased $CD4^+CD28^{null}$ T cells in COPD indicates that chronic antigen exposure, e.g. through contents of smoke, leads to loss of CD28 and up-regulation of NK cell receptors expression on T cells in susceptible patients.

Keywords: $CD4^+CD28^{null}$, COPD, granzyme, perforin, smoking

Introduction

Chronic obstructive pulmonary disease (COPD) is a leading cause of death worldwide [1]. By 2020, only ischaemic heart disease and cerebrovascular disease will account for a higher mortality among the world's population [2]. Prevalence and hospitalization rates have increased significantly over the past years [3,4]. Several studies were able to show a strong correlation between tobacco abuse and the development of COPD. However, not every smoker develops clinical features of COPD [5,6]. Pathogenesis of the disease is characterized by irreversible airflow obstruction because of constant remodelling of the airways and chronic inflammatory responses [7]. The impairment of the immune system is not restricted to the lungs, as COPD patients are also at higher risk for systemic failures including cardiovascular diseases [8]. Diagnosis of airway obstruction according to the guidelines of the Global Initiative for Chronic Obstructive Lung Diseases (GOLD) requires the use of spirometry. A post-bronchodilator forced expiratory volume in 1 s(FEV_1)/forced vital capacity ratio of less than 70% indicates an irreversible airflow obstruction, and is therefore considered to be the main parameter for the diagnosis of COPD [9].

Although smoking is accepted widely as the major risk factor for the development of the disease, descriptions of specific pathogenetic mechanisms remain vague. For decades, neutrophils and macrophages, as part of the innate immunity, were considered pivotal in the airway remodelling

process occurring in patients with COPD. Recent reports have challenged this pathognomonic concept by demonstrating increased $CD8^+$ and $CD4^+$ T cells as part of the adaptive immune system in bronchoalveolar lavage and sputum analyses of COPD patients. These T lymphocytes contained higher levels of perforin and revealed cytotoxic activity compared with cells of healthy donors or non-COPD smokers [10,11]. Furthermore, Di Stefano *et al.* presented two papers in which they were able to show that stable mild/moderate COPD is associated with an active T helper 1 cell/type-1 cytotoxic T cell inflammatory process involving activation of signal transducer and activator of transcription 4 and interferon (IFN)-γ production and natural killer (NK) cells in COPD lung tissue and bronchoalveolar lavage [12,13].

Based on the data of Hodge *et al.*, who described $CD8^+CD28^{null}$ in COPD and Di Stefano *et al.*'s data, we hypothesized that a specific chronic inflammatory reaction of the adaptive immune system is occurring in patients with COPD. Antigenic stimulation causes a rapid expansion of antigen-specific T cells that increase to large clonal size. This physiological increment is counterbalanced by a preprogrammed clonal contraction. This process is robust and usually suffices to maintain a diverse memory T cell compartment [14,15]. Chronic antigen exposure because of infections with human immunodeficiency virus or cytomegalovirus [16,17] and advanced age [15] also leads to expansion of monoclonal T cell populations.

Replicatively stressed $CD4^+$ T cells undergo multiple phenotypic and functional changes. The most widely acknowledged phenotypic change is the loss of the co-stimulatory surface marker CD28. Expansion of $CD4^+$ T cells and loss of CD28 are presumably senescent ($CD4^+CD28^{null}$). This has been described in several autoimmune diseases, such as diabetes mellitus, rheumatoid arthritis, Wegener's granulomatosis, multiple sclerosis and ankylosing spondylitis [18–20]. $CD4^+CD28^{null}$cells are clonally expanded and are known to include autoreactive T cells, implicating a direct role in autoimmune disease. These expanded $CD4^+$ clonotypes are phenotypically distinct from the classic T helper cells. Because of a transcriptional block of the CD28 gene, clonally expanded $CD4^+$ T cells lack surface expression of the major co-stimulatory molecule CD28. $CD4^+CD28^{null}$ T cells release large amounts of IFN-γ and contain intracellular perforin and granzyme B, providing them with the ability to lyse target cells. Their outgrowth into large clonal populations may be attributed partially to a defect in down-regulating Bcl-2 when deprived of T cell growth factors. In the absence of the CD28 molecule, these unusual $CD4^+$ T cells use alternative co-stimulatory pathways. Several of these functional features in $CD4^+CD28^{null}$ T-cells are reminiscent of NK cells. Like NK cells, $CD4^+CD28^{null}$ T cells are cytotoxic and can express NK cell receptors such as CD94 and CD158. NK cells are regulated closely by a family of polymorphic receptors that interact with major histocompatibility complex (MHC) class I molecules, resulting in signals that control NK-mediated cytotoxicity and cytokine production. MHC class I-mediated triggering of the full-length NK cell receptors transduces a dominant inhibitory signal that blocks the cytolytic activity and cytokine release of NK cells. These receptors also contain highly homologous members that have truncated cytoplasmatic domains and transmit activating signals [21–24].

Because chronic antigen exposure because of history of smoking can be assumed in most COPD patients, we hypothesized that chronic stimulation of the adaptive immune system leads to increased levels of systemic clonogenic $CD4^+CD28^{null}$ T cell populations. To prove this we included age- and sex-matched healthy non-smokers, smokers with a history of tobacco abuse and normal lung function and patients with diagnosed COPD according to GOLD classification (groups: mild, COPD I–II; severe, COPD III–IV respectively). We determined intracellular expression of cytolytic proteins perforin and granzyme B as well as surface expression of the NK cell receptors CD94 and CD158 on $CD4^+CD28^{null}$ T cells. We further designed *in vitro* experiments to explore whether peripheral blood mononuclear cells (PBMCs) obtained from each study group secrete augmented levels of IFN-γ, tumour necrosis factor (TNF)-α and interleukin (IL)-12 after T cell triggering. Because systemic inflammation is associated with systemic proinflammatory cytokines *in vivo*, we correlated serum levels of IL-1β, TNF-α, IFN-γ and IL-10 with lung function parameters. We conclude in this work that patients with COPD show increased circulating clonogenic T cells that have diagnostic potential for detection of COPD according to the GOLD classification.

Methods

Patients

The study protocol was approved by the ethics committee of the Medical University of Vienna (EK no. 091/2006) and was performed in accordance with the Declaration of Helsinki and current revisions of the Good Clinical Practice Guidelines of the Medical University of Vienna. A total number of 64 volunteers, at least 40 years old, participated in this trial. Healthy non-smokers ($n = 15$), smokers ($n = 14$) and smokers meeting the GOLD diagnostic criteria for COPD I–II ($n = 19$) and COPD III–IV ($n = 16$) [25] were recruited. COPD patients with acute exacerbation as defined by the guidelines from the WHO and GOLD [9,26] within 14 days before study entry were excluded. Additional exclusion criteria were a history of asthma, autoimmune diseases or other relevant lung diseases (e.g. lung cancer, known α1-anti-trypsin deficiency). Furthermore, all patients were free from known coronary artery disease, peripheral artery disease and carotid artery disease. All patients provided written, informed consent before collection of blood

samples and lung function. Height and weight (Seca; Vogel and Halke, Hamburg, Germany) were measured and body mass index was determined. Pulmonary function was measured using the same model spirometer (AutoboxV6200; SensorMedics, Vienna, Austria). Measurements were made before and – if criteria for airflow obstruction were met – 15–30 min after inhaling 200 μg salbutamol. Arterial blood gases (PaO_2, $PaCO_2$) were obtained at rest while breathing room air in a sitting position. Measurement of arterial blood gases was performed with an ABL 510 gas analyser (Radiometer, Copenhagen, Denmark). Results are expressed as absolute values and as percentages of predicted values for age, sex and height, according to the European Community for Steel and Coal prediction equations [27]. Predicted normal values were derived from the reference values of the Austrian Society of Pulmonary Medicine.

Flow cytometry analysis

Heparinized blood samples were incubated on ice with fluorochrome-labelled antibodies. Prior to antibody incubation, erythrocytes were lysed by addition of BD fluorescence activated cell sorter lysing solution (Becton Dickinson, Franklin Lakes, NJ, USA). Cells were then stained with fluorescein isothiocyanate-conjugated anti-CD4 (BD Biosciences Pharmingen, San Jose, CA, USA), phycoerythrin (PE)-labelled anti-CD158 (R&D Systems, Minneapolis, MN, USA), PE-Cy5-labelled anti-CD28 (Biolegend, San Diego, CA, USA) and PE-conjugated anti-CD94 (eBioscience, San Diego, CA, USA) at various combinations. Stained cells were analysed using a Cytomics FC 500 flow cytometer (Beckman Coulter, Fullerton, CA, USA). For intracellular staining, PE-conjugated antibodies directed against perforin and granzyme B (BD Biosciences Pharmingen; Serotec, Dusseldorf, Germany) were used and incubated with pre-stained cells after permeabilization of the cell membrane with saponin solution.

Enzyme-linked immunosorbent assays

The enzyme-linked immunosorbent assays (ELISA) technique (BenderMedSystems, Vienna, Austria) was used to quantify levels of IL-1β, TNF-α, IFN-γ and IL-10 in serum samples obtained after centrifugation of whole blood. Ninety-six-well plates were coated with a monoclonal antibody directed against the specific antigen and incubated overnight at 4°C. After a washing step, plates were blocked with assay buffer for 2 h. 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. Tetramethylbenzidine substrate solution was applied after the appropriate incubation time and another washing step. Colour development was then monitored using a Wallac Multilabel counter 1420 (PerkinElmer, Boston, MA, USA). The optical density values obtained were compared with the standard curve calculated from optical density values of standards with known concentrations of antigen.

Stimulation of freshly prepared PBMCs

Freshly prepared PBMCs were separated by standard Ficoll densitiy gradient centrifugation. Cells were then washed twice in phosphate-buffered saline, counted and transferred to a 96-well flat-bottomed plate at 1×10^5 cells per well in 200 μl serum-free ultra culture medium (Cambrex Corp., East Rutherford, NJ, USA) containing 0·2% gentamycinsulphate (Sigma, St Louis, MO, USA) and 0·5% β-mercaptoethanol (Sigma) 1% L-glutamine (Sigma). Anti-CD3 (CD3) (10 μg/ml) or phytohaemagglutinin (PHA) (7 μg/ml) were added and plates were transferred to a humidified atmosphere (5% CO_2, 37°C) for 18 h. Supernatants were harvested and stored at −20°C.

Quantification of IFN-γ, TNF-α and IL-12 in supernatants

The ELISA technique (BenderMedSystems) was used to quantify levels of IFN-γ, TNF-α and IL-12 in supernatants of stimulated cells, as described above.

Statistical methods

Comparison of the primary end-point $CD4^+CD28^{null}$% of $CD4^+$ and the second end-points (IFN-γ, TNF-α and IL-12 *ex vivo* CD3 and PHA, IL-1β, TNF-α, IFN-γ and IL-10 serum values) between healthy non-smokers, healthy smokers, COPD I–II and COPD III–IV patients was performed with the non-parametric Kruskal–Wallis test. Pairwise comparisons between groups were performed with Wilcoxon tests. For the six pairwise between-group comparisons of the primary end-point $CD4^+CD28^{null}$% of $CD4^+$ additionally adjusted critical values, according to Shaffer (1986) [28], were applied to control the familywise error rate in the strong sense.

Parametric 95% confidence intervals (CI) for the mean $CD4^+CD28^{null}$ percentages in each group were computed. Correlations of percentage of $CD4^+CD28^{null}$ cells and serum cytokine levels with parameters of lung function were calculated using the Spearman's correlation coefficient. These correlations were performed for all patients, the subgroup of smokers and the subgroup of COPD patients.

The prevalence of perforin, granzyme B and expression of CD94 and CD158 was compared between $CD4^+CD28^{null}$ and $CD4^+CD28^+$ cells using Wilcoxon's signed-rank tests. Additionally, parametric 95% CI for the mean percentages for each variable are given.

In the subgroup of smokers a logistic regression with dependent variable COPD (yes/no) and independent variable $CD4^+CD28^{null}$% was performed. To account for an

Table 1. Clinical characteristics. Severity of airflow obstruction was determined using lung function test in all subjects; chronic obstructive pulmonary disease (COPD) patients meeting the Global Initiative for Chronic Obstructive Lung Diseases diagnostic criteria for COPD.

Subject category	Healthy	Healthy smoker	COPD GOLD I–IV	COPD GOLD I–II	COPD GOLD III–IV
N	15	14	35	19	16
Male/female	10/5	7/7	20/15	10/9	10/6
Age	57·20 (12·50)	56·64 (9·17)	59·60 (8·01)	60·68 (7·39)	58·31 (8·75)
Lung function					
FVC (l)	4·55 (0·94)	3·84 (0·66)	2·80 (1·08)	3·33 (1·06)	2·14 (0·70)
FEV_1 (%)	105·37 (17·11)	94·40 (11·96)	52·76 (23·71)	70·21 (13·33)	30·67 (12·66)
FEV_1/VC (%)	76·80 (7·85)	75·95 (3·99)	51·18 (16·83)	61·74 (8·36)	37·80 (15·33)
MEF_{50} (%)	100·67 (28·92)	87·64 (21·45)	27·29 (18·68)	39·42 (15·93)	11·93 (6·60)
MEF_{25} (%)	103·53 (33·89)	75·71 (31·33)	29·71 (15·31)	37·37 (16·19)	20·00 (5·94)
Smoking history					
Never-smoker	15	0	0	0	0
Ex-smoker	0	3	7	4	3
Current-smoker	0	11	28	15	13
Pack years	0	34 (25·2)	45·8 (30·6)	47·3 (29·7)	44·0 (32·6)
Body weight (kg)	71·6 (13·9)	76·4 (8·6)	80·4 (21·6)	79·7 (16·7)	81·1 (27·2)
Body height (cm)	172·7 (10·9)	168·7 (8·1)	169·2 (10·5)	167·7 (12·1)	171·2 (7·9)

Data are given as mean (±standard deviation) if not otherwise stated. FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; MEF, maximum expiratory flow.

outlying observation, the square root of the percentages was used in this analysis. To assess the predictive capacity of the percentage of $CD4^+CD28^{null}$ a receiver operating characteristic (ROC) curve with its area under the curve (AUC) was computed.

Results

Demographic characteristics of study patients

Demographic characteristics of patients are depicted in Table 1. Healthy non-smokers, healthy smokers, GOLD-classified COPD I–II and COPD III–IV were included. In all groups a similar number of patients were included and age and sex were distributed equally.

$CD4^+CD28^{null}$ cells show increased occurrence in patients suffering from COPD

To test our hypothesis whether $CD4^+CD28^{null}$ cells are increased in patients with COPD, we evaluated blood samples using multi-stain flow cytometry. Figure 1a and Table 2 illustrate percentages of $CD4^+CD28^{null}$ cells of the total $CD4^+$ cell population. The COPD III–IV group showed significantly increased values compared with the healthy non-smoker and smoker groups (Wilcoxon test: $P = 0{\cdot}012$, $P = 0{\cdot}002$, Kruskal–Wallis test for the overall comparison: $P = 0{\cdot}005$). Additionally, we observed a significant difference between the COPD I–II group and the healthy smoker group (Wilcoxon test: $P = 0{\cdot}046$). Applying the Shaffer (1986) [28] multiplicity adjusted critical values, only the differences between the COPD III–IV and the healthy groups remained significant.

Unstimulated $CD4^+CD28^{null}$ cells contain cytolytic proteins perforin and granzyme B

To evaluate the intra-cytoplasmic content of cytolytic proteins perforin and granzyme B in $CD4^+$ cells, flow cytometric analysis of blood samples was performed after co-incubation with saponin solution and intracellular staining. Content of perforin was more prevalent in

Table 2. Percentage of $CD4^+CD28^{null}$ cells in the peripheral blood flow.

