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Pepsin From Bronchoalveolar Lavage (BAL) Fluid in Lung Diseases and Tumours

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Table of content

Zusammenfassung

Hintergrund und Zielsetzung der vorliegenden Dissertation

Eine Korrelation zwischen der wiederkehrenden Mikroaspiration beim gastroösophagealen Reflux und verschiedenen Lungenerkrankungen, insbesondere idiopathische Lungenfibrose (IPF), chronisch obstruktive Lungenerkrankungen (COPD) und Asthma, wurde bereits von anderen Wissenschaftlern in der Forschung beschrieben. Die Relevanz für andere Lungenerkrankungen ist noch unklar. Das Ziel der Studie bestand darin, den Zusammenhang zwischen der Pepsinkonzentration in der bronchoalveolären Lavage (BAL) und der Lungenfunktion sowie den Zusammenhang zwischen der Pepsinkonzentration und den Atemwegsentzündungen bei Patienten mit anderen nicht infektiösen, chronischen Lungenerkrankungen oder Tumoren zu untersuchen.

Methoden

Es wurden Patienten mit einer klinischen Indikation für eine BAL aus der Medizinischen Klinik und Poliklinik V, Klinik der Universität München (Sektion Pneumologie Innenstadt und Throakale Onkologie) untersucht. Die Konzentrationen von Pepsin, CRP und TNF alpha wurden durch ELISA bestimmt. Retrospektiv wurden spirometrische (FEV1, VC% Soll) und körperplethysmographische (Resistance, DC% Soll) Lungenfunktionswerte, Laborbefunde sowie biometrische (Geschlecht, Größe, Gewicht) und klinische Parameter (Krankheit, Medikamente, Raucherstatus) aufgezeichnet.

Ergebnisse

Insgesamt wurden 70 Patienten betrachtet, bei denen Pepsin in der BAL festgestellt wurde (Mittelwert 24589.51 µg/ml). BAL CRP und BAL TNF alpha unterschieden sich signifikant in der Gruppe mit einer chronischen, akuten Lungenerkrankung und der Gruppe ohne einen pathologischen Befund. Es zeigte sich, dass BAL-Pepsin in der Gruppe mit einer chronischen Lungenerkrankung mit den Kenngrößen BAL CRP, TNF alpha und TLC% (Spearman Korrelationskoeffizient jeweils 0,577 mit p-Wert 0,001; 0,469 mit p-Wert 0,010 und –0.430 mit p-Wert 0,041) signifikant korrelierte. Bei der Betrachtung aller teilnehmenden Patienten korrelierte BAL-Pepsin auch positiv mit CRP und TNF (Korrelationskoeffizienten waren 0,408, 0,346 mit $P<0,05$). Gleichzeitig konnte man zudem eine negative Korrelation von BAL-Pepsin mit Parametern der Lungenfunktion wie FEV1%, VC% und TLC%

(Korrelationskoeffizienten waren –0,386, –0,355 und –0,402 mit $P<0,05$) feststellen. Ferner wurde erkannt, dass die BAL-Pepsin-Konzentration bei Patienten mit verschiedenen Lungentumoren unterschiedlich war. Es gab allerdings keinen signifikanten Unterschied zwischen BAL-Pepsin, CRP und TNF alpha bei Patienten mit Raucheranamnese. Die PPI-Behandlung übte auch keinen Einfluss auf diese Indikatoren aus.

Diskussion

BAL-Pepsin korreliert mit Atemwegsentzündungen und auch mit der Schwere der Einschränkung bei Patienten mit nicht infektiösen Lungenerkrankungen und Tumoren ohne IPF. Reflux könnte daher ein ursächlicher Parameter bei verschiedenen Lungenerkrankungen sein, weshalb weitere Untersuchungen zu diesem Thema folgen sollten.

Abstract

Background and Objective

A correlation between recurrent microaspiration in gastro-oesophageal reflux and some lung diseases, especially IPF, COPD and asthma, has been previously described by other researchers. The relevance to other lung diseases is still unclear. The aim of the study was to investigate the relationship between pepsin concentration in bronchoalveolar lavage (BAL) and lung function as well as its relationship with respiratory tract inflammation in patients with other non-infectious, chronic lung diseases or tumours.

Methods

Patients with a clinical indication for BAL were examined in the clinic and outpatient clinic V of the university hospital LMU (section pneumology and thoracic oncology). The concentrations of pepsin, CRP and TNF alpha were determined by ELISA. Retrospectively, spirometric (FEV_1, VC% target), bodyplethysmographic (resistance, TLC% target), laboratory chemical, biometric (sex, height, weight) and clinical parameters (disease, medication, smoking status) were recorded.

Results

A total of 70 patients were enrolled, in whom pepsin was detected in the BAL fluid (mean 24589.51µg/ml). BAL CRP and BAL TNF alpha varied significantly among the analysis groups chronic, acute lung diseases and without pathologic findings. In the chronic lung disease group it showed a significant correlation of BAL pepsin level with BAL CRP, TNF alpha and TLC% respectively (correlation coefficients were 0.577, 0.469 and -0.430 with $P<0.05$). Positive correlation of BAL pepsin with CRP and TNF alpha could also be identified in the scope of all patients (correlation coefficients were 0.408, 0.346 with $P<0.05$). Meanwhile BAL pepsin correlated negatively with lung function parameters FEV_1%, VC% and TLC% (correlation coefficients were -0.386,-0.355 and -0.402 with $P<0.05$). Further it was also found that BAL pepsin level was significantly different in respect to different types of lung cancer. There was however no evident difference on BAL pepsin, BAL CRP and BAL TNF alpha in patients with smoking history. PPI treatment had no impact on these indicators either.

Discussion

BAL pepsin correlates with airway inflammation and also with severity of restriction in patients with non-infectious, non-IPF lung diseases and tumours. Reflux could therefore be a causative parameter in various lung diseases, and thus further investigations should follow on this topic.

List of figures

List of tables

List of abbreviations

AC:	adenocarcinoma
ALRI:	acute lower respiratory tract infection
BAL:	bronchoalveolar lavage
BALF:	bronchoalveolar lavage fluid
BOS:	Obliterative bronchiolitis syndrome
CD3+:	a protein complex and T cell co-receptor that is involved in activating both the cytotoxic T cell (CD8+ naive T cells) and T helper cells (CD4+naive T cells).
CD4+:	a T lymphocyte sub-population
CD8+:	cytotoxic T lymphocytes
CDC:	centers for disease control and prevention
COPD:	chronic obstructive pulmonary disease
CSS:	Churg-Strauss syndrome
EGFR:	epidermal growth factor receptor
ELF:	epithelial lining fluid
ELISA:	enzyme-linked immunosorbent assay
FCR:	function residual capacity
FEV1:	forced expiratory volume in one second
FRC:	functional residual volume
FVC:	forced vital capacity
GERD:	gastroesophageal reflux disease
H2RA:	H2 receptor antagonists
HPIV:	human parainfluenza virus
IL-8:	interleukin-8
ILD:	interstitial lung disease
IPF:	idiopathic pulmonary fibrosis
LES:	lower esophageal sphincter

NLST: national lung screening trial

NMA: network meta-analysis

NSCLC: non-small cell lung cancer

OB: obliterative bronchiolitis

PAP: pulmonary alveolar proteinosis

PAR-2: proteinase-activated receptors-2

PD-1: programmed cell death protein 1

PPI: proton pump inhibitors

RA: rheumatoid arthritis

SCLC: small cell lung cancer

SQCC: squamous cell lung cancer

TGF-β1: tumour growth factor-β1

TKI: tyrosine kinase inhibitors

TLC: total long capacity

TBB: transbronchial biopsy

VC: vital capacity

1. Introduction

Chronic lung disease is often a preventable and treatable disease with progressive persistent airflow limitation[1]. Recent studies have found that the incidence of gastroesophageal reflux disease is very high[2-3], it refers to the symptoms or complications caused by the backflow of gastric contents into the oesophagus. Patients with acute exacerbation are likely to experience more serious heartburn and acid reflux symptoms. Pepsin is the main substance enzyme that causes the symptoms of reflux[4]. Population-based studies have demonstrated that 44% of US adults experience GERD-related symptoms once a month and 20% of total adult population experience it once a week[5]. Studies have also found that GERD is one of the causes of many pulmonary diseases, such as chronic cough, asthma, aspiration pneumonia, bronchiectasis and pulmonary fibrosis. A correlation analysis could be demonstrated by the fact that 90% of patients with idiopathic pulmonary fibrosis (IPF) may also have GERD[6-7]. Its relevance to other lung diseases is still unclear. The aim of the study is to investigate relationships between pepsin concentration in bronchoalveolar lavage (BAL) and lung function as well as its relationship with respiratory tract inflammation in patients with other non-infectious, chronic lung diseases or tumours.

1.1 Lung Diseases

Lung diseases are one of the most common medical conditions in the world. In the United States, tens of millions of people suffer from lung disease. In Europe, interstitial lung diseases are one of the severest risk factors of death e.g. Idiopathic pulmonary fibrosis (IPF). Between 2011 and 2013, the UK

reported the highest IPF mortality rate, while Lithuania had the lowest[8]. In Finland, the median mortality rate for man was 7.36 per 100,000 and 3.62 per 100,000 for women ranking the second among the 27 EU countries[8]. In Germany according to WHO reports data, the lung diseases deaths reached 43,056 in total, which was 5.98% of the total deaths[9]. The situation is even worse in Asian countries. 51% of the total lung cancer cases occurs in Asian countries, especially China. China is the world's largest workforce that is devoted to tobacco farming, manufacturing and sales. If current rates of smoking continue unabated, in year 2030, two million people will die of lung diseases per year[10]. Apart from the obvious circumstances that may lead to the lung disease, e.g. smoking, infections and genetics, studies have also found reasonable correlation with other environmental factors such as radon and asbestos in some patients[11].

The early stage of lung diseases is often asymptomatic. Some patients start with a nagging cough, abnormalities breathing, haemorrhage in respiratory passages as well as pain in the throat and chest[12]. According to the different clinical symptoms, the severity and the specific cause of the disease will be determined by different testing methods including chest radiographs, computed tomography, magnetic resonance imaging combined with the laboratory testing, pulmonary function test, bronchoscopy and biopsy. Treatment may depend on the underlying cause of the disease and patient's health status. Medicine, surgery and respiratory therapy may be prescribed to help lungs to recover. Some patients with advanced lung disease may even require a lung transplant.

1.1.1 Chronic malignant lung diseases

Globally, lung cancer is one of the most common forms of cancer. Among the EU members, approximately every fifth cancer death is led back to the cause of lung cancer which is estimated for the year of 2020 (182 600 men, 99 800 women) [13]. The chance for men to develop lung cancers is higher than that of women but the rates of young white women and women without smoking history are now keeping up[14]. The small cell lung cancer (SCLC) and the non-small cell lung cancer (NSCLC) are the two main groups of the primary lung cancer. The small cell lung cancer with high malignancy, early and extensive metastasis is however sensitive to radiotherapy and chemotherapy. which makes up to 15-20% of the lung cancer cases in Germany. Whereas, the non-small cell lung cancer as the most common form of lung cancer with a rate around 80% of the total lung cancer cases[15] represents a biologically and clinically heterogeneous group of histologies ranging from squamous cell lung cancer (SQCC 25–30%), adenocarcinoma (AC 40%) and the mixed subtypes (adenosquamous), over large cell carcinoma (10–15%) to the most uncommon type sarcomatoid carcinoma[16]. For all people with all types of lung cancers the 5-year survival rate is observed to be 19%. For women it is around 22%, while for men it is about 16%. Compared to 6% for small cell lung cancer, the 5-year survival rate of NSCLC is around 24%[17].

In many cases it has been found that NSCLC is resistant to drug therapy so that a complete response is rarely to be obtained. In consequence of drug resistance patients with NSCLC suffer excessively in the treatment by chemotherapy or in combination with radiotherapy[16]. The basic methods of treatment in NSCLC are nowadays surgery, radiation therapy, chemotherapy, targeted therapy, and immunotherapy.

Surgery is the standard treatment for patients with stage I and II tumours and some with stage III tumours, on the condition that they had received pre- or postoperative radiation therapy or chemotherapy (or both) if the tumour invades the mediastinal lymph nodes. However, because the early symptoms of lung cancers are not obvious, 70% to 80% of patients are found in the locally advanced and advanced stages, which makes the survival rate of NSCLC patients still low. There are several types of surgery depending on the scope of removal. The most effective one is presently thought to be lobectomy, which means the removal of an entire lobe as a lung has 5 lobes (2 in the left lung and 3 in the right lung). Lobectomy is a type of surgery commonly used in NSCLC[18], even in case that the size of lung tumour is still small. If the removal of an entire lobe of the lung is due to comorbidities not possible, another type of surgery called wedge resection may be applied. It means only the tumour along with a small healthy portion of the lung has to be removed. Similarly, a segmentectomy aims to remove exclusively the portion of the lung with cancer[18]. It is a practical method if the feasibility to remove an entire lobe of the lung is low. In a pneumonectomy[18], the surgeon however has to remove the entire lung, when the tumour is closer to the centre of the chest.

Radiation therapy is a physical treatment using high energy x-rays or other particles. By damaging directly the DNA the cancer cells are destroyed. The power of damage is however restricted in the path of the radiation beam only. The healthy cells in the same path are damaged as well. Concerning this side effect radiation therapy cannot be applied frequently in large areas of the body. Patients with a white blood cell count of less than 3000 mm^3 and a platelet count of less than 70,000 mm^3 (relative contraindications) may not be treated by radiotherapy. Radiation pneumonitis may occur during and within 1 month after radiotherapy, but also the following months. The symptoms of an acute

radiation pneumonitis include high fever, chest pain, cough and shortness of breath. Patients can be treated with medications e.g. prednisone (Rayos) under severe conditions.

Chemotherapy uses drugs to destroy the cancer cells. Chemotherapy is varying in its efficacy with adenocarcinoma or squamous cell carcinoma NSCL in different situations[19]: Neoadjuvant chemotherapy may be used before surgery eliminate disseminated tumour cells and to shrink a tumour to remove it with less extensive surgery. Adjuvant chemotherapy may be used after the surgery to kill any possible residual cancer cells that might have been left behind or have spread but not visible on imaging. For locally advanced NSCLC chemotherapy together with radiation therapy is recommend as the main treatment for more advanced cancers that have grown into near tissue. For metastatic (stage IV) NSCLC the chemotherapy may be given for spread areas outside the lung such as liver and adrenal gland. The main chemotherapy drugs for NSCLC include: Cisplatin, Carboplatin, Pemetrexed, Gemcitabine, Paclitaxel & Docetaxel & Albumin-bound paclitaxel (nab-paclitaxel), Vinorelbine and Etoposide.

For patients with preserved function status, a two-drug chemotherapy combined with a single third generation drug like vinorelbine, docetaxel, or pemetrexed along with cisplatin or carboplatin is recommended. Patients who are unable to endure a platinum-based combination could be rendered a single-agent therapy with a third generation drug[20]. The first-line standard chemotherapy regimen has reached consensus that it can increase the survival rate of NSCLC patients to a certain extent, but the efficacy has reached a plateau, thus the molecular targeted therapies and immune

check-point inhibition have become new hot spots in NSCLC medical treatment[21].