Subject category	Healthy	Healthy smoker	COPD GOLD I–II	COPD GOLD III–IV
$CD4^+CD28^{null}$% of $CD4^+$	1·96 (1·07–2·84)	1·5 (0·41–2·59)	3·22 (1·83–4·62)	7·53 (2·67–12·39)
IFN-γ CD3 (pg/ml)	272 (188–356)	240 (178–301)	440 (286–594)	328 (214–442)
IFN-γ PHA (pg/ml)	116 (83–149)	91 (53–129)	375 (135–615)	134 (1–266)
TNF-α CD3 (pg/ml)	922 (368–1476)	731 (333–1128)	1234 (793–1674)	1508 (860–2157)
TNF-α PHA (pg/ml)	1096 (551–1641)	777 (411–1143)	2465 (1532–3398)	1144 (387–1901)
IL-12 CD3 (pg/ml)	93 (46–139)	63 (34–92)	72 (36–108)	42 (13–71)
IL-12 PHA (pg/ml)	44 (8–80)	33 (19–47)	78 (31–125)	17 (8–25)

Furthermore, cytokine expression in supernatants of peripheral blood mononuclear cells stimulated with either anti-CD3 or phytohaemagglutinin (PHA) is described. All date are given as mean (95% confidence interval). COPD, chronic obstructive pulmonary disease; GOLD, Global Initiative for Chronic Obstructive Lung Disease; IFN-γ, interferon-γ; IL, interleukin; TNF-α, tumour necrosis factor-α.

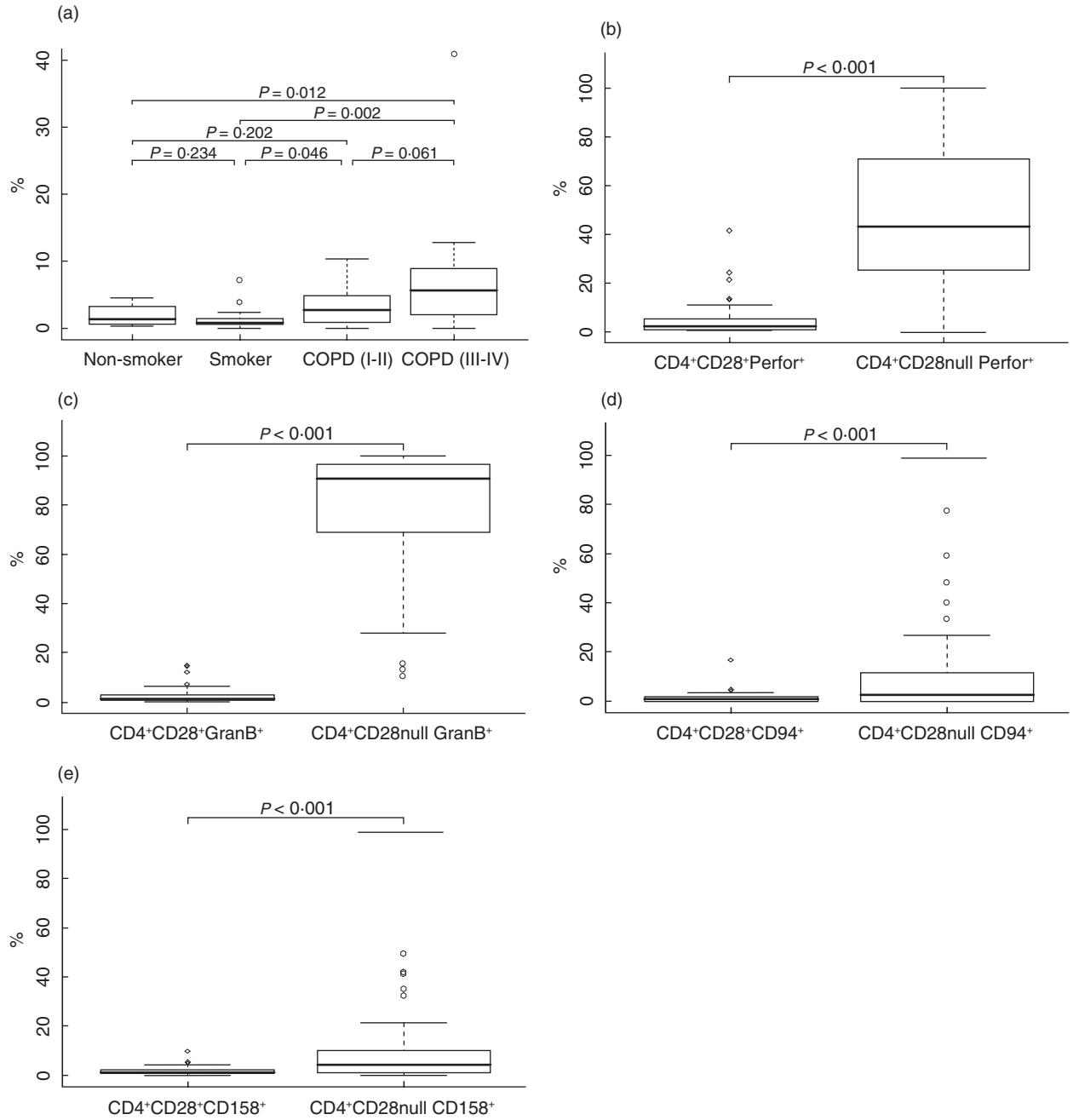

Fig. 1. (a) Boxplot showing percentage of $CD4^+CD28^{null}$ cells in the peripheral blood flow. (b,c) Subset of $CD4^+$ T cells lacking co-stimulatory CD28 contained intracellular cytolytic proteins perforin and granzyme B. (d,e) $CD4^+CD28^{null}$ cells showed significantly increased surface expression of natural killer (NK) cell receptors CD94 and CD158. Bars indicate medians; solid boxes show span between 25th and 75th percentiles; whiskers illustrate lowest and highest values. Outliers are marked as open circles.

$CD4^+CD28^{null}$ cells compared with $CD4^+CD28^+$ cells (Fig. 1b) [46·13% (39·34–52·91) *versus* 4·68% (3·04–6·32), $P < 0·001$; all means (95% CI)]. Positive staining for intracellular granzyme B in $CD4^+CD28^{null}$ cells was more frequent than in $CD4^+CD28^+$ cells (Fig. 1c) [78·63% (72·65–84·61) *versus* 2·36% (1·63–3·11), $P < 0·001$; all means (95% CI)].

Increased prevalence of NK cell receptors on $CD4^+CD28^{null}$ cells

Flow cytometry analysis was used to evaluate expression of CD94 and CD158 on the surface of $CD4^+$ cells. Figure 1d and e shows increased expression of surface antigens CD94 and CD158 on $CD4^+CD28^{null}$ cells [CD94, 10·00%

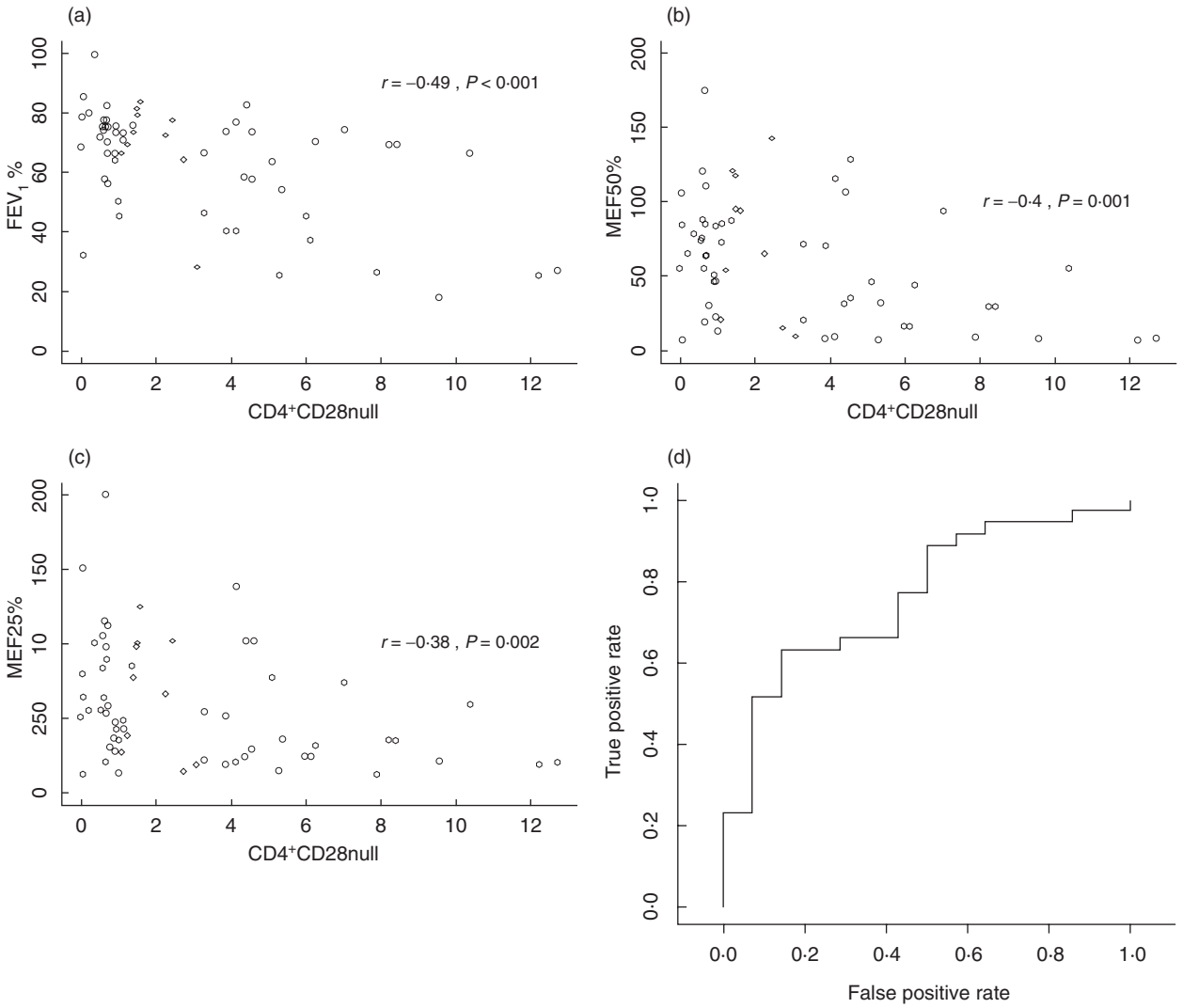

Fig. 2. (a,b,c) Scatterplots showing correlations of $CD4^+CD28^{null}$% of $CD4^+$ and Forced expiratory volume in 1 s, maximum expiratory flow ($MEF_{50\%}$), and $MEF_{25\%}$, Spearman's correlation coefficients and *P*-values are given. (d) Receiver operating characteristic curve for the prediction of chronic obstructive pulmonary disease in the subgroup of smokers based on the $CD4^+CD28^{null}$% measurement.

(6·04–13·97) *versus* 1·41% (0·85–1·97), $P < 0·001$; CD158, 9·35% (6·22–12·47) *versus* 2·00% (1·61–2·39), $P < 0·001$; all means (95% CI)].

Percentage of $CD4^+CD28^{null}$ cells correlates negatively with routine parameters of spirometry

For verification of our flow cytometry data with routine clinical data, we correlated the percentage of $CD4^+CD28^{null}$ with $FEV_{1\%}$ of vital capacity, 50% maximum expiratory flow ($MEF_{50\%}$) of predicted value and $MEF_{25\%}$ of predicted value. All parameters showed a statistically significant negative correlation with percentage of $CD4^+CD28^{null}$ cells (Spearman's correlation coefficients: $FEV_{1\%}$, $R = –0·49$, $P < 0·001$; $MEF_{50\%}$, $R = –0·40$, $P = 0·001$; $MEF_{25\%}$, $–0·38$, $P = 0·002$; Fig. 2a–c). Similarly, we observed significant correlations in the subgroup of smokers (Spearman's correlation coefficients: $FEV_{1\%}$, $R = –0·52$, $P < 0·001$; $MEF_{50\%}$, $R = –0·48$, $P = 0·001$; $MEF_{25\%}$, $R = –0·40$, $P = 0·004$). In the subgroup of COPD patients marginally significant correlations with $FEV_{1\%}$ and $MEF_{50\%}$ (Spearman's correlation coefficients: $FEV_{1\%}$, $R = –0·32$, $P = 0·068$; $MEF_{50\%}$, $R = –0·36$, $P = 0·04$) and no significant correlation with $MEF_{25\%}$ (Spearman's correlation coefficient: $MEF_{25\%}$, $R = –0·15$, $P = 0·38$) were found.

Prediction capacity of the percentage of $CD4^+CD28^{null}$ cells for COPD in smokers

In the logistic regression analysis for the subset of smokers the independent variable percentage of $CD4^+CD28^{null}$ cells showed a significant association with COPD ($P = 0·012$). The corresponding ROC curve (Fig. 2d) has an AUC = 0·76.

Table 3. Correlations of serum cytokine levels with parameters of lung function test.

	All patients			All smokers			All COPD		
Correlation	*n*	Coeff.	*P*-value	*n*	Coeff.	*P*-value	*n*	Coeff.	*P*-value
$CD4^+CD28^{null}$-$FEV_{1\%}$	62	−0·485	<0·001	48	−0·517	<0·001	34	−0·317	0·068
$CD4^+CD28^{null}$-$MEF_{50\%}$	62	−0·404	0·001	48	−0·479	<0·001	34	−0·355	0·04
$CD4^+CD28^{null}$-$MEF_{25\%}$	62	−0·38	0·002	48	−0·403	0·004	34	−0·154	0·384
IFN-γ-$FEV_{1\%}$	62	0·461	<0·001	48	0·613	<0·001	34	0·491	0·003
IFN-γ-$MEF_{50\%}$	62	0·556	<0·001	48	0·645	<0·001	34	0·541	<0·001
IFN-γ-$MEF_{25\%}$	62	0·489	<0·001	48	0·618	<0·001	34	0·492	0·003
TNF-α-$FEV_{1\%}$	62	0·374	0·003	48	0·336	0·019	34	0·226	0·198
TNF-α-$MEF_{50\%}$	62	0·337	0·007	48	0·275	0·058	34	0·123	0·489
TNF-α-$MEF_{25\%}$	62	0·309	0·014	48	0·249	0·087	34	0·039	0·828
IL-1β-$FEV_{1\%}$	62	0·344	0·006	48	0·287	0·048	34	0·066	0·709
IL-1β-$MEF_{50\%}$	62	0·282	0·026	48	0·256	0·079	34	0·078	0·663
IL-1β-$MEF_{25\%}$	62	0·266	0·037	48	0·22	0·133	34	0·002	0·993
IL-10-$FEV_{1\%}$	62	0·256	0·044	48	0·178	0·226	34	0·096	0·587
IL-10-$MEF_{50\%}$	62	0·328	0·009	48	0·278	0·055	34	0·329	0·058
IL-10-$MEF_{25\%}$	62	0·3	0·018	48	0·226	0·122	34	0·214	0·223

FEV_1, forced expiratory volume in 1 s; IFN-γ, interferon-γ; IL, interleukin; MEF, maximum expiratory flow; TNF-α, tumour necrosis factor-α.

Correlations of serum cytokine concentrations (IL-1β, TNF-α, IFN-γ and IL-10) with $FEV_{1\%}$, $MEF_{50\%}$ and $MEF_{25\%}$

Table 3 embraces the results of non-parametric correlations of serum cytokines IL-1β, TNF-α, IFN-γ and IL-10 with routine lung function parameters.