The epidermal growth factor receptor (EGFR) was the first molecule to be inhibited by a specific ‘targeted’ therapy in the treatment of advanced NSCLC. EGFR is a member of the transmembrane receptor Erb-B family. When a ligand binds with the extracellular domain of the receptor, EGFR activates to form of homo- or heterodimer, leading to the activation of the tyrosine kinase inhibitors (TKIs), and the tyrosine auto phosphorylates, eventually contributing to the abnormal cell proliferation, differentiation, increase angiogenesis, and inhibit tumour cell apoptosis[22]. If there is a mutation in the receptor a permanent activation of this pathway is present. EGFR inhibitors entering the clinical trial and application stage are mainly TKIs and humanized monoclonal antibodies. The former includes Gefitinib and Afatinib, and the typical representative of the humanized monoclonal antibodies is cetuximab[22]. A recent network meta-analysis (NMA) about the first-line treatments revealed that osimertinib is at the moment the best therapeutic agent among five major EGFR TKIs and helped patients with NSCLC achieve the longest progression-free survival (PFS) via EGFR-activating mutations[23]. There are further genetic alterations which can drive lung cancers and can be treated specifically like Alk- and RET-fusions. The precision therapies guided by biomarkers are still rapidly developing.

Immunological therapy for NSCLC: In recent years, immunotherapy has become increasingly prominent in the comprehensive treatment of NSCLC because of its high effectiveness and safety.

The programmed cell death protein 1 (PD-1) and programmed death ligand 1 (PD-L1) inhibitors have been widespread in first- and second-line treatment of advanced NSCLC[24-25]. They are effective in recurrent disease as monotherapy. In stage IV NSCLC chemotherapy and PD-L1 inhibitors and in high expressors of PD-L1 immuno-monotherapy can increase progression-free survival and overall survival compared with traditional chemotherapy. But further continuous optimization is required for advanced NSCLC. Immunotherapy combined with chemotherapy or radiation therapy should be applied to benefit a larger population.

1.1.2 Chronic non-malignant lung diseases

Typical non-malignant respiratory diseases are asthma, COPD (Chronic Obstructive Pulmonary Disease), interstitial lung disease, pulmonary sarcoidosis.

The incidence of asthma has been rising since 1970s. The WHO estimates that it has affected around 235 million people worldwide[26-27]. In the United States there are over 25 million people suffering from asthma[28]. It is also one of the most common chronic diseases in children, which has affected more than 6 million children[29-30]. The cause of asthma is multi-factorial, generally asthma is a result of the interaction between multiple susceptible genes and environmental factors. Most patients with asthma have symptoms of allergy such as anaphylactic rhinitis or atopic dermatitis. They may also be allergic to common allergens in the air (e.g. mites, pollen, pets and mold), certain foods (e.g. nuts, milk, peanuts, sea foods), or drugs[31].

Based on the clinical manifestations, the course of asthma can be characterized by acute exacerbation, chronic persistent phase and clinical remission[32]. In the chronic persistent phase symptoms (wheezing, shortness of breath, coughing) occur repeatedly. The clinical remission describes the disappearance of symptoms after or even without treatments and the recovery of lung functions equally to the status prior to onset. These conditions must be stable for at least 3 months[33-34]. For many patients it was misdiagnosed as chronic bronchitis or pharyngitis for a long time until the final diagnosis of asthma[35]. A severe acute onset of asthma can be fatal if not treated timely. The recurrence of asthma can lead furtherly to chronic obstructive pulmonary disease, emphysema, pulmonary heart disease, heart failure, respiratory failure and other complications[36-37].

Chronic obstructive pulmonary disease (COPD) is a kind of lung disease that causes dyspnea[52]. Chronic obstructive bronchitis and emphysema are two main forms of COPD[53]. The airways of patients with COPD become narrow as the result of chronic inflammatory damage over a long period of time. COPD the fourth leading cause of death in the United States and there are over 120,000 people dying from 2004[54-55]. Smoking is the most common cause of COPD and a large number of patients with COPD also have smoking history[56-57]. Therefore, quitting smoking is the first and the most important step to prevent and treat COPD. Passive smoking, dust, harmful gases in workplace, air pollution etc represent other risk factors[56-57]. The clinical manifestations of COPD are dyspnoea, shortness of breath, wheezing, coughing and fatigue, which occur initially after movement, but later on even at rest along with the disease progression[58]. The main diagnosis and evaluation method of COPD is the examination of lung function, especially spirometry[59]. Among COPD patients the rate of airflow becomes slow and the volume

decreases as a result of the airway inflammation and narrowing. In general, if the results out of the measurement fall below the normal value, the examination will be repeated after inhaling bronchodilators. In case of asthma the results usually turn to be normal afterwards, whereas only limited improvement can be observed among patients with COPD[59]. The severity of airway obstruction can predict the survival rate for patients with COPD. The 5-year survival rate would be 40%~60% when FEV1<1.25L. The rate would fall down to 30%~40% if FEV1<0.75L[60]. The BODE index can be applied to predict the risk more precisely, consisting of Body mass index (B), evaluation of Obstruction (O, namely FEV1), Dyspnoea (D, modified Medical Research Council Dyspnoea Scale) and Exercise capacity (E, assessed by 6-minute Walk Distance test). Drug, oxygen therapy, pulmonary rehabilitation and surgery are the common treatment of COPD[61]. Influenza and pneumonia can cause severe complications and even death in COPD patients. Hence, the preventive measure as vaccination against influenza and pneumonia is necessary for patients with COPD[62].

Interstitial lung disease (ILD) represents a group of diffuse lung disorders mainly involving the pulmonary interstitium, alveoli and bronchioles of the lung[63]. ILD is not a specific disease, it includes more than 200 diseases[63]. Although the clinical manifestations, laboratory and pathology changes of each disease have its own characteristics, there are some common clinical features in respiratory pathophysiology and pattern of chest X-ray, as progressive exertional dyspnoea, restrictive ventilation impairment associated with the decrease of diffused function, hypoxemia and diffuse double lung lesions on radiology[64]. The diseases develop progressively with gradual loss of alveolar-capillary function units to diffuse pulmonary fibrosis and honeycomb-formed lung pattern that finally cause respiratory failure. There are

around 20%~30% patients with non-small cell lung cancer (NSCLC) and 4.5% small cell lung cancer (SCLC) accompanied by ILD[65-66]. Epidemiological studies showed that IPF developed to lung cancer in 22% patients with a risk approximately 5 times as the normal population[67]. There are common pathogenic mechanisms between ILD and lung cancer. In the course of fibroblast formation, genes mutation (p53、SFTPA1、SFTPA2), release of soluble mediators (transforming growth factor (TGF), Nitric Oxide (NO), reactive oxygen species (ROS)) and imbalance of cell apoptosis lead to carcinogenic transformation of epithelial cells[68]. Although there are extensive correlations between ILD and lung cancer in terms of epidemiology and mechanism, the treatments for these particular patients have not been widely studied in depth. Nintedanib (Ofev™),is the only drug that relieves lung dysfunction in case of SSc-ILD and the only drug that has been approved for treating PF-ILD as well. It is a multi-target tyrosine-kinase inhibitor, which suppresses the key pathway involving in the course of pulmonary fibrosis. So far, Ofev has been approved to be applied in idiopathic pulmonary fibrosis (IPF), systemic scleroderma-interstitial lung disease (SSc-ILD) and progressive fibrosing interstitial lung disease (PF-ILD) other than IPF[69].

Pulmonary sarcoidosis is a granulomatous disease with unknown aetiology, which is often observed in lung, bilateral hilar lymph nodes, eyes, skin etc[70]. The invasion rate in chest is as high as 80%~90% [71]. The morbidity of pulmonary sarcoidosis is higher in Europe and the United States than in Asian countries[72]. It occurs mostly between 25 to 40 years old and slightly more frequently in women than men[73]. As the aetiology of sarcoidosis is yet unknown, the diagnosis relies on the corresponding clinical history, histologically the presence of extensive non-caseating granulomas on more than one organ, as well as rule out other possible diagnosis, such as

tuberculosis, fungal infection and malignant tumour[70]. Clinical, epidemiological and familial studies showed that sarcoidosis may be caused by exposure to a certain environmental factor and there is hereditary susceptibility to it at the same time[74]. Thoracic sarcoidosis presents clinically systemic symptoms that are not specific to certain aetiology, cough without sputum, shortness of breath, occasionally mild chest pain and haemoptysis[75]. The variety of manifestations of sarcoidosis makes it difficult in the diagnosis. Therefore, only the combination of clinical, imaging and pathological evaluation can ensure a deterministic diagnosis[75.] Sarcoidosis is often associated with granulomatous inflammation, which is accumulated in bronchopulmonary vessels and central lobular structure. Transbronchial biopsy (TBB) is routinely used to obtain histological specimen for clinical imaging diagnosis[76]. The diagnosis sensitivity of TBB to most of ILD range from 29% to 79%[77]. Corticosteroids remain the main treatment when there is risks of organ failure or the disease is progressing chronically[78]. Meanwhile, immunosuppressants as methotrexate (MTX) and the new anti-inflammatory therapies (infliximab) can also be applied[79].

1.1.3 Acute Lung diseases

Acute pulmonary diseases mainly include acute pneumonia, acute tuberculosis, acute pulmonary embolism, acute pulmonary heart disease and so on. According to the Centers for Disease Control and Prevention (CDC) in 2015 more than 50,000 people in the United States died from pneumonia, which is the leading cause of death worldwide for children under 5 years of age[80]. Pneumonia occurs frequently regardless of seasons. Children under 15 years old are more likely to suffer from pneumonia in winter and spring. If they don’t receive thorough treatments, it is easy to recur and cause other serious

complications, affecting children's healthy development. The clinical symptoms of pneumonia are fever, cough, shortness of breath, dyspnoea and moist rales in the lungs. Some children may experience severe cough and asthma without fever. Children's pneumonia has typical symptoms, but there are also atypical ones, especially neonatal pneumonia. Pneumonias are usually caused by bacteria and viruses including Staphylococcus aureus, Klebsiella pneumoniae, human parainfluenza virus (HPIV) infection and corona virus and so on. The disease should be treated with reasonable comprehensive measures. It is of immense significance to control infection effectively, keep the respiratory tract free from obstruction, correct hypoxia, prevent and cure complications and promote rehabilitation by enhancing the body resistance.

Pulmonary tuberculosis is the most common form of tuberculosis, which is a chronic infectious disease caused by mycobacterium tuberculosis. An important source of a tuberculosis infection are the excretors[81]. Mycobacterium tuberculosis is capable to invade many organs of human and animals. Human tuberculosis may lurk after infection until the resistance decreases or the cell-mediated allergy increases with the risk of clinical morbidity. Therefore, the essential way to avoid the progression to tuberculosis disease is screening and prophylactic therapy especially among the high risk individuals[81]. The most recommended methods for treatment of active tuberculosis are multiple drugs including isoniazid, rifampin, ethambutol and pyrazinamide[82].

In addition to the two main diseases mentioned above, the acute lung disease pertains among others also patients

1. after a lung transplantation surgery and suspected for organ rejection;
2. with rheumatic disease and potential lung affection;

3. with eosinophilic disorder which may lead to asthma or Churg-Strauss-syndrome.

Acute allograft rejection is an important problem in lung transplantation. Although the immunosuppressant medications have made progress to prevent the rejection, more than 1/3 of lung transplant recipients still receive treatment for acute rejection in the first year after the transplantation[83-84]. Among patients who died within 30 days after transplantation, about 4% patients died because of acute rejection[85]. Rejection means that the immune system recognizes the new lung as foreign invader and generates antibodies to attack it, the same way as a virus is "rejected". Affecting around 10% of the patients, chronic rejection is more severe than the acute rejection that occurs with quick symptoms. While muscle weakness (31.9%) and Cushingoid appearance (38.6%) have been found to be the most distressing symptoms, hirsutism (68.1%) and tremors (70%) are the symptoms reported most frequently[86]. Although the success rate of lung transplantation is getting higher and higher yet lung transplantation is still the most difficult of all organ transplants and moreover, it is not suitable for all patients in the end phase.

Rheumatoid arthritis (RA) is a chronic, inflammatory and systemic autoimmune disease with symptoms primarily in the joints. The cause lies in autoantibodies that target the various molecules along with modified self-epitopes. Lung diseases associated with RA have been observed frequently. RA can cause pulmonary complications by affecting the lung parenchyma, the airways and the pleura which result in 10 to 20% of all mortalities[87]. Scarring of the lungs is the most often associated lung disease with rheumatoid arthritis[88]. Similar to acute lung diseases, long-term inflammation related scars could lead to shortness of breath, chronic dry cough, fatigue, weakness and loss of appetite[89]. There are also some respiratory diseases that may affect the

quality of life and prognosis of patients with RA include: interstitial lung disease (ILD), obliterative bronchiolitis (OB), drug response, and infection. Therefore, chest HRCT and lung function tests are necessary for high-risk RA patients.

Churg-Strauss syndrome (CSS), also known as "EGPA" (eosinophilic granulomatosis with polyangiitis or allergic granulomatosis), is a kind of autoimmune disease[90]. Most people with Churg-Strauss syndrome have chronic diseases, such as asthma, blood and tissue eosinophilia. Patients may also have other symptoms like cough, shortness of breath, blood in the urine and stools etc..

1.2 Gastroesophageal reflux diseases

Gastroesophageal reflux disease (GERD) refers to excessive reflux of gastric and duodenal contents into the oesophagus which may cause heartburn and can lead to esophagitis and tissue damage outside the oesophagus such as pharynx, larynx and airway[91]. It is a common disease in western countries. About 7%-15% of the population have gastroesophageal reflux symptoms[92]. The incidence of gastroesophageal reflux increases with age reaching its peak at 40-60 years old[93]. There is no difference of the incidence between men and women, but men are more likely to have reflux oesophagitis than women (2:1 to 3:1). Compared with western countries, the incidence of gastroesophageal reflux disease in China is lower and the symptoms are milder[94]. The most common symptoms of gastroesophageal reflux disease are heart burning and acid regurgitation. Heart burning refers to the burning sensation behind the sternum or under the xiphoid. It usually extends upward from the lower sternum and occurs one hour after meal. It can be aggravated when lying,

bending or abdominal pressure increases. The gush of gastric contents into the oral cavity is collectively associated with nausea[95]. The reflux is mostly acidic. Some patients had dysphagia, swallowing pain and retrosternal pain. Such pain occurs behind the sternum or under the xiphoid. It can be as severe as intense tingling, and it can radiate to the back, chest, shoulder, neck, ear, resembling angina pectoris[96]. Exceptions without such typical symptoms of heartburn and acid reflux commonly related to gastroesophageal reflux disease represent difficulty in the diagnosis of GERD[97].

There are different approaches to ease or treat GERD. To reduce reflux during night-time physically, bed feet can be raised by 15-20 cm in order to adjust the sleeping position [98]. Reflux often occurs after meals. Hence it is not advisable to eat before bedtime or lie in bed immediately after meals during the day[99]. Patients may pay attention to reducing the factors that generally affect the increase of abdominal pressure, such as obesity, constipation and tight belts. Drug treatments include H2 receptor antagonists (H2RA), gastrointestinal motility drugs, proton pump inhibitors (PPIs)[100]. Anti-reflux surgery is a different type of fundoplication, which aims to prevent the stomach contents from flowing back into the oesophagus. Anti-reflux surgery is applied when: 1. strict medical treatment is ineffective; 2. the patient cannot tolerate the precipitation medication; 3. recurrent oesophageal stricture after dilatation treatment, especially in young patients; 4 confirmed serious respiratory illness by reflux[101].

1.3 Diagnostic tools in pneumology

With the advancement and update of technology, the examination methods for lung diseases have also become diverse, which have significantly improved the accuracy of diagnosis especially for the acute and potentially curable disease in the early stage. Many diseases can be cured if they are accurately diagnosed and effectively treated in the early stages. This is also true with cancer.