Stimulated PBMCs of patients suffering from early-stage COPD produce increased levels of IFN-γ and TNF-α *ex vivo*

To verify the functional activity of PBMCs we performed blastogenesis assays using lymphocyte-specific anti-CD3 and PHA. This analysis was performed for seven patients per group (except for the COPD III–IV group, where only five patients were included). Groupwise means and 95% CI are given in Table 2. Supernatants of patients with COPD I–II showed increased levels of IFN-γ compared with healthy smokers (Wilcoxon test: CD3 $P = 0{\cdot}026$, PHA: $P = 0{\cdot}038$); however, the differences failed to reach significance after correcting for multiple testing (Kruskal–Wallis test: CD3: $P = 0{\cdot}06$; PHA: $P = 0{\cdot}09$). Concentrations of the healthy group and of patients with COPD III–IV were lower but showed no significant difference to the COPD I–II group. None of the remaining pairwise comparisons was statistically significant. Significant difference of TNF-α (PHA) levels between groups were observed (Kruskal–Wallis test: $P = 0{\cdot}007$). The COPD I–II group showed significantly elevated levels of TNF-α (PHA) levels compared with healthy smokers (Wilcoxon test $P = 0{\cdot}001$) and non-smokers (Wilcoxon test: $P = 0{\cdot}007$) and marginally significant elevated levels compared with COPD III–IV patients (Wilcoxon test: $P = 0{\cdot}03$). None of the remaining pairwise comparisons was statistically significant. For TNF-α (CD3) no significant differences between groups were observed. For IL-12 (PHA) we observed marginally significant between-group differences (Kruskal–Wallis test: $P = 0{\cdot}048$). There were higher IL-12 (PHA) levels in the COPD I–II group compared with the other groups. However, only the difference to the COPD III–IV group reached statistical significance (Wilcoxon test: $P = 0{\cdot}018$). Additionally, the difference between non-smokers and COPD III–IV patients was marginally significant (Wilcoxon test: $P = 0{\cdot}048$). Concentrations of IL-12 (CD3) showed no significant between group differences.

Patients with severe COPD (GOLD III–IV) patients show decreased serum levels of IFN-γ

Significant differences of IFN-γ serum levels between groups have been observed (Kruskal–Wallis test: $P = 0{\cdot}002$). COPD III–IV patients showed lower IFN-γ serum levels than healthy smokers (Wilcoxon test: $P < 0{\cdot}001$) and healthy non-smoker (Wilcoxon test: $P = 0{\cdot}002$) patients. Additionally, marginally significantly lower values were observed in the COPD I–II group compared with the healthy smoker group. Note that in 94% of COPD III–IV and 74% in COPD I–II patients (compared with 40% in healthy controls and 21% in healthy smokers) no serum IFN-γ could be detected. For serum TNF-α, serum IL-10 and serum IL-1β no significant between-group differences were found.

Discussion

The total number of lymphocytes circulating in the blood and their subset distribution is under strict homeostatic control. We report for the first time that patients with

COPD show a profound change in the representation of functionally and phenotypically distinct subsets of CD4+ T cells. We propose that clonogenic CD4+ T cells with characterized loss of co-stimulatory CD28 and intracellular storage of the cytolytic proteins granzyme B and perforin might be causal for continuing systemic inflammatory state in COPD patients. The basic mechanisms causing replacement of other CD4+ T cells by CD4+CD28null clonotypes are incompletely understood. However, phenotypic and functional analyses of CD4+CD28null T cells have suggested that they are related to NK cells and represent a population of NK-like T cells [29]. In support of this hypothesis, we found that CD4+CD28null T cells express MHC class I-recognizing receptors of the immunoglobulin superfamily (CD94, CD158) [30,31]. Our data corroborate the concept that CD4+CD28null T cells share multiple features with NK cells and may combine functional properties of innate and adaptive immunity in COPD patients.

To prove relevant immune functions we separated PBMCs of the study groups and activated them via specific and unspecific T cell stimulation *in vitro*. We were able to show that systemic white blood cells derived from COPD GOLD I–II secreted augmented levels of IFN-γ and TNF-α – cytokines that are known to increase macrophage and dendritic cell activity – compared with controls and severe COPD (GOLD III–IV). This observation is particularly interesting, as this *in vitro* phenomenon was observed only in patients at the initial stage of COPD progression (GOLD stages I–II), indicating a specific role of NK-like T cells in triggering initial lung tissue destruction. Our data confirm and corroborate the pathophysiological speculation by Hodge *et al.* [11] and Di Stefano *et al.* [12,13], who argued that T cell activation is leading to enhanced secretion of IFN-γ, a cytokine that activates macrophages and enhances innate immunity, and is thus causing tissue destruction in COPD-susceptible patients. [32,33]

This *in vitro* finding led us to explore whether systemic serum levels of IL-1β, TNF-α, IFN-γ and IL-10 were elevated in COPD patients without recent exacerbation of COPD disease. Contrary to our assumption, the level of inflammatory cytokine IFN-γ correlated negatively with spirometric parameters. This finding underlines the importance of a local interaction of cell-based immune system and lung tissue interphase in the presence of T cell-triggering noxious substances (e.g. inhaled smoke). In a final attempt we investigated whether systemic presence of clonogenic CD4+CD28null T cells is relevant for diagnosing COPD by means of flow cytometry analysis *ex vivo*. We performed a logistic regression analysis and were able to show that presence of systemic CD4+CD28null T cells was highly predictive for diagnosing COPD. Because of these data we are currently designing a clinical trial to evaluate whether systemic determination of CD4+CD28null by means of flow cytometry analysis is an appropriate tool to identify COPD patients at risk.

Clinical perspective in comparison with other aetiologies

Whatever competing mechanism is causative for COPD, the presence of systemic chronic inflammation in COPD has been associated with a variety of co-morbidities including cachexia [34], osteoporosis [35] and cardiovascular diseases. The relationship between COPD and cardiovascular diseases is especially germane, as more than half of patients with COPD die of cardiovascular causes [36–38]. Nakajima *et al.* demonstrated that patients with acute ischaemic heart disease are characterized by a perturbation of functional T cell repertoire (CD4+CD28null) with a bias towards increased IFN-γ production compared with controls [39,40]. Of particular importance is a study by Pingiotti *et al.* They were able to show that patients with rheumatoid arthritis (RA) show increased circulating CD4+CD28null T cells that are related directly to pre-clinical atherosclerotic changes, such as arterial endothelial dysfunction and carotid artery wall thickening [41]. Our observation of T cell pool perturbation in COPD might be relevant in explaining the previously observed long-term cardiovascular risk in this disease entity. However, it remains unclear whether the higher percentage of CD4+CD28null T cells is the result of the inflammatory process, i.e. prematurely senescent CD4+ cells that are unable to go into cell death but still secrete cytokines, or if it represents a subset of COPD subjects whose pathogenetic process includes generation of this T cell subset at an early stage of the disease.

If we interpret our data correctly, a detailed picture is emerging. Chronic antigen exposure, e.g. through contents of tobacco smoke, leads to loss of CD28 and up-regulation of NK cell receptors expression on T cells in potentially genetically susceptible patients. This induced immunological 'senescence' is accompanied by a dysregulation of apoptosis-inducing signals, e.g. Bcl-2, fostering longevity of cytotoxic T cells and increased secretion of IFN-γ and TNF-α upon T cell triggering [15]. In conclusion, we believe that the appearance of clonogenic T cells in COPD patients is partially causative for the progressive cell-based inflammatory process in lung tissue irrespective of smoking status.

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Disclosures

The Medical University of Vienna claims financial interest.

References

1 Murray CJ, Lopez AD. Mortality by cause for eight regions of the world: Global Burden of Disease study. Lancet 1997; **349**:1269–76.
2 Murray CJ, Lopez AD. Alternative projections of mortality and disability by cause 1990–2020: Global Burden of Disease Study. Lancet 1997; **349**:1498–504.
3 Hurd S. The impact of COPD on lung health worldwide: epidemiology and incidence. Chest 2000; **117**:1S–4S.
4 Mannino DM. COPD: epidemiology, prevalence, morbidity and mortality, and disease heterogeneity. Chest 2002; **121**:121S–6S.
5 Lange P, Groth S, Nyboe GJ *et al.* Effects of smoking and changes in smoking habits on the decline of FEV1. Eur Respir J 1989; **2**:811–6.
6 Higenbottam T, Clark TJ, Shipley MJ, Rose G. Lung function and symptoms of cigarette smokers related to tar yield and number of cigarettes smoked. Lancet 1980; **1**:409–11.
7 Mercer PF, Shute JK, Bhowmik A, Donaldson GC, Wedzicha JA, Warner JA. MMP-9, TIMP-1 and inflammatory cells in sputum from COPD patients during exacerbation. Respir Res 2005; **6**:151.
8 Sin DD, Man SF. Why are patients with chronic obstructive pulmonary disease at increased risk of cardiovascular diseases? The potential role of systemic inflammation in chronic obstructive pulmonary disease. Circulation 2003; **107**:1514–9.
9 Global Strategy for the Diagnosis, Management and Prevention of COPD, Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2007. Available at: http://www.goldcopd.org.
10 Hodge S, Hodge G, Nairn J, Holmes M, Reynolds PN. Increased airway granzyme b and perforin in current and ex-smoking COPD subjects. COPD 2006; **3**:179–87.
11 Hodge G, Nairn J, Holmes M, Reynolds PN, Hodge S. Increased intracellular T helper 1 proinflammatory cytokine production in peripheral blood, bronchoalveolar lavage and intraepithelial T cells of COPD subjects. Clin Exp Immunol 2007; **150**:22–9.
12 Di Stefano A, Caramori G, Capelli A *et al.* STAT4 activation in smokers and patients with chronic obstructive pulmonary disease. Eur Respir J 2004; **24**:78–85.
13 Di Stefano A, Capelli A, Lusuardi M *et al.* Severity of airflow limitation is associated with severity of airway inflammation in smokers. Am J Crit Care Med 1998; **158**:1277–85.
14 Campisi J. Replicative senescence: an old lives' tale? Cell 1996; **84**:497–500.
15 Goronzy JJ, Weyand CM. Aging, autoimmunity and arthritis: T-cell senescence and contraction of T-cell repertoire diversity – catalysts of autoimmunity and chronic inflammation. Arthritis Res Ther 2003; **5**:225–34.
16 Choremi-Papadopoulou H, Viglis V, Gargalianos P, Kordossis T, Iniotaki-Theodoraki A, Kosmidis J. Downregulation of CD28 surface antigen on CD4+ and CD8+ T lymphocytes during HIV-1 infection. J Acquir Immune Defic Syndr 1994; **7**:245–53.
17 Hooper M, Kallas EG, Coffin D, Campbell D, Evans TG, Looney RJ. Cytomegalovirus seropositivity is associated with the expansion of CD4+CD28- and CD8+CD28- T cells in rheumatoid arthritis. J Rheumatol 1999; **26**:1452–7.
18 Moosig F, Csernok E, Wang G, Gross WL. Costimulatory molecules in Wegener's granulomatosis (WG): lack of expression of CD28 and preferential up-regulation of its ligands B7-1 (CD80) and B7-2 (CD86) on T cells. Clin Exp Immunol 1998; **114**:113–8.
19 Markovic-Plese S, Cortese I, Wandinger KP, McFarland HF, Martin R. CD4+CD28- costimulation-independent T cells in multiple sclerosis. J Clin Invest 2001; **108**:1185–94.
20 Schirmer M, Goldberger C, Wurzner R *et al.* Circulating cytotoxic CD8(+) CD28(-) T cells in ankylosing spondylitis. Arthritis Res 2002; **4**:71–6.
21 Griffiths GM, Alpert S, Lambert E, McGuire J, Weissman IL. Perforin and granzyme A expression identifying cytolytic lymphocytes in rheumatoid arthritis. Proc Natl Acad Sci USA 1992; **89**:549–53.
22 Schirmer M, Vallejo AN, Weyand CM, Goronzy JJ. Resistance to apoptosis and elevated expression of Bcl-2 in clonally expanded CD4+CD28- T cells from rheumatoid arthritis patients. J Immunol 1998; **161**:1018–25.
23 Lopez-Botet M, Bellon T. Natural killer cell activation and inhibition by receptors for MHC class I. Curr Opin Immunol 1999; **11**:301–7.
24 Lanier LL. NK cell receptors. Annu Rev Immunol 1998; **16**:359–93.
25 *Global strategy for the diagnosis, management and prevention of chronic pulmonary disease (GOLD)*. NIH Publication no. 2701. National Heart, Lung, and Blood Institute/World Health Organization, 2005. Available at: http://www.goldcopd.org.
26 Rabe KF, Beghe B, Luppi F, Fabbri LM. Update in chronic obstructive pulmonary disease 2006. Am J Respir Crit Care Med 2007; **175**:1222–32.
27 Quanjer PH, Tammeling GJ, Cotes JE, Pedersen OF, Peslin R, Yernault JC. Lung volumes and forced ventilatory flows. Report of the Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. Eur Respir J Suppl 1993; **16**:5–40.
28 Shaffer JP. Modified sequentially rejective multiple test procedures. J Am Stat Assoc 1986; **81**:826–31.
29 Lanier LL. Back to the future – defining NK cells and T cells. Eur J Immunol 2007; **37**:1424–6.
30 Warrington KJ, Takemura S, Goronzy JJ, Weyand CM. CD4+,CD28- T cells in rheumatoid arthritis patients combine features of the innate and adaptive immune systems. Arthritis Rheum 2001; **44**:13–20.
31 Speiser DE, Valmori D, Rimoldi D *et al.* CD28-negative cytolytic effector T cells frequently express NK receptors and are present at variable proportions in circulating lymphocytes from healthy donors and melanoma patients. Eur J Immunol 1999; **29**:1990–9.
32 Gallin JI, Farber JM, Holland SM *et al.* Interferon-gamma in the management of infectious diseases. Ann Intern Med 1995; **123**:216–24.
33 Antoniou KM, Ferdoutsis E, Bouros D. Interferons and their application in the diseases in the lung. Chest 2003; **123**:209–16.
34 Agusti AG. Systemic effects of chronic obstructive pulmonary disease. Proc Am Thorac Soc 2005; **2**:367–70; discussion 71–2.
35 Ionescu AA, Schoon E. Osteoporosis in chronic obstructive pulmonary disease. Eur Respir J Suppl 2003; **46**:64s–75s.
36 Calverley PM, Scott S. Is airway inflammation in chronic obstructive pulmonary disease (COPD) a risk factor for cardiovascular events? COPD 2006; **3**:233–42.
37 Friedman GD, Klatsky AL, Siegelaub AB. Lung function and risk of

myocardial infarction and sudden cardiac death. N Engl J Med 1976; **294**:1071–5.

38 Camilli AE, Robbins DR, Lebowitz MD. Death certificate reporting of confirmed airways obstructive disease. Am J Epidemiol 1991; **133**:795–800.

39 Nakajima T, Schulte S, Warrington KJ *et al.* T-cell-mediated lysis of endothelial cells in acute coronary syndromes. Circulation 2002; **105**:570–5.

40 Nakajima T, Goek O, Zhang X *et al. De novo* expression of killer immunoglobulin-like receptors and signaling proteins regulates the cytotoxic function of CD4 T cells in acute coronary syndromes. Circ Res 2003; **93**:106–13.

41 Pingiotti E, Cipriani P, Marrelli A *et al.* Surface expression of fractalkine receptor (CX3CR1) on CD4+/CD28 T cells in RA patients and correlation with atherosclerotic damage. Ann NY Acad Sci 2007; **1107**:32–41.

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ORIGINAL ARTICLE

Elevated HSP27, HSP70 and HSP90α in Chronic Obstructive Pulmonary Disease: Markers for Immune Activation and Tissue Destruction

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SUMMARY

Chronic obstructive pulmonary disease (COPD) is one of the leading causes of death. Although the underlying pathomechanism remains poorly understood, COPD is accompanied by increased cellular stress and inflammation. We investigated serum contents of heat shock proteins (HSP 27, 60, 70, 90alpha), 20S proteasomes, C-reactive protein (CRP), and interleukin-6 (IL-6) in patients with mild or severe COPD, healthy smokers and non-smokers. HSP27, HSP70 and HSP90α were significantly altered in patients suffering from COPD as compared to controls. HSP27 and HSP70 are potential novel serum markers for the diagnosis of COPD in the smoking population. This is the first study to demonstrate elevated serum levels of the described heat shock proteins in patients with COPD. We showed sensitivity and specificity of serum HSP27 and HSP70 as diagnostic markers for COPD. (Clin. Lab. 2009;55:31-40)

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a significant burden to health care systems worldwide. Despite COPD being one of the leading causes of death and disability (1), well designed epidemiological studies remain vague leading to the impression of COPD as a rare disease (2). Contrary, prevalence has inclined dramatically over the past years (3). Although active tobacco smoking is thought to be the predominant risk factor (4) only a fraction of smokers develop the clinical features of COPD (5). Currently, the diagnostic process requires the assessment of lung function parameters. In several recent studies genetic factors were considered to contribute to the susceptibility to develop COPD (6). Results of these evaluations varied and did not reveal a definite answer. The only accepted genetic cause of emphysema to date is severe α1-antitrypsin deficiency (AATD).

Like the many possible etiologies triggering the development of COPD, the underlying pathogenic pathways remain poorly understood. The disease is characterized by irreversible airflow obstruction - the current diagnostic criterion - due to remodelling and an aberrant inflammatory response (7). Chronic bronchitis and lung emphysema are pathologic characteristics of COPD and both conditions result from progressive inflammatory destruction of the lung parenchyma. Airflow limitation is slowly progressive, leading to dyspnoea and limitations of physical exercise capacities (8). However, immune activation is not restricted to the lungs, as patients suffering from COPD are also at higher risk for cardiovascular diseases (9) and autoimmune diseases such as Ulcerative Colitis and Crohn's Disease (10) Interestingly, some authors presented data of persisting inflam-

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matory reactions in COPD patients despite cessation of smoking and without other forms of exogenous triggering of an inflammatory response. These findings are suggesting an autoimmune aspect contributing to the disease progression (11).

Heat shock proteins (HSPs) are a group of highly preserved stress proteins that are ubiquitously expressed in all cells. The main functions of these proteins comprise a process referred to as chaperoning - the conservation of the correct protein structure under stress conditions - as well as the regulation of death pathways (12). The classification of these stress proteins follows their molecular weight, e.g. the HSP70 has an approximate molecular weight of 70 kDa. Under normal physiological conditions HSPs usually account for up to 5% of the total protein content of a cell. Expression of HSPs is upregulated under stressful events like heat, bacterial or viral infections (13, 14, 15). Newly synthesized HSPs are thought to fold denatured proteins and prevent activation of caspases otherwise leading to active cell death, e.g. apoptosis. Besides known intracellular chaperoning, HSPs may also be released into the extracellular space following massive trauma or stress (16, 17). This spillage of proteins serves as "danger signal" leading to cytokine transcription and release (18). Furthermore, extracellular stress proteins are able to induce the adaptive immune system through binding to antigenic peptides. These HSP–peptide complexes are then processed by antigen presenting cells via MHC class I molecules and lead to activation of cytotoxic T-lymphocytes (19). Extracellular and intravascular HSPs seem to play a key role in the activation of the immune response following stressors like trauma, heat or infection.

The 20S proteasome is a multicatalytic protease complex, localized in the cytosol as well as in the nucleus of all eukaryotic cells. It is crucially involved in the enzymatic degradation of ubiquitinated proteins. The proteasome consists of a cylindrical-shaped core particle, the 20S proteasome, which itself contains two sets of seven different α and ß subunits assembled in four heptameric rings. Spillage of intracellular 20S proteasome was recently shown to occur during degradative processes of cells, e.g. sepsis, trauma, acute liver failure, and on-pump coronary artery bypass grafting (20, 21, 22).

The aim of the present study was to evaluate in a well-defined study cohort whether the serum levels of various heat shock proteins and 20S proteasome as markers for immune activation and cellular destruction are elevated in patients with COPD. Furthermore, we tested the ability of serum HSPs and 20S proteasome to serve as diagnostic markers for the detection of COPD. The diagnostic use of serum proteins prior to lung function testing may support the diagnostic process and lead to an earlier treatment of patients with COPD.

MATERIALS AND METHODS

Patients

A total number of 64 patients and controls were included in this case control study. The study protocol was approved by the ethics committee of the Medical University of Vienna (EK Nr.: 091/2006). All clinical and laboratory tests were performed in accordance with the Declaration of Helsinki and the guidelines for Good Clinical Practice of the Medical University of Vienna. All patients and controls provided written, informed consent before entering the study. Healthy non-smoking volunteers (n=15), smokers without COPD (n=14), patients with mild to moderate COPD (n=19) and patients with severe or very severe COPD (n=16) were evaluated in four study groups. Patient characteristics are depicted in Table 1. Exclusion criteria were acute exacerbation as defined by the guidelines of the WHO and the Global Initiative for Chronic Obstructive Lung Disease (GOLD) or use of immunomodulatory drugs – including steroids - within the past 14 days, history of asthma, autoimmune diseases, other relevant lung diseases (e.g., lung cancer, known α1-antitrypsin deficiency), or any known cardiopulmonary co-morbidity. Height and weight (Seca; Vogel and Halke, Hamburg, Germany) were measured and the body mass index (BMI) was determined. Pulmonary function parameters (FEV1, FVC, FEV1:FVC ratio) were measured using the same model spirometer (AutoboxV6200, SensorMedics, Vienna, Austria). Measurements were made before and – if criteria for airflow obstruction were met – 15-30 minutes after inhaling of 200 µg salbutamol. Arterial blood gases (PaO_2, $PaCO_2$) were obtained at rest while breathing room air in a sitting position. Measurement of arterial blood gases was performed with an ABL 510 gas analyzer (Radiometer, Copenhagen, Denmark). Results are expressed as absolute values and as percentages of predicted values for age, sex and height, according to the European Community for Steel and Coal prediction equations. Predicted normal values were derived from the reference values of the Austrian Society of Pulmonary Medicine. Blood samples were collected at the time of pulmonary evaluation. Serum was acquired after centrifugation and aliquots were kept frozen at -20° Celsius until further testing.

Heat Shock Proteins 27, 60, and 70

Levels of HSP27, HSP60, and HSP70 were determined using adapted enzyme-linked immunosorbent assay (ELISA) kits for the quantification of intracellular HSP (Duoset IC; R&D Systems, Minneapolis, MN, USA). Ninety-six–well microtiter plates were coated overnight at 4°C with the capture antibody at a concentration of 1 µg/mL. After blocking of plates, serum samples and standard protein in different concentrations were added to the wells. After a washing step, a biotin-labelled antibody was added to each well and incubated for 1 hour. Plates were washed and Streptavidin-HRP was added. Color reaction was achieved using tetramethylbenzidine

Table 1: Clinical characteristics (severity of airflow obstruction was determined using lung function test in all subjects; COPD patients meeting the GOLD diagnostic criteria for COPD). Data are given as mean (+/- standard deviation) if not otherwise stated.

Subject Category	Healthy Non-Smoker	Healthy Smoker	COPD GOLD I&II	COPD GOLD III&IV
N	15	14	19	16
Male/Female	10/5	7/7	10/9	10/6
Age	57.20 (12.50)	56.64 (9.17)	60.68 (7.39)	58.31 (8.75)
Body Weight (kg)	71.6 (13.9)	76.4 (8.6)	79.7 (16.7)	81.1 (27.2)
Body Height (cm)	172.7 (10.9)	168.7 (8.1)	167.7 (12.1)	171.2 (7.9)
Lung Function				
FVC (L)	4.55 (0.94)	3.84 (0.66)	3.33 (1.06)	2.14 (0.70)
FEV1 (%)	105.37 (17.11)	94.40 (11.96)	70.21 (13.33)	30.67 (12.66)
FEV1/VC (%)	76.80 (7.85)	75.95 (3.99)	61.74 (8.36)	37.80 (15.33)
MEF 50 (%)	100.67 (28.92)	87.64 (21.45)	39.42 (15.93)	11.93 (6.60)
MEF 25 (%)	103.53 (33.89)	75.71 (31.33)	37.37 (16.19)	20.00 (5.94)
Smoking History				
Never-smoker	15	0	0	0
Ex-smoker	0	3	4	3
Current-smoker	0	11	15	13
Pack Years	0	34 (25.2)	47.3 (29.7)	44.0 (32.6)

Abbrevations used: COPD: Chronic obstructive pulmonary disease, FEV1: Forced expiratory volume in one second, FVC: Forced vital capacity, GOLD: Global Initiative for Chronic Obstructive Lung Disease MEF: Maximum expiratory flow.

(TMB; Sigma, St. Louis, MO, USA) and was stopped by an acid stop solution. Optical density was measured at 450 nm on an ELISA reader.

Heat Shock Protein 90alpha

Serum levels of HSP90alpha (HSP90α) were measured with a commercially available ready-to-use ELISA kit (Stressgen, Ann Arbor, MI, USA). In brief, serum samples and standards were incubated in 96-well microtiter plates, precoated with anti-human HSP90α antibody. After a washing step, anti-HSP90α:HRP-conjugated antibody was added, and plates were incubated for 24 hours. Plates were washed and TMB substrate was added. Color development was stopped by an acid stop solution, and optical density was determined at 450 nm. The amount of protein in each sample was calculated according to a standard curve of optical density values constructed for known levels of HSP90α. The sensitivity of the ELISA has been determined to be 50 pg/mL; the intra-assay variability is stated to be less than 10% by the manufacturer.

20S Proteasome

Microtiter plates were incubated overnight at 4°C with a monoclonal antibody against the C6-subunit of the 20S proteasome (Biomol, Plymouth Meeting, PA, USA). Plates were washed and blocked for 1 hour with 1% BSA in phosphate-buffered saline. Serum samples and different concentrations of a standard protein (Biomol) were added, then plates were sealed and incubated for 24 hours at 4°C. A rabbit polyclonal antibody to 20S proteasome α/β subunits (Biomol), serving as the detection antibody, was added; and after a washing step, plates were incubated with a peroxidase-labeled donkey anti-rabbit IgG (Jackson ImmunoResearch, Soham, United Kingdom) for another 2 hours. Tetramethylbenzidine served as color substrate. The reaction was stopped by adding 1N sulphuric acid. Plates were read at 450 nm using a Wallac Multilabel counter 1420 (PerkinElmer, Boston, MA, USA).

Interleukin-6

Serum levels of IL-6 were determined by a commercially available ELISA kit (BenderMedSystems, Vienna, Austria). Assays were performed ac-

cording to the manufacturer's instructions. Plates were read at 450 nm on an ELISA reader, and IL-6 contents were calculated comparing optical density values of samples with optical density values of known IL-6 concentrations.

C-reactive Protein
Serum levels of C-reactive protein were routinely analyzed by the Department of Laboratory Medicine at the Medical University of Vienna.

Statistical Methods
SPSS Software (SPSS Inc., Chicago, IL, USA) was used to calculate all results. A p-value <0.05 was considered statistically significant. Pair-wise comparisons between groups were performed using the Mann-Whitney-U-Test. Correlations were calculated using the Spearman-Correlation-Coefficient. Univariate logistic regression models in a subgroup excluding healthy non-smokers were calculated for HSPs and 20S proteasome. Receiver operating characteristic (ROC) curves were plotted to demonstrate sensitivity and specificity of the evaluated serum proteins. Results were not corrected for multiple testing.

RESULTS

HSP27
Serum levels of HSP27 were 2042.57 pg/mL [1599.58 - 2485.57] (mean [95% confidence interval]) in healthy controls, 2199.64 [1641.52 - 2757.75] in healthy smokers, 2862.62 [2280.49 - 3444.74] in COPD GOLD I-II, and 3717.58 [3079.35 - 4355.81] in COPD GOLD III-IV. Statistically significant differences were found between healthy controls and COPD I-II (p=0.025), healthy controls and COPD III-IV (p<0.001), healthy smokers and COPD III-IV (p=0.001), and COPD I-II and COPD III-IV (p=0.026). Serum levels of HSP27 did not correlate with body weight. Figure 1a.

HSP60
Serum levels of HSP60 were 1836.69 pg/mL [153.30 - 3520.08] in healthy controls, 4378.40 [-3851.48 - 12608.27] in healthy smokers, 3497.42 [-1561.38 - 8556.23] in COPD GOLD I-II, and 531.81 [132.57-931.05] in COPD GOLD III-IV. No statistically significant differences were found between the groups. Serum levels of HSP60 showed a weak correlation with body weight (R=-0.269; p= 0.034). Figure 1b.

HSP70
Serum levels of HSP70 were 140.50 pg/mL [67.97 - 213.04] in healthy controls, 108.50 [43.30 - 173.69] in healthy smokers, 454.29 [327.05 - 581.52] in COPD GOLD I-II, and 437.92 [143.41 - 732.42] in COPD GOLD III-IV.
Statistically significant differences were found between healthy controls and COPD I-II (p<0.001), healthy controls and COPD III-IV (p=0.009), healthy smokers and COPD I-II (p<0.001), and healthy smokers and COPD III-IV (p<0.001). Serum levels of HSP70 did not correlate with body weight. Figure 1c.

HSP90alpha
Serum levels of HSP90alpha were 13133.78 pg/mL [9791.40 - 16476.15] in healthy controls, 12827.91 [10838.21 - 14817.62] in healthy smokers, 17884.50 [13307.14 - 22461.85] in COPD GOLD I-II, and 17273.02 [12573.96 - 21972.08] in COPD GOLD III-IV. Statistically significant differences were found between healthy controls and COPD I-II (p=0.025), and healthy controls and COPD III-IV (p=0.049). Serum levels of HSP90alpha showed a weak correlation with body weight (R=0.257; p= 0.044). Figure 1d.

20S Proteasome
Serum levels of 20S proteasome were 194.78 ng/mL [164.94 - 224.62] in healthy controls, 188.25 [159.26 - 217.25] in healthy smokers, 172.33 [133.78 - 210.88] in COPD GOLD I-II, and 187.50 [145.54 - 229.46] in COPD GOLD III-IV. No statistically significant differences were found between the groups. Serum levels of 20S proteasome did not correlate with body weight. Figure 1e.

Interleukin-6
Serum levels of IL-6 were 5.18 pg/mL [0.17 - 10.19] in healthy controls, 1.65 [-0.11 - 3.42] in healthy smokers, 7.14 [1.19 - 13.09] in COPD GOLD I-II, and 2.99 [0.99 - 5.00] in COPD GOLD III-IV. A statistically significant difference was found between healthy smokers and COPD I-II (p=0.017). Serum levels of IL-6 did not correlate with body weight.

C-reactive Protein
Serum levels of CRP were 0.19 mg/dL [0.00 – 0.37] in healthy controls, 0.20 [0.11 - 0.29] in healthy smokers, 1.10 [0.54 - 1.66] in COPD GOLD I-II, and 0.71 [0.37 - 1.05] in COPD GOLD III-IV.
Statistically significant differences were found between healthy controls and COPD I-II (p<0.001), healthy controls and COPD III-IV (p=0.001), healthy smokers and COPD I-II (p<0.001), and healthy smokers and COPD III-IV (p=0.004). Serum levels of CRP did not correlate with body weight.

Regression Models
In univariate logistic regression models including only healthy smokers and patients with COPD, HSP27 had an area under the curve (AUC) in the receiver operating characteristic (ROC) curve of 0.763 (0.624 – 0.902; 95% CI; p=0.004), and HSP70 showed an AUC of 0.885 (0.786 – 0.983: 95% CI; p<0.001). All other variables showed no significant result in the univariate logistic regression analysis. Figure 2.