Currently, the main technical detection method of pneumonology apart from lung function tests is imaging examination including computed tomography, X-ray, bronchoscopy and radionuclide examination. Chest X-ray and CT provide a clearer image of the morphology and properties (e.g. size, shape and position) of lung shadows and tumours. Clinical screening trials are studies that help determine the extent to which screening methods are proven to reduce mortality (death rate) with the corresponding cost evaluation. The use of low dose computed tomography is better than the use of X-ray in the early diagnosis of lung cancer. According to the National Lung Screening Trial (NLST), the application of routine annual chest radiography for screening of high-risk patients for lung cancer, is in contrast to low-dose chest CT concluded not to be beneficial in terms of improving mortality although it helps detect a significant number of cases[102]. Fibreoptic bronchoscopy and through skin biopsy of lung biopsy can gain material for pathological, bacterial, and biochemical tests, which greatly improves the diagnosis rate of aetiology. The bronchoalveolar lavage fluid from bronchoscopy is used to count, classify and measure cells, immune antibodies, complement and enzymes in the liquid especially in the diagnosis of bronchopulmonary diseases. Radionuclide examination is to inject a radionuclide-labelled substance into a vein and use a

gamma camera or ECT to take an image of the distribution of radioactive substances in the lungs. It is mainly used for the diagnosis of pulmonary embolism like COPD. Moreover, the radionuclide examination can also determine the lung perfusion and ventilation function.

1.3.1 Spirometry and Body Plethysmography

Spirometry is a physiological test that measures the volume of breathed air as a function of time, which is the simplest and effective test method to assess the lung function[103]. Pulmonary function tests can detect the lung disease or monitor the severity of disease (such as COPD). The spirometry routine testing can help doctors diagnose the disease at early stages and propose the best treatment plan in time. It is a rapid and painless test. Currently there are several different models of equipment, which require cooperation between the subject and the examiner. The results depend on technical and personal factors[104].

Before the examination, the staff needs to explain the purpose of the test and demonstrate the procedure. Subjects will be asked about their medical history in details in order to judge the indication of the testing and exclude the contraindications. After recording the basic information e.g. height, weight, age and gender, subjects will sit upright without the support of a chair back, put the feet on the ground without raising legs, maintain the head at a natural level or slightly upward without bowing or bending. A correct sitting posture is essential to maximize the breathing capacity. Considering the special circumstances, other positions such as standing or lying should also be noted in the report. Subjects will breathe through a mouth piece, inspire maximally and hold the

breath for a while, afterwards exhale as hard as they can. The test will be repeated at least three times to ensure that the result is consistent. If there is significant variation the test is to be repeated and the doctor will take the highest value from three close test readings as the final result of the subject[105]. The results of the spirometry test are different from individual to individual depending on the characteristics e.g. gender, age, height. The main measurement items of spirometry are: expiratory forced vital capacity (FVC) which is the maximum amount of air that a subject can breathe out after breathing in as deeply as possible. Forced expiratory volume in one second (FEV_1) is the amount of the air that a subject can force out of his or her lungs in one second. According to the American Thoracic Society the FEV_1 value can help the doctor evaluate how severe the abnormalities are. FVC and FEV_1 will normally be analyzed separately and a FEV_1/FVC ratio can be calculated. The ratio presents the percentage of the lung vital capacity which can be exhaled in one second. A normal ratio is about > 70% for adults and > 85% for children[106].

Lung plethysmography test is another advanced technique to determine lung function which is also called body plethysmography. It is more accurate than the standard spirometry testing. The measurement has been proved to be able to provide clinical information that is independent from other functional information, especially in cases of obstructive airway disease[107]. During the test, subjects will take seat in an enclosed transparent plastic box. With a nose clip on they will be instructed how to breathe through the mouthpiece. The duration of the whole test is about a quarter hour. By body plethysmography airway resistance can be measured. A further difference between plethysmography test and the most pulmonary function tests is that the former can determine residual volume or the amount of air left in the lungs after subjects exhale as much air as possible. This test can be applied to: functional

residual volume (FRV), function residual capacity (FRC) and total lung capacity (TLC), vital capacity (VC). Body plethysmography is recommended for young children with acute lower respiratory tract infection (ALRI) aged 1-36 months[108].

1.3.2 Bronchoalveolar lavage

Bronchoalveolar lavage or BAL, is a minimally invasive procedure in which doctors during bronchoscopy drip sterile physiological saline into the small airways and alveoli, then aspirate and collect samples of the epithelial lining fluid (ELF). A study has showed the bronchoscopy are the safest and most accurate tools to evaluate both central and distal airway mucosa[109]. The procedure uses a bronchoscope tube with a small camera inserted into the subject's nose or mouth, down from throat into the trachea and to the bronchi of the lung. An official guideline published by American Thoracic Society introduced the clinical utility of bronchoalveolar lavage[110]. BAL fluid cell patterns reflect inflammatory cell profiles in affected lung tissues and provide important information that can support the diagnosis[111]. Differential cell count has been performed with identification of alveolar macrophages, lymphocytes, neutrophils and eosinophils, or other findings like tumoral cells, foreign body, mastocytes, basophils, or red blood cells [112].

In order to analyze the cell profile changes in the bronchoalveolar lavage fluid (BALF) in lung cancer patients and find out the specific immunologic reactions of the lung, Dr. Domagala-Kukawik[113] and colleagues conducted routine analysis and lymphocyte phenotyping by an immunoperoxidase technique with the monoclonal antibodies such as CD3, CD8, the results of which were

compared with a control group. The results showed that compared with healthy controls, BALF macrophage (60%) decreased significantly, lymphocytes, neutrophils (24%, 13%) as well as T lymphocytes (86%) increased significantly and CD4 + / CD8 + relative declined[114]. Subsequent studies also used enzyme-linked immunosorbent assay (ELISA) to detect primary lung cancer and tumour growth factor-β1 (TGF-β1) of healthy subjects and patients. The observation of higher levels of TGF-beta1 in the BALF of the patients compared with the healthy subjects confirmed the active participation of the TGF-beta in the local response in the development of primary lung cancer [115].

BAL may also be applied to measure the concentration of substances which are considered to causally relate to pulmonary disease states[116]. Dr. Lee and his colleagues tested BAL pepsin among 24 patients with acute exacerbations of IPF and 30 patients with stable disease control. They found patients with acute exacerbations had apparently a higher pepsin level than those under stable controls. These results indicate that there may impact from occult aspiration in some cases of acute exacerbation of idiopathic pulmonary fibrosis[116]. Dr. Dong also did the same testing and he said that if the positive results were obtained, the initial pepsin level of the BAL could predict the progression of the acute exacerbation episode in the future, and could at least help to find the effective treatment criteria for some patients[117].

1.4 Pepsin

As an endopeptidase generated in the stomach, pepsin breaks down proteins into polypeptides. It is the chief digestive enzyme in the stomach[118]. Pepsinogen, the precursor of pepsin, is secreted by the main cells of the

gastric glands. By action of gastric acid or activated pepsin an inactive pepsinogen is converted into an active pepsin. Pepsin acts to break down protein under appropriate conditions (pH<3.0). The primary product is hydrazine, rarely accompanied with small molecular peptides or amino acids[119]. Pepsin acts mainly on the peptide bond composed of aromatic amino acid or an amino group of acidic amino acid.

Gastroesophageal reflux is a common problem of non-cystic fibrotic bronchiectasis and COPD[120]. A study found that the positive salivary pepsin was related to the acute exacerbation of COPD patients with gastroesophageal reflux in stable stage. Salivary pepsin test should be considered before treating patients with chronic obstructive pulmonary disease who experience gastroesophageal reflux with proton pump inhibitor (PPI)[121]. Lee[122] detected pepsin in the samples of bronchiectasis and COPD exhaust gas condensate. The results showed that although the concentration of pepsin was not related to the diagnosis of gastroesophageal reflux, the relationship between the concentration of pepsin in sputum and exhaled gas condensate indicated that the detection of pepsin in exhaled gas condensate might be a useful non-invasive marker for the detection of related lung diseases. Recent data suggested that anti-gastroesophageal reflux surgery can effectively prevent lung disease progression in patients with idiopathic pulmonary fibrosis or lung transplantation[123]. Measuring biomarkers of gastric reflux inhaled into the lower respiratory tract (e.g., pepsin and bile acid concentrations in bronchoalveolar lavage fluid) can be helpful in diagnosis and treatment. Timms et al[124] also found that new methods such as expiratory condensation analysis and electronic nose technology can improve the accuracy of diagnosis of gastroesophageal reflux.