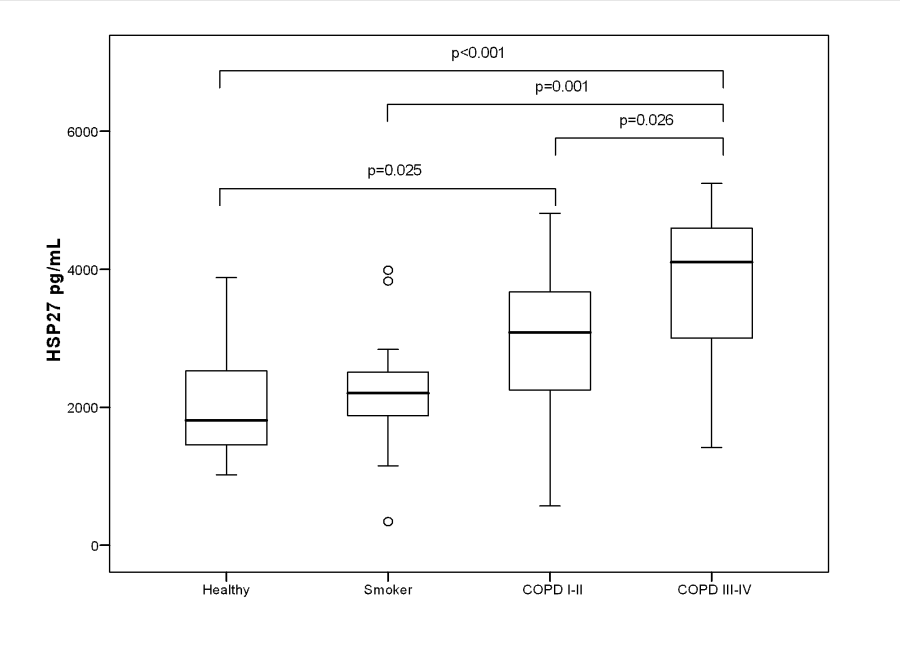

Figure 1a

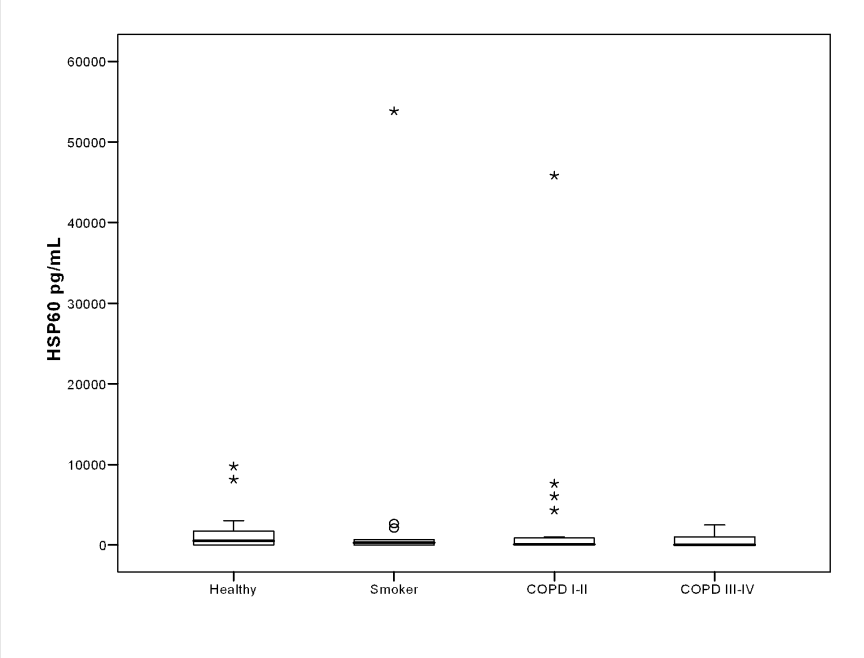

Figure 1b

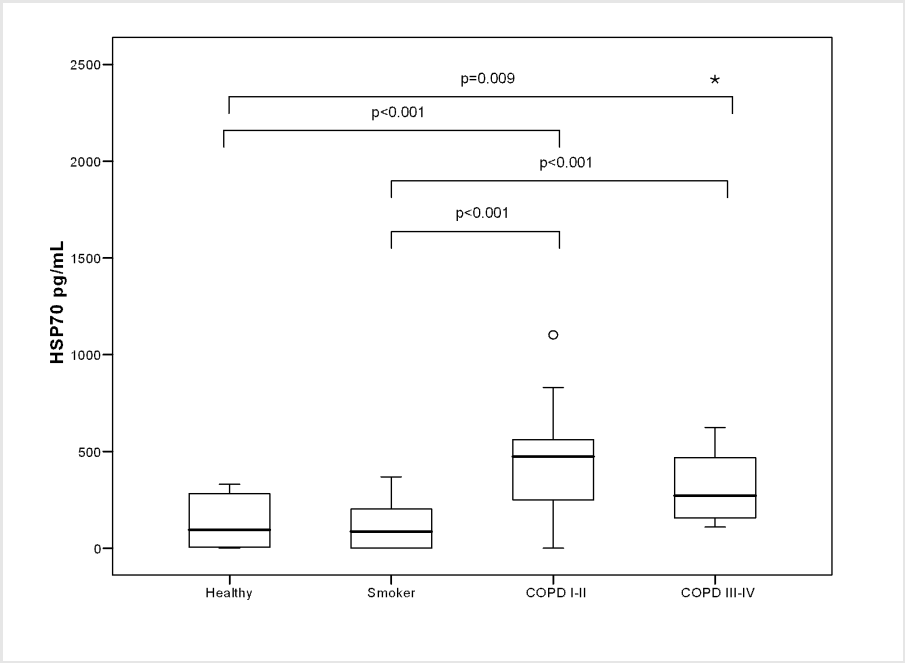

Figure 1c

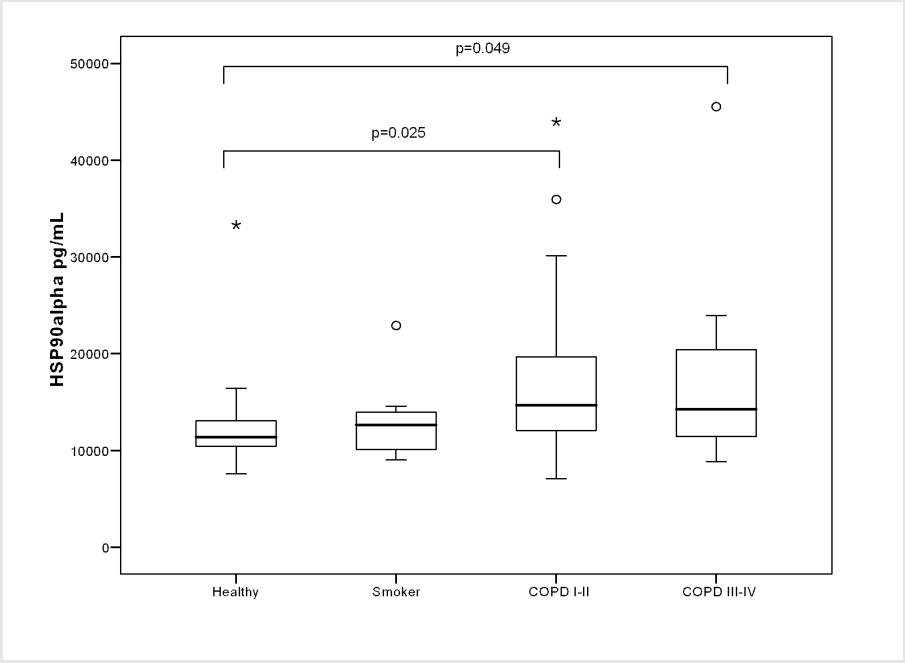

Figure 1d

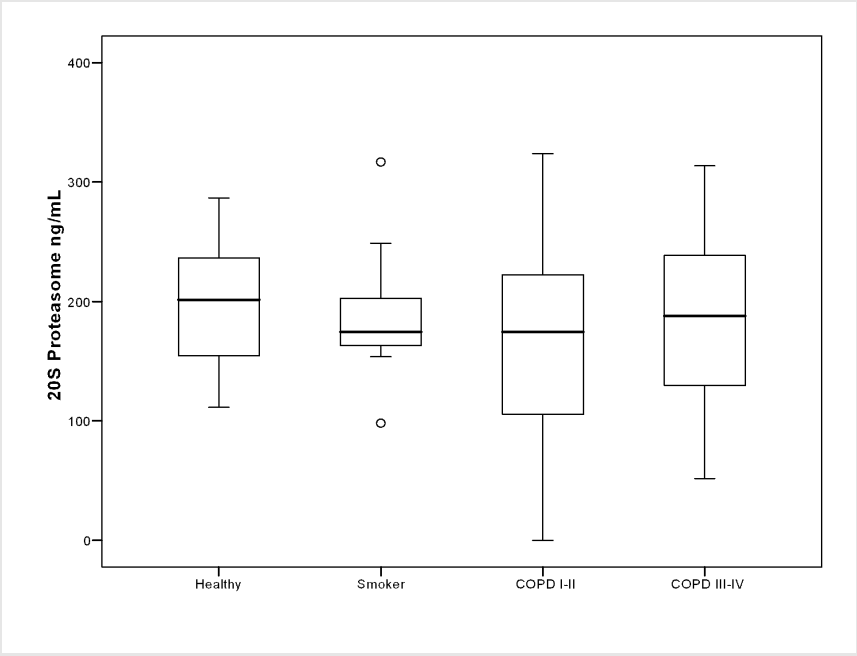

Figure 1e

Figure 1 (a, b, c, d, e): Serum levels of heat shock proteins and 20S proteasome were determined in the systemic blood flow of patients and controls. Bars indicate medians; solid boxes show span between 25th and 75th percentile; whiskers illustrate lowest and highest values; outliers are marked. P-values indicate significant differences.

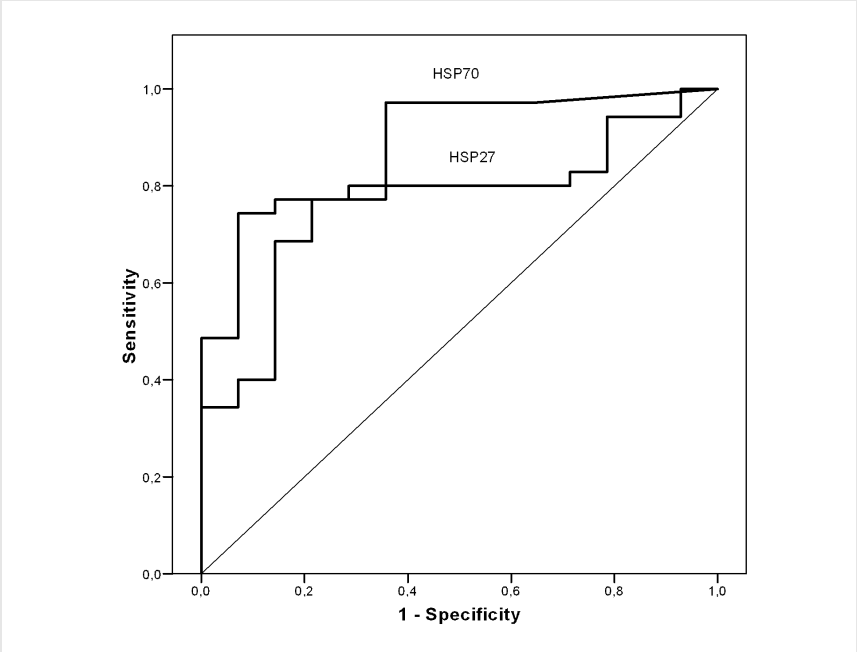

Figure 2: Receiver operating characteristic (ROC) curve indicating sensitivity and specificity of HSP27 and HSP70 to diagnose COPD in the smoking study population.

DISCUSSION

Patients suffering from chronic obstructive pulmonary disease present progressive inflammation of the bronchial airways, small airways, and lung parenchyma. Lung biopsies revealed massive infiltration of the peribronchial tissue with neutrophils, macrophages, and lymphocytes as part of the innate and adaptive immune system (7, 23). Activation of these cells is believed to lead to remodelling of the lung tissue (24). In COPD, both endogenous factors including neutrophils and cytotoxic T-cells (25) as well as exogenous stimuli like tobacco smoke (26) are thought to contribute towards tissue destruction. However, cessation of smoking does not alter impairment of the inflammatory response. The constant induction of inflammatory signals and increased cellular turnover result in upregulation of intracellular heat shock proteins and augmented release into the extracellular environment. We were able to show a significant increase of HSP27 in serum samples taken from the peripheral blood flow of patients suffering from COPD as compared to healthy smokers. HSP27 functions as repair mechanism aiming at the stability and correct posttranslational folding of intracellular proteins as well as the prevention of apoptotic cell death. Elevated serum levels of HSP27 were reported in inflammatory disorders including acute coronary syndrome and chronic allograft nephropathy (27, 28). Expression of HSP27 is transiently induced as a response to stress events. Termination of the acute triggering results in an immediate downregulation of HSP27 concentrations to normal levels. Thus, HSP27 is only upregulated when its cytoprotective properties are required (22). Interestingly, our results demonstrate a continuous increase of serum HSP27 concentrations with disease severity. This effect may be due to increased tissue devastation especially in late stages of COPD and spreading of the inflammatory disease to other organ systems resulting in a systemic spillage of HSP27 into the vascular bed. HSP27 generally acts as anti-apoptotic mediator (29) and can be seen as an endogenous immunosuppressive attempt to control excessive inflammation in COPD. Serum contents of HSP27 showed diagnostic potential to determine the occurrence of COPD in a logistic regression model and may serve as marker for diagnosis and prediction of disease severity. Further explorations are needed to determine optimal cut-off values and improve the proposed sensitivity and specificity of serum HSP27 in a clinical setting.

The role of extracellular HSP60 has not been well defined. Some authors have described pro-inflammatory features, primarily in atherosclerosis (12, 30). Our data did not provide any support for HSP60 being a key element in the pathogenesis of COPD. Serum concentrations of HSP60 did not correlate with levels of other HSPs. However, as we did not include any patients with known coronary artery disease in our study, further investigations are needed to define the role of soluble HSP60 in patients with COPD at increased risk for coronary events.

Serum levels of HSP70 were elevated in patients at early and late stages of COPD. We evidenced a four-fold increase in the GOLD I-II group compared to non-symptomatic smokers. HSP70 is an intracellular chaperone that is released into the extracellular space upon cell death (31) or by means of various secreting pathways (32). Extracellular HSP70 has been reported to activate cells of the innate and adaptive immune system and to stimulate cytokine production (18, 32). We found increased concentrations of soluble HSP70 in COPD samples. Values peaked in the COPD I-II group indicating a state of vast immune activation primarily at the early stages of the disease. Furthermore, serum contents of HSP70 showed high sensitivity and specificity to determine the occurrence of COPD in a logistic regression model and could serve as diagnostic marker. Because there was no significant difference between the COPD groups, HSP70 – unlike HSP27 – might not be suitable as marker for disease progression or response to therapy. Soluble HSP90α was significantly upregulated in the peripheral blood flow in the COPD groups as compared to healthy non-smokers. Elevated levels of HSP90α have previously been described in on-pump coronary artery bypass grafting (22) and wound healing after hypoxia (33). Rajagopal et al. characterized HSP90 as central factor in antigen presentation to T-lymphocytes via major histocompatibility complex class II molecules (MHC II) (34). In synopsis with our data, we hypothesize that elevated levels of extracellular HSP90α in COPD are an essential elicitor of the adaptive immune system, triggering a possible autoreactive response to self-antigen. This function of HSP90α may also change the immunogenicity of the associated antigen. Therefore, HSP90α has immunomodulatory effects through cross-presentation of associated peptides in the context of major histocompatibility complex molecules. The exact immunological role of increased serum HSP-90α in COPD has to be addressed in further studies. The extracellular content of 20S proteasome was not statistically increased or decreased in the investigated study cohorts. Levels remained under 200 ng/mL in all groups and appear to be of subordinate importance in the progression of COPD.

In conclusion, we were able to demonstrate elevated serum concentrations of soluble heat shock proteins 27, 70 and 90α in patients with COPD. This spillage into the vascular bed may be caused by continuous activation of the immune system in the deterioration of COPD through endogenous and exogenous trigger mechanisms. This is the first study to demonstrate elevated serum levels of the described HSPs in patients with COPD at stable stages of the disease. Furthermore, HSP27 and HSP70 showed statistical trends to serve as diagnostic markers or markers for disease progression. Further investigations employing higher numbers of patients are needed to establish diagnostic algorithms using serum levels of HSPs.

References

1. Lopez AD, Mathers CD, Ezzati M, Jamison DT and Murray CJ. Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. Lancet 2006; 367: 1747-57.

2. Halbert RJ, Isonaka S, George D and Iqbal A. Interpreting COPD prevalence estimates: what is the true burden of disease? Chest 2003; 123: 1684-92.

3. Mannino DM. COPD: epidemiology, prevalence, morbidity and mortality, and disease heterogeneity. Chest 2002; 121: 121S-6S.

4. Higenbottam T, Clark TJ, Shipley MJ and Rose G. Lung function and symptoms of cigarette smokers related to tar yield and number of cigarettes smoked. Lancet 1980; 1: 409-11.

5. Fairclough L, Urbanowicz RA, Corne J and Lamb JR. Killer cells in chronic obstructive pulmonary disease. Clin Sci (Lond) 2008; 114: 533-41.

6. DeMeo DL, Hersh CP, Hoffman EA et al. Genetic determinants of emphysema distribution in the national emphysema treatment trial. Am J Respir Crit Care Med 2007; 176: 42-8.

7. Barnes PJ. Immunology of asthma and chronic obstructive pulmonary disease. Nat Rev Immunol 2008; 8: 183-92.

8. Celli BR, Cote CG, Marin JM et al. The body-mass index, airflow obstruction, dyspnea, and exercise capacity index in chronic obstructive pulmonary disease. N Engl J Med 2004; 350: 1005-12.

9. Sin DD and Man SF. Why are patients with chronic obstructive pulmonary disease at increased risk of cardiovascular diseases? The potential role of systemic inflammation in chronic obstructive pulmonary disease. Circulation 2003; 107: 1514-9.

10. Ekbom A, Brandt L, Granath F, Lofdahl CG and Egesten A. Increased Risk of Both Ulcerative Colitis and Crohn's Disease in a Population Suffering from COPD. Lung 2008; 186: 167-72.

11. Willemse BW, ten Hacken NH, Rutgers B, Lesman-Leegte IG, Postma DS and Timens W. Effect of 1-year smoking cessation on airway inflammation in COPD and asymptomatic smokers. Eur Respir J 2005; 26: 835-45.

12. Calderwood SK, Mambula SS, Gray PJ, Jr. and Theriault JR. Extracellular heat shock proteins in cell signaling. FEBS Lett 2007; 581: 3689-94.

13. Feder ME and Hofmann GE. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu Rev Physiol 1999; 61: 243-82.

14. Sedger L and Ruby J. Heat shock response to vaccinia virus infection. J Virol 1994; 68: 4685-9.

15. Zheng L, He M, Long M, Blomgran R and Stendahl O. Pathogen-induced apoptotic neutrophils express heat shock proteins and elicit activation of human macrophages. J Immunol 2004; 173: 6319-26.

16. Basu S, Binder RJ, Suto R, Anderson KM and Srivastava PK. Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kappa B pathway. Int Immunol 2000; 12: 1539-46.

17. Hightower LE and Guidon PT, Jr. Selective release from cultured mammalian cells of heat-shock (stress) proteins that resemble glia-axon transfer proteins. J Cell Physiol 1989; 138: 257-66.

18. Asea A, Kraeft SK, Kurt-Jones EA et mal. HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. Nat Med 2000; 6: 435-42.

19. Arnold-Schild D, Hanau D, Spehner D et al. Cutting edge: receptor-mediated endocytosis of heat shock proteins by professional antigen-presenting cells. J Immunol 1999; 162: 3757-60.

20. Roth GA, Moser B, Krenn C et al. Heightened levels of circulating 20S proteasome in critically ill patients. Eur J Clin Invest 2005; 35: 399-403.

21. Hetz H, Hoetzenecker K, Hacker S et al. Caspase-cleaved cytokeratin 18 and 20 S proteasome in liver degeneration. J Clin Lab Anal 2007; 21: 277-81.

22. Szerafin T, Hoetzenecker K, Hacker S et al. Heat shock proteins 27, 60, 70, 90alpha, and 20S proteasome in on-pump versus off-pump coronary artery bypass graft patients. Ann Thorac Surg 2008; 85: 80-7.

23. Hogg JC, Chu F, Utokaparch S et al. The nature of small-airway obstruction in chronic obstructive pulmonary disease. N Engl J Med 2004; 350: 2645-53.

24. Mikko M, Fredriksson K, Wahlstrom J, Eriksson P, Grunewald J and Skold CM. Human T cells stimulate fibroblast-mediated degradation of extracellular matrix in vitro. Clin Exp Immunol 2008; 151: 317-25.

25. Majo J, Ghezzo H and Cosio MG. Lymphocyte population and apoptosis in the lungs of smokers and their relation to emphysema. Eur Respir J 2001; 17: 946-53.

26. Kang MJ, Homer RJ, Gallo A et al. IL-18 is induced and IL-18 receptor alpha plays a critical role in the pathogenesis of cigarette smoke-induced pulmonary emphysema and inflammation. J Immunol 2007; 178: 1948-59.

27. Park HK, Park EC, Bae SW et al. Expression of heat shock protein 27 in human atherosclerotic plaques and increased plasma level of heat shock protein 27 in patients with acute coronary syndrome. Circulation 2006; 114: 886-93.

28. Djamali A, Reese S, Oberley T, Hullett D and Becker B. Heat shock protein 27 in chronic allograft nephropathy: a local stress response. Transplantation 2005; 79: 1645-57.

29. Vander Heide RS. Increased expression of HSP27 protects canine myocytes from simulated ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 2002; 282: H935-41.

30. Xu Q. Role of heat shock proteins in atherosclerosis. Arterioscler Thromb Vasc Biol 2002; 22: 1547-59.

31. Madach K, Molvarec A, Rigo J et al. Elevated serum 70kDa heat shock protein level reflects tissue damage and disease severity in the syndrome of hemolysis, elevated liver enzymes, and low platelet count. Eur J Obstet Gynecol Reprod Biol 2008; 139: 133-8.

32. Vega VL, Rodriguez-Silva M, Frey T et al. Hsp70 Translocates into the Plasma Membrane after Stress and Is Released into the Extracellular Environment in a Membrane-Associated Form that Activates Macrophages. J Immunol 2008; 180: 4299-307.

33. Li W, Li Y, Guan S, Fan J et al. Extracellular heat shock protein-90alpha: linking hypoxia to skin cell motility and wound healing. EmboJ 2007; 26: 1221-33.

34. Rajagopal D, Bal V, Mayor S, George A and Rath S. A role for the Hsp90 molecular chaperone family in antigen presentation to T lymphocytes via major histocompatibility complex class II molecules. Eur J Immunol 2006; 36: 828-41.

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EDUCATION

2008/07 – 2008/08	Visiting Research Fellow at the Department of Surgery, Division of Plastic Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA
2007/11 – Present	Student Research Fellow at the Department of Plastic and Reconstructive Surgery, General Hospital Vienna, Medical University of Vienna, Vienna, Austria
2005/04 – Present	Student Research Fellow at the Department of Cardio-Thoracic Surgery, General Hospital Vienna, Medical University of Vienna, Vienna, Austria
2003/10 – Present	Medical Student at the Medical University of Vienna, Austria
2002/10 – 2003/09	Social Service at the Red Cross Austria
2002/06	Matura (High School Graduation) with Distinction
2000/08 – 2001/06	Special Visiting Student at Pine Crest Preparatory High School, Fort Lauderdale, Florida, USA
1994 – 2002	Bundesgymnasium (High School), Wieselburg, Austria
1990 – 1994	Lower School, Baden and Purgstall an der Erlauf, Austria

CLINICAL TRAINING

2009/03 – 2009/05	Clinical Clerkship at the Department of Neurology, Univ. Prof. Dr. Eduard Auff, General Hospital Vienna, Medical University of Vienna, Austria (5 weeks)
2009/03	Clinical Clerkship at the Department of Dermatology, Prim. Univ. Prof. Dr. Wolfgang Jurecka, Wilhelminenspital Vienna, Austria (3 weeks)
2009/01	Clinical Clerkship at the Department of Otorhinolaryngology, Univ. Prof. Dr. Wolfgang Gstöttner, General Hospital Vienna, Medical University of Vienna, Austria (3 weeks)
2008/12 – 2009/01	Clinical Clerkship at the Department of Ophthalmology, Univ. Prof. Dr. Ursula Schmidt-Erfurth, General Hospital Vienna, Medical University of Vienna, Austria (3 weeks)
2008/11 – 2008/12	Clinical Clerkship at the Department of Obstetrics and Gynecology, Prim. Univ. Prof. Dr. Heinrich Salzer, Wilhelminenspital Vienna, Austria (5 weeks)
2008/10 – 2008/11	Clinical Clerkship at the Department of Pediatrics, Prim. Univ. Prof. Dr. Andreas Lischka, Wilhelminenspital Vienna, Austria (5 weeks)

2008/04 – 2008/05	Clinical Clerkship at the Department of Transplantation, Intensive Care Unit, O. Univ. Prof. Dr. Ferdinand Mühlbacher, General Hospital Vienna, Medical University of Vienna, Austria (3 weeks)
2008/02 – 2008/04	Clinical Clerkship at the Department of Internal Medicine and Rheumatology, Prim. Univ. Doz. Dr. Peter Fasching, Wilhelminenspital Vienna, Austria (5 weeks)
2007/11	Clinical Clerkship at the Department of Pediatric Surgery, O. Univ. Prof. Dr. Ernst Horcher, General Hospital Vienna, Medical University of Vienna, Austria (3 weeks)
2007/10	Clinical Clerkship at the Departments of Surgery and Trauma Surgery, Prim. Univ. Prof. Dr. Karl Glaser, Prim. Univ. Prof. Dr. Michael Wagner, Wilhelminenspital Vienna, Austria (3 weeks)
2007/09	Clinical Clerkship at the Department of Plastic and Reconstructive Surgery, O. Univ. Prof. Dr. Manfred Frey, General Hospital Vienna, Medical University of Vienna, Austria (4 weeks)
2007/06	Clinical Clerkship at the Department of Plastic and Reconstructive Surgery, Prim. Univ. Doz. Dr. Rupert Koller, Wilhelminenspital Vienna, Austria (4 weeks)
2006/07	Clinical Clerkship at the Department of Trauma Surgery, O. Univ. Prof. Dr. Vilmos Vecsei, General Hospital Vienna, Medical University of Vienna, Austria (4 weeks)
2006/02	Clinical Clerkship at the Department of Clinical Pathology, Prim. Dr. Felix Pantucek, Hospital of Amstetten, Austria (2 weeks)
2005/08	Clinical Clerkship at the Department of Cardio-Thoracic Surgery, O. Univ. Prof. Dr. Ernst Wolner, General Hospital Vienna, Medical University of Vienna, Austria (4 weeks)
2005/07	Clinical Clerkship at the Department of Internal Medicine, Prim. Dr. Friedrich Steger, Hospital of Scheibbs, Austria (4 weeks)

CONTINUING EDUCATION

2008/12	Biometrie III: Klinische Studien – Biometry III: Clinical Trials, Vienna, Austria
2008/02	Anatomische Fertigkeiten – "Anatomie des zentralen Nervensystems" – "Advanced Anatomy of the Central Nervous System", Dr. Stefan Meng, Vienna, Austria
2007/09	Fortbildungskurs "Mikrochirurgische Reanimation des gelähmten Gesichtes" – Workshop "Microsurgical Reanimation after Facial Palsy", O. Univ. Prof. Dr. Manfred Frey, Vienna, Austria
2006/12 – 2007/09	Anatomia practica – Advanced course in anatomy, A.o. Univ. Prof. Dr. Wolfgang Weninger, Vienna, Austria
2007/05	Methodenseminar "Planung klinischer Studien" – Methods Seminar "Designing Clinical Trials", A.o. Univ. Prof. Dr. Brigitte Blöchl-Daum, Vienna, Austria
2007/05	Biometrie II: Statistische Tests und Lebensdaueranalyse bei medizinischen Fragestellungen – Biometry II: Statistical Tests and Analysis of Survival in Medical Research, Vienna, Austria

2007/03	Methodenseminar “Zellbiologie und Biochemie“ – Methods Seminar “Cell Biology and Biochemistry“, A.o. Univ. Prof. Dr. Harald Sitte, Vienna, Austria
2006/10	Biometrie I: Beschreibung und Visualisierung medizinischer Daten – Biometry I: Description and Visualization of Medical Data, Vienna, Austria

TEACHING ACTIVITY

2007/10 – Present	Teaching Assistant at the Department of Cardio-Thoracic Surgery, General Hospital Vienna, Medical University of Vienna, Univ. Doz. Dr. Hendrik Jan Ankersmit
2007/10 – Present	Teaching Assistant at the Department of Systematic Anatomy, Medical University of Vienna, A.o. Univ. Prof. Dr. Wolfgang Weninger

MEETINGS

2008/11	1st EACTS Meeting on Cardiac and Pulmonary Regeneration, Bern, Switzerland
2008/10	Austrotransplant – 22nd Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, Zell am See, Austria
2008/10	Gemeinsame Jahrestagung der DGPRÄC/ VDÄPC und ÖGPÄRC – Joint Meeting of the Austrian and German Societies of Plastic, Aesthetic and Reconstructive Surgery, Stuttgart, Germany
2008/09	Jahrestagung 2008 der Österreichischen Gesellschaft für Pneumologie – Annual Meeting 2008 of the Austrian Society of Pneumology, Vienna, Austria
2007/12	Jahrestagung der Österreichischen Gesellschaft für Allergologie und Immunologie – Annual Meeting of the Austrian Society of Allergology and Immunology, Alpbach, Austria
2007/10	Austrotransplant – 21st Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, St. Wolfgang, Austria
2007/10	45. Jahrestagung der Österreichischen Gesellschaft für Plastische, Ästhetische und Rekonstruktive Chirurgie – 45th Annual Meeting of the Austrian Society of Plastic, Aesthetic and Reconstructive Surgery, Linz, Austria
2007/06	48. Österreichischer Chirurgenkongress – 48th Annual Meeting of the Austrian Society of Surgery, Graz, Austria
2007/01	Kardiovaskuläre Forschungstage 2007 – 2nd Joint Meeting of the Austrian, German and Swiss Societies for Cardio-Thoracic Surgery and Cardiology (A, G), Weissensee, Austria
2006/10	Austrotransplant – 20th Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, Hof bei Salzburg, Austria
2006/10	44. Jahrestagung der Österreichischen Gesellschaft für Plastische, Ästhetische und Rekonstruktive Chirurgie – 44th Annual Meeting of the Austrian Society of Plastic, Aesthetic and Reconstructive Surgery, Vienna, Austria
2006/06	47. Österreichischer Chirurgenkongress – 47th Annual Meeting of the Austrian Society of Surgery, Vienna, Austria
2005/10	Austrotransplant – 19th Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, Alpbach, Austria

PUBLICATIONS

Articles:

Hacker S, Langenberger H, Plank C, Gorlitzer M, Ehrlich M, Dolak W, Kreuzer S, Loewe C, Klepetko W, Ankersmit HJ.
Management of aortobronchial fistula developing 27 years after open aortic surgery by means of endovascular stent grafting.
J Thorac Cardiovasc Surg. In Press.

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.
Lab Medicine 2009;40:290-293.

Ankersmit HJ, Hoetzenecker K, Dietl W, Soleiman A, Horvat R, Wolfsberger M, Gerner C, **Hacker S**, Mildner M, Moser B, Lichtenauer M, Podesser BK.
Irradiated cultured apoptotic peripheral blood mononuclear cells regenerate infarcted myocardium.
Eur J Clin Invest. 2009 Apr 16. [Epub ahead of print]

Hacker S*, Lambers C*, Hoetzenecker K, Pollreisz A, Aigner C, Lichtenauer M, Mangold A, Niederpold T, Zimmermann M, Taghavi S, Klepetko W, Ankersmit HJ.
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.
* contributed equally

Lambers C*, **Hacker S***, Posch M, Hoetzenecker K, Pollreisz A, Lichtenauer M, Klepetko W, Ankersmit HJ.
T cell senescence and contraction of T cell repertoire diversity in patients with chronic obstructive pulmonary disease.
Clin Exp Immunol. 2009 Mar;155(3):466-75.
* contributed equally

Klinger M, Eipeldauer S, **Hacker S**, Herberger B, Tamandl D, Dorfmeister M, Koelblinger C, Gruenberger B, Gruenberger T.
Bevacizumab protects against sinusoidal obstruction syndrome and does not increase response rate in neoadjuvant XELOX/FOLFOX therapy of colorectal cancer liver metastases.
Eur J Surg Oncol. 2009 May;35(5):515-20. Epub 2009 Feb 5.