1.4.1 Pepsin in the gastroesophageal reflux disease

Essentially, the pathogenesis of gastroesophageal reflux disease is the imbalance between the defence barrier against oesophageal reflux and the attack of reflux food on oesophageal mucosa[125]. Specific mechanisms include the following aspects: (1) lower oesophageal sphincter (LES). LES is a barrier against gastroesophageal reflux. Previously it was once considered that the conditions for a complete LES should be: LES pressure > 6 mmHg, abdominal LES length > 1 cm and LES length > 2 cm. But in recent years, high resolution oesophageal manometry system showed that the conditions are: LES pressure integral > 400 mmHg/(s cm), LES length > 2 cm and abdominal LES length > 1 cm as an important factor for LES to maintain effective resistance[126].

Oesophageal epithelial permeability changes. Electron microscopy showed that the gap between oesophageal epithelial cells widened in patients with gastroesophageal reflux, which deepened the understanding of the aetiology of gastroesophageal reflux at the cellular and molecular level. Considering the role of E-cadherin in maintaining epithelial cell junction and strengthening epithelial barrier, Western blot detection showed that E-cadherin was degraded. In addition, the decrease of oesophageal epithelial resistance monitoring and the increase of fluorescein monitoring flow support that the degradation of cadherin leads to the increase of oesophageal epithelial permeability, which results in epithelial dysfunction[127]. Besides the mechanical barrier of LES and oesophageal epithelium, saliva secretion is also an important component of oesophageal defence mechanism[128]. Salivary hypopharynx clears the oesophagus, neutralizes and dilutes the reflux that

enters the oesophagus. Saliva also contains epidermal growth factors that promote the growth of oesophageal epidermal cells[129-130].

Proteinase-activated receptors-2 (PAR-2): Kandulski et al. found duodenal contents in reflux of patients with severe gastroesophageal reflux disease, especially when trypsin left the normal functional environment, which could cause obvious mucosal damage. Its direct mechanism of destruction was related to proteinase-activated receptors-2 (PAR-2), which would lead to oesophageal mucositis. The expression of interleukin-8 (IL-8) is increased, which participates in the inflammatory reaction of oesophageal mucosa and causes epithelial injury. PAR-2 and interleukin-8 pathways explain the molecular and inflammatory changes of gastroesophageal reflux disease[131].

Helicobacter pylori infection: The impact of Helicobacter pylori on gastroesophageal reflux disease is still controversial, and the eradication of Helicobacter pylori needs further weighing of the pros and cons before any individual treatment. Helicobacter pylori infection causes gastric mucosal inflammation, increases local related cytokines, decreases gastric acid secretion, and atrophy of gastric mucosa. After eradication of Helicobacter pylori, acid secretion rebounds without increasing, although the recovery of gastric acid secretion may cause symptoms due to asymptomatic reflux. The risk of not eradicating Helicobacter pylori is that patients may develop gastric cancer, and therefore the treatment needs to vary from person to person. Gastric antral inflammation caused by Helicobacter pylori causes an increase in gastrin and gastric acid secretion, so eradication of Helicobacter pylori can alleviate the symptoms of gastroesophageal reflux[132-133].

1.5 Aim of the dissertation

Previous studies have estimated that as many as 90% of patients with Idiopathic pulmonary fibrosis (IPF) may also have GERD[134] and pepsin could be taken as an indicator of worsening lung function. There is few analysis about the correlation between the pepsin and lung diseases other than IPF. The aim of this study is to investigate the relationship between pepsin concentration in bronchoalveolar lavage (BAL) fluid and lung function as well as its relationship with respiratory tract inflammation markers in acute/chronic disease and normal groups.

2. Patients and methods

2.1 Patients

We examined patients from our pneumological ward with a clinical indication for BAL. A total of 72 patients were involved in the study, and two patients with IPF pulmonary fibrosis were removed. Numerous previous studies have shown that 90% of IPF patients may also have GERD[6-7]. In order to analyze the relationship between GERD and other lung diseases, two patients with IPF were excluded. The remaining 70 patients were divided into three large groups, the acute lung disease group, the chronic lung disease group, and the control group. Acute lung diseases include tuberculosis and pneumonia, chronic lung diseases include pulmonary nodular lesions, interstitial lung disease excluding IPF, squamous cell carcinoma, non-squamous cell carcinoma (such as small cell lung cancer, adenocarcinoma and undifferentiated large cell carcinoma). The control group mainly includes patients without specific diagnosis who were however clinically symptomatic (cough, shortness of breath).

The concentrations of pepsin, CRP and TNF alpha were determined by ELISA. Retrospectively, spirometric (FEV_1, VC% target), and bodyplethysmographic (resistance, TLC% target) values, laboratory chemical, biometric (sex, height, weight) and clinical parameters (disease, medication, smoking status) were recorded.

2.2 Bronchoscopic procedures and sample collection

2.2.1 Bronchoscopic procedures

Bronchoalveolar lavage (BAL) is performed in accordance with an official clinical practice guideline that was published by American thoracic society [80,135]. First, the patient's nostrils and pharynx are locally anesthetized with lidocaine, and a titrated sedation with midazolam is started, followed by the placement of the flexible bronchoscope in wedge position within the relevant bronchopulmonary segment. Thereafter, 100-300 ml physiologic saline at room-temperature is instilled via the bronchoscope by 3-5 equal portions. After each procedure, the portion of saline is usually retrieved by a negative suction pressure less than 100 mm Hg, which is constantly adjusted for avoiding any distinct airway collapse. Typically, the minimal retrieved volume is above 30% of the total instillation. When only < 5% of each instilled portion is retrieved with most of fluid left in the lung, the procedure is to aborted to avoid additional risk. A pooled BAL sample with a volume no less than 5 ml (optimal volume 10-20 ml) is required to conduct BAL cellular analysis. All the retrieved BAL fluid out of the aliquots can be gathered to carry out routine analysis[134]. The BAL cellular components are analyzed in terms of morphology and immunology. Standard items are total cell count, differential count of macrophages, lymphocytes and neutrophils along with flow cytometry analysis of the lymphocyte subsets such as BAL CD4/CD8 T-cell ratio. Subgroups of different cell counts (lymphocytes, leukocytes, macrophages neutrophils, CD4/CD8 ratio) are defined in reference to the standard threshold in interpreting the BAL fluid[135].

The diagnosis may also partially refer to the appearance of the BAL fluid. For instance, extremely bloody BAL fluid with enhanced intensity in the sequence of aliquots suggests the possibility of acute diffuse alveolar haemorrhage, whereas BAL fluid with extremely cloudy pattern (i.e., milky or light brown-beige) containing flocculent deposition on container bottom formed within 15-20 minutes is highly suggestive of pulmonary alveolar proteinosis (PAP)[135]. Meanwhile, the blood oxygen saturation and heart rate are monitored continuously during bronchoscopy as well as the non-invasive blood pressure monitored at regular intervals.

2.2.2 Sample processing

After recording the retrieved volume of lavage fluid, samples were divided in the bronchoscopy suite depending on clinical indication. Aliquots were marked for the purpose of microbiological and pathological analyses respectively. The remaining lavage fluid was sent immediately to the on-site pneumology laboratory at ambient temperature for further processing. A triple-layer of sterile gauze was used to filter the fluid so that mucous and large particles were finally removed. 15ml BAL filtered fluid was prepared to carry out fluorescence activated cell sorting (FACS) analysis. Furthermore, at least 2 aliquots (500 ml) had to be stored under -20 C° to perform enzyme-linked immunosorbent assay (ELISA) analysis [115].

2.2.2.1 TNF alpha

Tumour necrosis factor α (TNF-α) is an active inflammatory cytokine, which plays an important role in numerous lung inflammatory diseases[136]. The TNF-α

concentrations in plasma and serum were determined by the Quantikine HS TNF-alpha Immunoassay kit, which was a 6.5 hour solid phase containing recombinant human TNF-α derived from *E. coli* as well as antibodies generated against recombinant factor. This kit can be used to precisely quantify the recombinant natural human TNF-α.