Szerafin T, Niederpold T, Mangold A, Hoetzenecker K, **Hacker S**, Roth G, Lichtenauer M, Dworschak M, Wolner E, Ankersmit HJ.
Secretion of Soluble ST2 - Possible Explanation for Systemic Immunosuppression after Heart Surgery.
Thorac Cardiovasc Surg. 2009 Feb;57(1):25-9. Epub 2009 Jan 23.

Hoetzenecker K, Ankersmit HJ, Aigner C, Lichtenauer M, Kreuzer S, **Hacker S**, Hoetzenecker W, Lang G, Klepetko W.
Consequences of a wait-and-see strategy for benign metastasizing leiomyomatosis of the lung.
Ann Thorac Surg. 2009 Feb;87(2):613-4.

Roth GA, Faybik P, Hetz H, **Hacker S**, Ankersmit HJ, Bacher A, Thalhammer T, Krenn CG.
Pro-inflammatory Interleukin-18 and Caspase-1 serum levels in liver failure are unaffected by MARS treatment.

Dig Liver Dis. 2009 Jun;41(6):417-23. Epub 2008 Nov 18.

Soleiman A, Lukschal A, **Hacker S**, Aumayr K, Hoetzenecker K, Lichtenauer M, Moser B, Untersmayr E, Horvat R, Ankersmit HJ.
Myocardial lipofuscin-laden lysosomes contain the apoptosis marker caspase-cleaved cytokeratin-18.
Eur J Clin Invest. 2008 Oct;38(10):708-12.

Pollreisz A, Assinger A, **Hacker S**, Hoetzenecker K, Schmid W, Lang G, Wolfsberger M, Steinlechner B, Bielek E, Lalla E, Klepetko W, Volf I, Ankersmit HJ.
Intravenous immunoglobulins induce CD32-mediated platelet aggregation in vitro.
Br J Dermatol. 2008 Sep;159(3):578-84.

Ankersmit HJ, Loewe C, **Hacker S**, Hoetzenecker K, Grimm M.
Gigantic coronary fistula.
Wien Klin Wochenschr. 2008;120(5-6):152. Erratum in: Wien Klin Wochenschr. 2009;121(5-6):225.

Kandutsch S, Klinger M, **Hacker S**, Wrba F, Gruenberger B, Gruenberger T. Patterns of hepatotoxicity after chemotherapy for colorectal cancer liver metastases.
Eur J Surg Oncol. 2008 Nov;34(11):1231-6. Epub 2008 Feb 12.

Szerafin T, Hoetzenecker K, **Hacker S**, Horvath A, Pollreisz A, Arpád P, Mangold A, Wliszczak T, Dworschak M, Seitelberger R, Wolner E, Ankersmit HJ.
Heat shock proteins 27, 60, 70, 90alpha, and 20S proteasome in on-pump versus off-pump coronary artery bypass graft patients.
Ann Thorac Surg. 2008 Jan;85(1):80-7.

Hoetzenecker K, **Hacker S**, Hoetzenecker W, Sadeghi K, Sachet M, Pollreisz A, Mangold A, Wliszczak T, Bielek E, Muehlbacher F, Klepetko W, Ankersmit HJ.
Cytomegalovirus hyperimmunoglobulin: mechanisms in allo-immune response in vitro.
Eur J Clin Invest. 2007 Dec;37(12):978-86.

Hetz H, Hoetzenecker K, **Hacker S**, Faybik P, Pollreisz A, Moser B, Roth G, Hoetzenecker W, Lichtenauer M, Klinger M, Krenn CG, Ankersmit HJ. Caspase-cleaved cytokeratin 18 and 20 S proteasome in liver degeneration.
J Clin Lab Anal. 2007;21(5):277-81.

Adlbrecht C, Hoetzenecker K, Posch M, Steiner S, Kopp C, **Hacker S**, Auer J, Horvath R, Moser B, Roth G, Wolner E, Lang IM, Ankersmit HJ.
Elevated levels of interleukin-1β-converting enzyme and caspase-cleaved cytokeratin-18 in acute myocardial infarction.
Eur J Clin Invest. 2007 May;37(5):372-380.

Szerafin T, Horvath A, Moser B, **Hacker S**, Hoetzenecker K, Steinlechner B, Brunner M, Roth G, Boltz-Nitulescu G, Peterffy A, Wolner E, Ankersmit HJ. Apoptosis-specific activation markers in on- versus off-pump coronary artery bypass graft (CABG) patients.
Clin Lab 2006;52(5-6):255-61.

Ankersmit HJ, Ullrich R, Moser B, Hoetzenecker K, **Hacker S**, German P, Krenn C, Horvat R, Grimm M, Wolner E, Zuckermann A.
Recovery from giant cell myocarditis with ECMO support and utilisation of polyclonal antithymocyte globulin: a case report.
Thorac Cardiov Surg 2006;54:278–280.

Letters: Ankersmit HJ, **Hacker S**, Hoetzenecker K, Moser B, Wolner E.

The impact of hypogammagammaglobulinemia on infection outcome in patients undergoing ventricular assist device implantation.
J Heart Lung Transplant 2006 Nov;25(11):1387-8.

Abstracts:

Hacker S, Lambers C, Hoetzenecker K, Lichtenauer M, Niederpold T, Klepetko W, Ankersmit HJ.
Elevated HSP27, HSP70 and HSP90α in COPD: Markers for immune activation and tissue destruction.
22nd Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, Zell am See, Austria. 2008/10.
European Surgery 2008;40:Suppl 226:17.

Hacker S, Lambers C, Hoetzenecker K, Pollreisz A, Mangold A, Klepetko W, Ankersmit HJ.
Increased soluble serum markers ICE, ST2, caspase-cleaved cytokeratin-18 and histones indicate apoptotic turnover and chronic immune response in COPD.
22nd Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, Zell am See, Austria. 2008/10.
European Surgery 2008;40:Suppl 226:16.

Hacker S, Lambers C, Posch M, Hoetzenecker K, Pollreisz A, Klepetko W, Ankersmit HJ.
T-cell senescence and contraction of T-cell repertoire diversity in patients with chronic obstructive pulmonary disease.
22nd Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, Zell am See, Austria. 2008/10.
European Surgery 2008;40:Suppl 226:16.

Schmidt M, Aszmann OC, Beck H, **Hacker St**, Frey M.
Die anatomischen Grundlagen des Arteria mammaria interna-Perforator (IMAP)-Lappen.
Joint Meeting of the Austrian and German Societies of Plastic, Aesthetic and Reconstructive Surgery, Stuttgart, Germany. 2008/10.
Plastische Chirurgie 8 (Suppl. 1): 3 (2008):48

Hacker S, Lambers C, Posch M, Hoetzenecker K, Pollreisz A, Nickl S, Klepetko W, Ankersmit HJ.
T-cell senescence and contraction of T-cell repertoire diversity in patients with chronic obstructive pulmonary disease.
Annual Meeting 2008 of the Austrian Society of Pneumology, Vienna, Austria. 2008/09.
Wien Klin Wochenschr (2008) 120/13–14:A21.

Hacker S, Lambers C, Hoetzenecker K, Pollreisz A, Aigner C, Mangold A, Lichtenauer M, Nickl S, Klepetko W, Ankersmit HJ.
Elevated HSP27, HSP70 and HSP90α in COPD: Markers for immune activation and tissue destruction.
Annual Meeting 2008 of the Austrian Society of Pneumology, Vienna, Austria. 2008/09.
Wien Klin Wochenschr (2008) 120/13–14:A20.

Hacker S, Lambers C, Pollreisz A, Hoetzenecker K, Mangold A, Niederpold T, Lichtenauer M, Lang G, Nickl S, Klepetko W, Ankersmit HJ.
Increased soluble serum markers ICE, ST2, caspase-cleaved cytokeratin-18 and histones indicate apoptotic turnover and chronic immune response in COPD.
Annual Meeting 2008 of the Austrian Society of Pneumology, Vienna, Austria. 2008/09.
Wien Klin Wochenschr (2008) 120/13–14:A20.

Klinger M, Kandutsch S, **Hacker S**, Gruenberger B, Gruenberger T.
Patterns of hepatotoxicity after chemotherapy for colorectal cancer metastases: Effect of bevacizumab.
44th ASCO Annual Meeting 2008, Chicago, IL, USA. 2008/05.
J Clin Oncol 26: 2008 (May 20 suppl; abstr 4082)

Klinger M, Kandutsch S, **Hacker S**, Gruenberger T.
Patterns of hepatotoxicity after chemotherapy for colorectal cancer metastases: effect of bevacizumab.
49th Annual Meeting of the Austrian Society of Surgery, Innsbruck, Austria. 2008/05.
European Surgery 2008;40:Suppl 224:94.

Niederpold T, Hoetzenecker K, **Hacker S**, Mangold A, Pollreisz A, Lichtenauer M, Szerafin T, Krenn C, Ankersmit HJ.
Th1 and Th2 cytokine response in coronary artery bypass graft (CABG) patients.
49th Annual Meeting of the Austrian Society of Surgery, Innsbruck, Austria. 2008/05.
European Surgery 2008;40:Suppl 224:72.

Pollreisz A, **Hacker S**, Hoetzenecker K, Assinger A, Kebschull M, Volf I, Ankersmit HJ.
Intravenous Immunoglobulins Induce CD32-Mediated Platelet Aggregation in Vitro.
Arteriosclerosis, Thrombosis and Vascular Biology Annual Conference 2008, Atlanta, GA, USA. 2008/04.
Arterioscler. Thromb. Vasc. Biol. 2008;28;e71

Mangold A, Hoetzenecker K, **Hacker S**, Pollreisz A, Wliszczak T, Lichtenauer M, Wolner E, Klepetko W, Gollackner B, Szerafin T, Auer J, Ankersmit HJ. Alpha-Gal Specific Humoral Immune Response after Implantation of Bioprostheses in Cardiac Surgery.
6th EAACI-GA^{2}LEN Davos Meeting, Pichl, Austria. 2008/02.
published in Abstractbook.

Pollreisz A, Assinger A, **Hacker S**, Hoetzenecker K, Schmid W, Steinlechner B, Bielek E, Klepetko W, Volf I, Ankersmit HJ.
Intravenous Immunoglobulins Induce CD32-mediated Platelet Aggregation *in vitro.*
6th EAACI-GA^{2}LEN Davos Meeting, Pichl, Austria. 2008/02.
published in Abstractbook.

Hacker S, Soleiman A, Hoetzenecker K, Lukschal A, Pollreisz A, Mangold A, Wliszczak T, Lichtenauer M, Horvat R, Muehlbacher F, Wolner E, Klepetko W, Ankersmit HJ.
Degenerative Cardiac Pigment Lipofuscin Contains Cytokeratin-18 and Caspase-cleaved Cytokeratin-18.
Annual Meeting of the Austrian Society of Allergology and Immunology, Alpbach, Austria. 2007/12.
published in Abstractbook.

Pollreisz A, Assinger A, **Hacker S**, Hoetzenecker K, Schmid W, Steinlechner B, Bielek E, Klepetko W, Volf I, Ankersmit HJ.
Intravenous Immunoglobulins Induce CD32-mediated Platelet Aggregation *in vitro.*
Annual Meeting of the Austrian Society of Allergology and Immunology, Alpbach, Austria. 2007/12.
published in Abstractbook.

Hoetzenecker K, **Hacker S**, Hoetzenecker W, Sadeghi K, Sachet M, Pollreisz A, Mangold A, Wliszczak T, Bielek E, Muehlbacher F, Wolner E, Klepetko W, Ankersmit HJ.

CMV Hyperimmunoglobulin Influence NK cell Viability and Function in vitro. Annual Meeting of the Austrian Society of Allergology and Immunology, Alpbach, Austria. 2007/12.
published in Abstractbook.

Mangold A, Hoetzenecker K, **Hacker S**, Pollreisz A, Wliszczak T, Lichtenauer M, Wolner E, Klepetko W, Gollackner B, Szerafin T, Auer J, Ankersmit HJ. Alpha-Gal Specific Humoral Immune Response after Implantation of Bioprostheses in Cardiac Surgery.
Annual Meeting of the Austrian Society of Allergology and Immunology, Alpbach, Austria. 2007/12.
published in Abstractbook.

Hacker S, Soleiman A, Lukschal A, Hoetzenecker K, Horvat R, Ankersmit HJ. Degenerative cardiac pigment lipofuscin contains cytokeratin-18 and caspase-cleaved cytokeratin-18.
21st Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, St. Wolfgang, Austria. 2007/10.
European Surgery 2007;39:Suppl 218:15.

Pollreisz A, **Hacker S**, Hoetzenecker K, Wliszczak T, Volf I, Ankersmit HJ. CMVIg and IVIg induce CD32-mediated platelet aggregation in vitro: implication of therapy induced thrombocytopenia and thrombosis in vivo.
21st Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, St. Wolfgang, Austria. 2007/10.
European Surgery 2007;39:Suppl 218:28.

Mangold A, Hoetzenecker K, **Hacker S**, Szerafin T, Auer J, Ankersmit HJ. Alpha-Gal specific humoral immune response after implantation of bioprostheses in cardiac surgery.
21st Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, St. Wolfgang, Austria. 2007/10.
European Surgery 2007;39:Suppl 218:25.

Hoetzenecker K, **Hacker S**, Hoetzenecker W, Sachet M, Klepetko W, Ankersmit HJ.
CMV hyperimmunoglobulin: mechanisms in allo-immune response in vitro. 21st Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, St. Wolfgang, Austria. 2007/10.
European Surgery 2007;39:Suppl 218:17.

Ankersmit HJ, **Hacker S**, Hoetzenecker K, Klepetko W, Wolner E, Wollenek G, Grimm M.
Gigantic coronary fistula: rare finding without clinical symptom.
48th Annual Meeting of the Austrian Society of Surgery, Graz, Austria. 2007/06.
European Surgery 2007;39:Suppl 215:118.

Hoetzenecker K, **Hacker S**, Hoetzenecker W, Sachet M, Sadeghi K, Pollreisz A, Mangold A, Wliszczak T, Moser B, Muehlbacher F, Klepetko W, Wolner E, Ankersmit HJ.
CMV Hyperimmunoglobulin evidences anti-proliferative properties and reduces natural occuring cell mediated cytotoxicity in vitro.
48th Annual Meeting of the Austrian Society of Surgery, Graz, Austria. 2007/06.
European Surgery 2007;39:Suppl 215:38.

Hoetzenecker K, Szerafin T, **Hacker S**, Pollreisz A, Mangold A, Wliszczak T, Moser B, Muehlbacher F, Klepetko W, Wolner E, Ankersmit HJ.
Heat shock proteins 27/60/70/90 - and 20S proteasome in on- versus off-pump coronary artery bypass graft patients.

48th Annual Meeting of the Austrian Society of Surgery, Graz, Austria. 2007/06.
European Surgery 2007;39:Suppl 215:16.

Hoetzenecker K, **Hacker S**, Hoetzenecker W, Sadeghi K, Pollreisz A, Mangold A, Moser B, Grimm M, Muehlbacher F, Klepetko W, Wolner E, Ankersmit HJ. 376: Anti-proliferative properties of CMV hyperimmunoglobulin are related to activation induced cell death in vitro: Possible role in tolerance induction.
ISHLT 27th Annual Meeting and Scientific Sessions, San Francisco, CA, USA. 2007/04.
J Heart Lung Transplant 2007 Feb;26(2):Suppl 1:S195.

Adlbrecht C, Hoetzenecker K, Posch M, Steiner S, **Hacker S**, Moser B, Roth G, Wolner E, Lang IM, Ankersmit HJ.
Elevated levels of interleukin-1ß-converting enzyme and caspase-cleaved cytokeratin-18 in acute myocardial infarction.
2nd Joint Meeting of the Austrian, German and Swiss Societies for Cardio-Thoracic Surgery and Cardiology (A, G), Weissensee, Austria. 2007/01. European Surgery 2007;39:Suppl 213:5.

Hötzenecker K, **Hacker S**, Hötzenecker W, Sadeghi K, Pollreisz A, Mangold A, Moser B, Grimm M, Zuckermann A, Mühlbacher F, Klepetko W, Wolner E, Ankersmit HJ.
CMV hyperimmunoglobulin evidence anti-proliferative activity in vitro triggered by the induction of apoptosis: Possible role in tolerance induction in allograft recipients.
20th Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, Hof bei Salzburg, Austria. 2006/10.
European Surgery 2006;38:Suppl 211:2.

Ankersmit HJ, Ullrich R, Moser B, Hoetzenecker K, **Hacker S**, German P, Krenn C, Grimm M, Wolner E, Zuckermann A.
Case report: antithymocyte globuline (rATG) and ECMO bridge as new options in the treatment of giant cell myocarditis.
47th Annual Meeting of the Austrian Society of Surgery, Vienna, Austria. 2006/06.
European Surgery 2006;38:Suppl 209:83.

Hötzenecker K, **Hacker S**, Hoetzenecker W, Polreisz A, Bohle B, Roth G, Moser B, Krenn C, Grimm M, Bolz-Nituescu G, Klepetko W, Zuckermann A, Wolner E, Ankersmit HJ.
Pooled human IgG and IgM has immunosuppressive properties in vitro and is partly triggered by Fc blockade.
19th Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, Alpbach, Austria. 2005/10.
European Surgery 2005;37:Suppl 205:26.

Poster Presentations:

Hacker S, Lambers C, Hoetzenecker K, Pollreisz A, Posch M, Mangold A, Niederpold T, Lichtenauer M, Moser B, Nickl S, Klepetko W, Ankersmit HJ.
T-cell Senescence And Contraction of T-cell Repertoire Diversity In Patients With Chronic Obstructive Pulmonary Disease.
22nd Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, Zell am See, Austria. 2008/10.

Hacker S, Lambers C, Hoetzenecker K, Pollreisz A, Aigner C, Mangold A, Niederpold T, Lichtenauer M, Moser B, Nickl S, Klepetko W, Ankersmit HJ.
Elevated HSP27, HSP70 And HSP90α in COPD: Markers for Immune Activation And Tissue Destruction.
22nd Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, Zell am See, Austria. 2008/10.

Hacker S, Lambers C, Hoetzenecker K, Pollreisz A, Mangold A, Niederpold T, Lichtenauer M, Moser B, Nickl S, Lang G, Klepetko W, Ankersmit HJ.

Increased Soluble Serum Markers ICE, ST2, Caspase-Cleaved Cytokeratin-18 And Histones Indicate Apoptotic Turnover and Chronic Immune Response in COPD.
22nd Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, Zell am See, Austria. 2008/10.

Hacker S, Lambers C, Hoetzenecker K, Pollreisz A, Posch M, Mangold A, Niederpold T, Lichtenauer M, Moser B, Nickl S, Klepetko W, Ankersmit HJ.
T-cell Senescence And Contraction of T-cell Repertoire Diversity In Patients With Chronic Obstructive Pulmonary Disease.
Annual Meeting 2008 of the Austrian Society of Pneumology, Vienna, Austria. 2008/09.

Hacker S, Lambers C, Hoetzenecker K, Pollreisz A, Aigner C, Mangold A, Niederpold T, Lichtenauer M, Moser B, Nickl S, Klepetko W, Ankersmit HJ.
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Annual Meeting 2008 of the Austrian Society of Pneumology, Vienna, Austria. 2008/09.

Hacker S, Lambers C, Hoetzenecker K, Pollreisz A, Mangold A, Niederpold T, Lichtenauer M, Moser B, Nickl S, Lang G, Klepetko W, Ankersmit HJ.
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Annual Meeting 2008 of the Austrian Society of Pneumology, Vienna, Austria. 2008/09.

Klinger M, Kandutsch S, **Hacker S**, Gruenberger B, Gruenberger T.
Patterns of hepatotoxicity after chemotherapy for colorectal cancer metastases: Effect of bevacizumab.
44th ASCO Annual Meeting 2008, Chicago, IL, USA. 2008/05.

Klinger M, Kandutsch S, **Hacker S**, Gruenberger T.
Patterns of hepatotoxicity after chemotherapy for colorectal cancer metastases: effect of bevacizumab.
49th Annual Meeting of the Austrian Society of Surgery, Innsbruck, Austria. 2008/05.

Pollreisz A, **Hacker S**, Hoetzenecker K, Assinger A, Kebschull M, Volf I, Ankersmit HJ.
Intravenous Immunoglobulins Induce CD32-Mediated Platelet Aggregation in Vitro.
Arteriosclerosis, Thrombosis and Vascular Biology Annual Conference 2008, Atlanta, GA, USA. 2008/04.

Mangold A, Hoetzenecker K, **Hacker S**, Pollreisz A, Wliszczak T, Lichtenauer M, Wolner E, Klepetko W, Gollackner B, Szerafin T, Auer J, Ankersmit HJ. Alpha-Gal Specific Humoral Immune Response after Implantation of Bioprostheses in Cardiac Surgery.
6th EAACI-GA^{2}LEN Davos Meeting, Pichl, Austria. 2008/02.

Pollreisz A, Assinger A, **Hacker S**, Hoetzenecker K, Schmid W, Steinlechner B, Bielek E, Klepetko W, Volf I, Ankersmit HJ.
Intravenous Immunoglobulins Induce CD32-mediated Platelet Aggregation *in vitro.*
6th EAACI-GA^{2}LEN Davos Meeting, Pichl, Austria. 2008/02.

Hacker S, Soleiman A, Hoetzenecker K, Lukschal A, Pollreisz A, Mangold A, Wliszczak T, Lichtenauer M, Horvat R, Muehlbacher F, Wolner E, Klepetko W, Ankersmit HJ.
Degenerative Cardiac Pigment Lipofuscin Contains Cytokeratin-18 and Caspase-cleaved Cytokeratin-18.
Annual Meeting of the Austrian Society of Allergology and Immunology, Alpbach, Austria. 2007/12.

Pollreisz A, Assinger A, **Hacker S**, Hoetzenecker K, Schmid W, Steinlechner B, Bielek E, Klepetko W, Volf I, Ankersmit HJ.
Intravenous Immunoglobulins Induce CD32-mediated Platelet Aggregation *in vitro.*
Annual Meeting of the Austrian Society of Allergology and Immunology, Alpbach, Austria. 2007/12.

Hoetzenecker K, **Hacker S**, Hoetzenecker W, Sadeghi K, Sachet M, Pollreisz A, Mangold A, Wliszczak T, Bielek E, Muehlbacher F, Wolner E, Klepetko W, Ankersmit HJ.
CMV Hyperimmunoglobulin Influence NK cell Viability and Function in vitro. Annual Meeting of the Austrian Society of Allergology and Immunology, Alpbach, Austria. 2007/12.

Mangold A, Hoetzenecker K, **Hacker S**, Pollreisz A, Wliszczak T, Lichtenauer M, Wolner E, Klepetko W, Gollackner B, Szerafin T, Auer J, Ankersmit HJ. Alpha-Gal Specific Humoral Immune Response after Implantation of Bioprostheses in Cardiac Surgery.
Annual Meeting of the Austrian Society of Allergology and Immunology, Alpbach, Austria. 2007/12.

Hacker S, Soleiman A, Hoetzenecker K, Lukschal A, Pollreisz A, Mangold A, Wliszczak T, Lichtenauer M, Horvat R, Muehlbacher F, Wolner E, Klepetko W, Ankersmit HJ.
Degenerative Cardiac Pigment Lipofuscin Contains Cytokeratin-18 and Caspase-cleaved Cytokeratin-18.
21st Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, St. Wolfgang, Austria. 2007/10.

Hoetzenecker K, **Hacker S**, Hoetzenecker W, Sadeghi K, Sachet M, Pollreisz A, Mangold A, Wliszczak T, Bielek E, Muehlbacher F, Wolner E, Klepetko W, Ankersmit HJ.
CMV Hyperimmunoglobulin Influence NK cell Viability and Function in vitro. 21st Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, St. Wolfgang, Austria. 2007/10.

Mangold A, Hoetzenecker K, **Hacker S**, Pollreisz A, Wliszczak T, Lichtenauer M, Wolner E, Klepetko W, Gollackner B, Szerafin T, Auer J, Ankersmit HJ. Alpha-Gal Specific Humoral Immune Response after Implantation of Bioprostheses in Cardiac Surgery.
21st Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, St. Wolfgang, Austria. 2007/10.

Ankersmit HJ, **Hacker S**, Hoetzenecker K, Klepetko W, Wolner E, Wollenek G, Grimm M.
Gigantic coronary fistula: rare finding without clinical symptom.
48th Annual Meeting of the Austrian Society of Surgery, Graz, Austria. 2007/06.

Hoetzenecker K, **Hacker S**, Hoetzenecker W, Sadeghi K, Pollreisz A, Mangold A, Moser B, Grimm M, Muehlbacher F, Klepetko W, Wolner E, Ankersmit HJ. Anti-proliferative properties of CMV hyperimmunoglobulin are related to activation induced cell death in vitro: Possible role in tolerance induction. ISHLT 27th Annual Meeting and Scientific Sessions, San Francisco, CA, USA. 2007/04.

Ankersmit HJ, Ullrich R, Moser B, Hoetzenecker K, **Hacker S**, German P, Krenn C, Grimm M, Wolner E, Zuckermann A.
Case Report: Antithymocyte globuline (rATG) and ECMO bridge as new options in the treatment of giant cell myocarditis.
47th Annual Meeting of the Austrian Society of Surgery, Vienna, Austria. 2006/06.

Ankersmit HJ, Ullrich R, Moser B, Hoetzenecker K, **Hacker S**, German P, Krenn C, Grimm M, Wolner E, Zuckermann A.
Case Report: Antithymocyte globuline (rATG) and ECMO bridge as new options in the treatment of giant cell myocarditis.
Poster Presentations - "vfwf Universitätsvorlesung 2006" at the Medical University of Vienna, Vienna, Austria. 2006/06.

Hetz H, Hoetzenecker K, Brunner M, Faybik P, Moser B, Roth G, Klinger M, **Hacker S**, Wolner E, Krenn C, Ankersmit HJ.
Caspase-cleaved cytokeratin 18 and 20S proteasome in liver degeneration. Poster Presentations - "vfwf Universitätsvorlesung 2006" at the Medical University of Vienna, Vienna, Austria. 2006/06.

Oral Presentations:

"COPD - A Systemic Autoimmune Disease?" (Oral Presentation)
Division of Pulmonary Medicine, Hospital of Hochegg, Grimmenstein, Austria. 2009/01.

"COPD - A Systemic Autoimmune Disease?" (Oral Presentation)
Division of Cardiothoracic Surgery, General Hospital of Vienna, Medical University of Vienna, Vienna, Austria. 2008/12.

"T-cell Senescence And Contraction of T-cell Repertoire Diversity In Patients With Chronic Obstructive Pulmonary Disease" (Oral Presentation)
Division of Thoracic Surgery, General Hospital of Vienna, Medical University of Vienna, Vienna, Austria. 2008/11.

"T-cell Senescence And Contraction of T-cell Repertoire Diversity In Patients With Chronic Obstructive Pulmonary Disease" (Poster Presentation)
22nd Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, Zell am See, Austria. 2008/10.

"Elevated HSP27, HSP70 And HSP90α in COPD: Markers for Immune Activation And Tissue Destruction" (Poster Presentation)
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"Increased Soluble Serum Markers ICE, ST2, Caspase-Cleaved Cytokeratin-18 And Histones Indicate Apoptotic Turnover and Chronic Immune Response in COPD" (Poster Presentation)
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Annual Meeting 2008 of the Austrian Society of Pneumology, Vienna, Austria. 2008/09.

"Elevated HSP27, HSP70 And HSP90α in COPD: Markers for Immune Activation And Tissue Destruction" (Poster Presentation)
Annual Meeting 2008 of the Austrian Society of Pneumology, Vienna, Austria. 2008/09.

"Increased Soluble Serum Markers ICE, ST2, Caspase-Cleaved Cytokeratin-18 And Histones Indicate Apoptotic Turnover and Chronic Immune Response in COPD" (Poster Presentation)
Annual Meeting 2008 of the Austrian Society of Pneumology, Vienna, Austria. 2008/09.

"Shock Waves & Wound Healing" (Oral Presentation)
Division of Plastic Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA. 2008/08.

"Micro-Array Chamber Device MACD" (Oral Presentation)
Division of Plastic Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA. 2008/08.

"Intravenous Immunoglobulins Induce CD32-mediated Platelet Aggregation *in vitro*" (Poster Presentation)
Annual Meeting of the Austrian Society of Allergology and Immunology, Alpbach, Austria. 2007/12.

"Degenerative Cardiac Pigment Lipofuscin Contains Cytokeratin-18 and Caspase-cleaved Cytokeratin-18" (Poster Presentation)
Annual Meeting of the Austrian Society of Allergology and Immunology, Alpbach, Austria. 2007/12.

"Writing a Diploma Thesis at the Medical University of Vienna". (Invited Lecture)
Medical University of Vienna, Vienna, Austria. 2007/12.

"Degenerative Cardiac Pigment Lipofuscin Contains Cytokeratin-18 and Caspase-cleaved Cytokeratin-18". (Poster Presentation)
21st Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, St. Wolfgang, Austria. 2007/10.

"CMVIg and IVIg induce CD32-mediated platelet aggregation in vitro: implication of therapy induced thrombocytopenia and thrombosis in vivo". (Oral Presentation)
21st Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, St. Wolfgang, Austria. 2007/10.

"Characterization of a Cytolytic CD4+ T-cell Subset in Patients with COPD". (Project Presentation)
Medical University of Vienna, Vienna, Austria. 2007/05.

Book Chapters: Ankersmit HJ, **Hacker S**, Wolner E, Klepetko W. Ethische und psychologische Aspekte der Explantation (Ethical and Psychological Aspects of Explantation) In: Fischer M, Zänker KS, eds. Medizin- und Bioethik. 1st ed. Frankfurt, Germany: Peter Lang Verlagsgruppe. 2006.

Clinical Trials: **Sub-Investigator:** Multicenter, double-blind, randomized, placebo-controlled, parallel-group study to assess the efficacy, safety and tolerability of tezosentan in patients with pre-operative pulmonary hypertension, due to left heart disease, undergoing cardiac surgery (EudraCT 2006-002907-15). Investigator: A.o. Univ. Prof. Dr. Rainald Seitelberger

High School Graduation Thesis: **Hacker S**. Rastafarianism: a view of an unknown culture.

AWARDS AND GRANTS

2008/12 Leistungsstipendium – Scholarship for Outstanding Academic Achievement, Medical University of Vienna

2008/09 Poster Prize – Annual Meeting 2008 of the Austrian Society of Pneumology, Vienna, Austria

2008/01	Studentenstipendium – Student Scholarship for the „Health Economic Forum", St. Anton am Arlberg, Austria
2007/12	Leistungsstipendium – Scholarship for Outstanding Academic Achievement, Medical University of Vienna
2006/12	Leistungsstipendium – Scholarship for Outstanding Academic Achievement, Medical University of Vienna
2006/06	Förderungsstipendium – Student Research Scholarship, Medical University of Vienna
2005/12	Leistungsstipendium – Scholarship for Outstanding Academic Achievement, Medical University of Vienna
2004/12	Leistungsstipendium – Scholarship for Outstanding Academic Achievement, Medical University of Vienna
2002/10	Special Award for Outstanding Academic Achievement, Austrian Minister of Education
2002/06	Matura (High School Graduation) with Distinction

MEMBERSHIPS

2007/11	Austrian Society of Allergology and Immunology
2005/10	Austrian Society of Transplantation, Transfusion and Genetics

OTHER OCCUPATIONS

1999/10 – Present	Technical Assistant at a Dentist's Office
2001/08 – 2004/01	Journalist at a Local Newspaper

LANGUAGE SKILLS

Native German Speaker
Proficient in English
Good Knowledge of French, Latin

REFERENCES

Associate Professor Hendrik Jan Ankersmit, M.D.
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General Hospital Vienna
Währinger Gürtel 18-20
1090 Vienna, Austria
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