The specific operation procedures could be summarized as the following steps[137]:

- Prepare all reagents, samples wells and move excessive microplate strips away from the plate frame, then add them into a foil pouch that contains desiccant pack, and seal again.
- Place Assay Diluent RD1F (50 μL) containing precipitates into each well and mix it thoroughly before use.
- Add 200 μL of sample covered by the given adhesive strip to culture under ambient temperature for 3 hours.
- Washing the plate: eliminate the liquid around the wells, hold the plate firmly, then rap the inverted plate onto the clean paper towel for 5 times or more. Add Wash Buffer (400 μL) into each well. Repeat the steps at least 5 times.
- Place natural human TNF-α HS Conjugate (200 μL) containing a precipitate into each well. Make sure it is well mixed. Cover the wells with new adhesive strips. Incubate for 2 hours and repeat the washing steps.

- Place Substrate Solution (50 µL) into each well. Use the adhesive strip to cover the wells. Keep the plate for 1 hour of incubation under ambient temperature.

- Place the Amplifier Solution (50 µL) into each well. Use the adhesive strip to cover the wells, followed by 30 minutes of incubation under ambient temperature.

- Place the Stop Solution (50 µL, which makes no difference to the well color) into each well.

- Measure the optical density in all wells within 30 minutes by means of the microplate reader at the level of 490 nm.

2.2.2.2 C- Reactive protein (CRP)

The Enzyme Immunoassay for Hs C-Reactive Protein was provided by DRG International Inc., USA. In comparison with the other testing methods, the ELISA provides highest sensitivity and specificity of detection CRP value[138]. The CRP assay can be conducted to assist in the diagnosis, treatment and monitoring of inflammation and related diseases. In the presence of acute tissue injury, the CRP contents in the serum or plasma would increase within 24-48 hours, which peak in the acute stage (about 1000x constitutive level) and reduce after the alleviation of trauma and inflammation. Normal CRP levels are below 3.0 mg/L.

The specific operation procedures could be summarized by the following steps[138]:

- Dilute BAL fluid by 1:100 before use.
- Keep the required number of coated wells firmly in the holder.
- Distribute the diluted samples (10 µL) into corresponding wells.
- Distribute the CRP Enzyme Conjugate Reagent (100 µL) into each well.
- Blend sufficiently for 30 seconds.
- Incubate for 45 minutes under ambient temperature (18- 25 °C).
- Flick plate contents to the waste container to move away incubation mixture. Use deionized water to wash and flick the microtiter wells 5 times.
- Drain each well using the paper towels or absorbent paper to remove the remaining water drops.
- Add TMB solution (100 µL) to each well and blend gently for 5 seconds.
- Incubate for 20 minutes under ambient temperature.
- Add the Stop Solution (100 µL) into each well to abort the reaction.
- Blend gently for 30 seconds till the solution color changes from blue to yellow completely.
- Determine the absorbance values by means of the microtiter well reader at the level of 450 nm within 15 minutes.

2.2.2.3 Pepsin by ELISA

The amount of pepsin in the specimen was determined by enzyme-linked immunosorbent assay (ELISA), and the buffer in the kit was used as a blank control. The kit is produced by USCN life science Inc. in the USA.

The specific operation procedure could be summarized as follows:

- Prepare 7 wells for dilute samples and 1 well as control. Place 100 µL sample into corresponding wells. Use the sealers to cover the plate and incubate the samples for 2 hours under 37 °C.
- Remove the liquid of each well without washing.
- Place the detection reagent A working solution (100 µL) into each well, use plate sealers to cover the wells and incubate the samples for 1 hour at 37 °C.
- Absorb the solution and wash each well with solution (350 µL) using a squirt bottle, multi-channel pipette and manifold dispenser, followed by standing for 1-2 minutes. Remove the residual liquid from all wells completely with the absorbent paper and rinse the wells 3 times.
- Place the Detection Reagent B working solution (100 µL) into each well, use plate sealers to cover the wells and incubate the samples for 30 minutes at 37 °C.
- Repeat the water absorption and rinsing process for 5 times according to the instruction in step 4.
- Place Substrate Solution (90 µL) into each well, use new plate sealers to cover the wells and incubate the samples for 15 - 25 minutes at 37 °C.

(no longer than 30 minutes). Keep the wells away from light. When Substrate Solution is added, the liquid will turn blue.

- Place the Stop Solution (50 µL) into each well. When Stop solution is added, the liquid will turn yellow. Blend the liquid by tapping the plate side.
- Remove fingerprints and water drops on the plate bottom and ensure no bubble on the surface of liquid. Afterwards, use the microplate reader to take the measurement at the level of 450 nm as soon as possible.

2.2.2.4 Lung function

Pulmonary function tests were performed according to the American Thoracic Society and European Respiratory Society statements[139] which have been also described previously. Patients were guided by an experienced technician, and at least 3 to 8 trials were performed within a test. Indices of the best manoeuvre for FEV_1, VC MAX, FEV_1/ VC MAX ratio, and maximum mid-expiratory flow volume at 25, 75 percent of the forced vital capacity (MMFF75/25) and TLC were analyzed. The subject's biometric (sex, height, weight, age) information were recorded.

2.3 Statistical Methods

The analysis of data was performed using SPSS version 23.0. Descriptive analysis (mean ± SD). Spearman correlation coefficient was used for establishing a correlation between pepsin and distributed continuous variables. The groups and subgroups were compared by non-parametric Mann/Whitney U test and Kruskal-Wallis test as appropriate. All tests were

assumed at two-tailed and performed at a significant level of 0.05. GraphPad Prism Software, version 6.0 was used to generate the figures.

3. Results

3.1 Demographics

A total of 70 patients were enrolled in the study. The age of the patients ranged from 18 to 84 years old with an average age of 55.94 years. The average BMI was 25.09 ± 5.89 kg/m^2. The patients were divided into three groups. Group I included 29 patients who experienced chronic lung diseases, e.g. sarcoidosis, interstitial lung disease or tumours between 2011-2012. Patients with lung cancer were divided into two subgroups, namely squamous cell carcinoma (PLECA) and non-PLECA because of the potential pathophysiology. We included SCLC (small cell lung carcinoma) and LCC (large-cell carcinoma) to the range of non-PLECA to enlarge the groups. Other cancers referred to patients with tumours in other parts, such as breast cancer. There were also 29 patients in Group II who had acute lung diseases, e.g. tuberculosis and pneumonia for over 2 years. Group III consisted of individuals who reported clinical symptoms but without specific diagnosis after testing, which was referred as normal group.

Table1 presents the basic characteristics of the patients. The average CRP of the whole patients was 0.14 ± 1.35 mg/L. The mean BAL pepsin values of Group I (27925.50 ± 29513.55) and Group II (24233.44 ± 17522.12) were higher than those in Group III (16772.67±10837.13). In chronic lung disease group (Group I) the patients who were diagnosed with interstitial lung disease (ILD) showed the lowest BAL pepsin value throughout all diseases in the study, whereas the patients who had pneumonia in the acute lung disease group (Group II) demonstrated the highest pepsin value of 25642.53 ± 19708.12.

Table 1: Characteristics of patients.

Characteristics	Number of patients (%)	Mean ± SD
All patients	70	
Years		55.94 ± 16.86
Male%	40 (57.1%)	
Female%	30 (42.9%)	
BMI	57	25.09 ± 5.89
CRP	70	0.14± 1.35
VC%	50	74.48 ± 21.04
BAL pepsin level ($pg.ml^{-1}$)		
all patients	70	24589.51 ± 22995.80
acute patients	29 (41.4%)	23662.15 ± 17243.75
chronic patients	29 (41.4%)	28751.41 ± 30242.81
Normal patients	12 (17.1%)	16772.67 ± 10837.13
Chronic Lung disease		
Sarcoidosis	6 (8.6%)	16486.82 ± 12168.50
ILD	5 (7.1%)	8006.43 ± 9113.41
PLECA	6 (8.6%)	70484.26 ± 38554.56
Non-PLECA	4 (5.7%)	18203.32 ± 9621.30
Other cancers	8 (11.4%)	24889.87 ± 19851.61
Acute Lung disease		
TBC	2 (2.9%)	23661.60 ± 9526.32
Pneumonia	20 (28.6%)	25642.53 ± 19708.12
Others	7 (10%)	18004.09 ± 9597.18
Normal Patients	12 (18.6%)	16772.67 ± 10837.13

Data are presented as n (%) or mean ± SD.

BMI: Body-Mass-Index; CRP: C-reactive protein; VC: vital capacity; BAL: Bronchoalveolar lavage; ILD: interstitial lung disease; PLECA: squamous cell carcinoma; Non-PLECA: tumour other than PLECA such as adenocarcinoma, SCLC (small cell lung carcinoma) and LCC (large-cell carcinoma); TBC: tuberculosis.

Participants were separated into three groups concerning the disease characteristics in Table 2. In the population more male patients were enrolled than female patients. BAL CRP, BAL Pepsin and BAL TNFa in chronic group (Group I) are higher than those in acute and normal groups (Group II and III), among which BAL pepsin (20424.903$pg.ml^{-1}$) is especially noticeable. The lymphocytes and neutrophils are relatively higher in the patients who had

acute respiratory diseases. The lung function values among different groups are comparable.

Table 2: Demographic descriptive analysis of each group.

Group	Chronic	Acute	Normal
Ages	58.23 ± 16.28	51.81 ± 17.32	59.23 ± 16.75
Sex (%)			
Male	18 (62.1%)	15 (51.7%)	7 (58.3%)
Female	11 (37.9%)	14 (48.3%)	5 (41.7%)
Smoking			
non-smoker	11 (68.8%)	6 (66.7%)	0 (0%)
smoker	1 (6.3%)	2 (22.2%)	2 (66.7%)
ex-smoker	4 (25%)	1 (11.1%)	1 (33.3%)
BMI (kg/m^2)	25.42 ± 5.25	24.84 ± 6.53	24.64 ± 5.06
BAL CRP	0.027 (0.002-0.467)	0.003 (0-0.17)	0 (0-0.006)
BAL Pepsin	20424.90 (7629.31-34753.79)	19099.38 (16925.47-28868.20)	13431.68 (6767.24-18400.62)
BAL TNFa	0.60 (0.18-1.72)	0.43 (0.10-2.03)	0.17 (0.04-0.21)
CD4	15.25 (4.71-35.04)	9.9 (3.05-29.58)	19.75 (5.47-28.61)
CD8	7.44 (5.28-13.98)	13.35 (4.31-25.08)	11.01 (10.71-28.57)
Platelets	255.24 ± 119.70	271 ± 119.27	216.33 ± 97.77
Lymphocytes absolute	0.80 (0.64-1.26)	1.76 (1.20-2.29)	0.95 (0.83-1.22)
Neutrophils absolute	4.8 (3.52-8.65)	6.09 (4.14-7.73)	4.56 (2.76-5.11)
FEV_1	2.33 ± 0.91	2.1 ± 0.7	2.43 ± 1.13
VC	3.04 ± 1.19	2.93 ± 0.74	3.31 ± 1.22
TLCO/VA%	1.17 ± 0.42	1.23 ± 0.4	1.17 ± 0.25

Data are presented as n (%) or median (lower and upper quartile).
Group I: chronic lung disease group: sarcoidosis, ILD, PLECA, non-PLECA and other cancers.
Group II: acute lung diseases group: TBC, pneumonia.
Group III: normal group: patients who were reported with clinical symptoms but without specific diagnosis after testing.

3.2 Impact of smoking history

The BAL pepsin value was compared between the patients with and without smoking history. There were 11 patients with active or ex-smoking history compared with 17 absolutely non-smoking patients as "non-smoker" group. Figures 1-3 indicate that smoking history has no impact on BAL pepsin, BAL CRP and BAL TNF among these subgroups with provided smoking behaviour, which made up however only 40% of the total population.

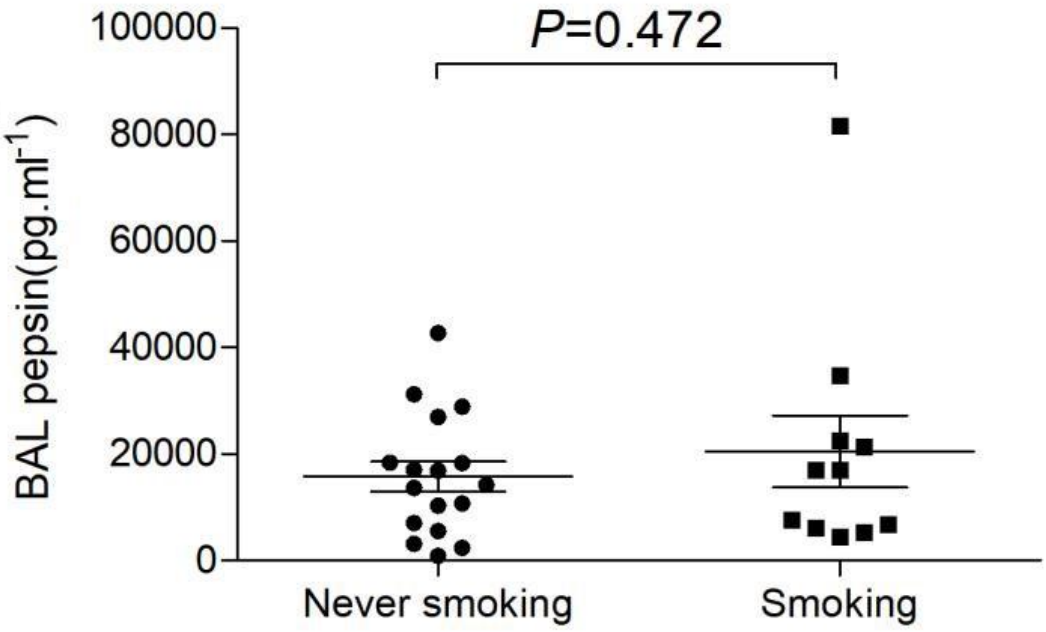

Figure 1: No significant difference of BAL pepsin between patients with and without smoking history.

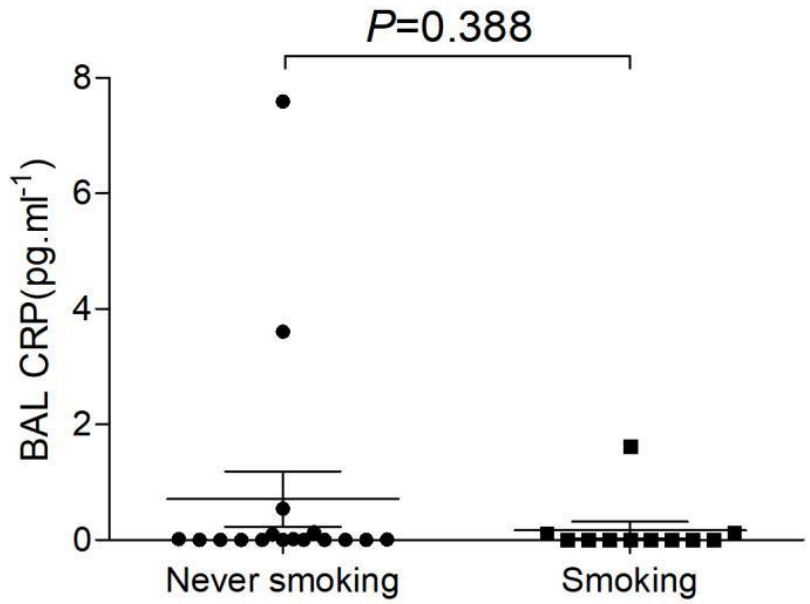

Figure 2: No significant difference of BAL CRP between patients with and without smoking history.

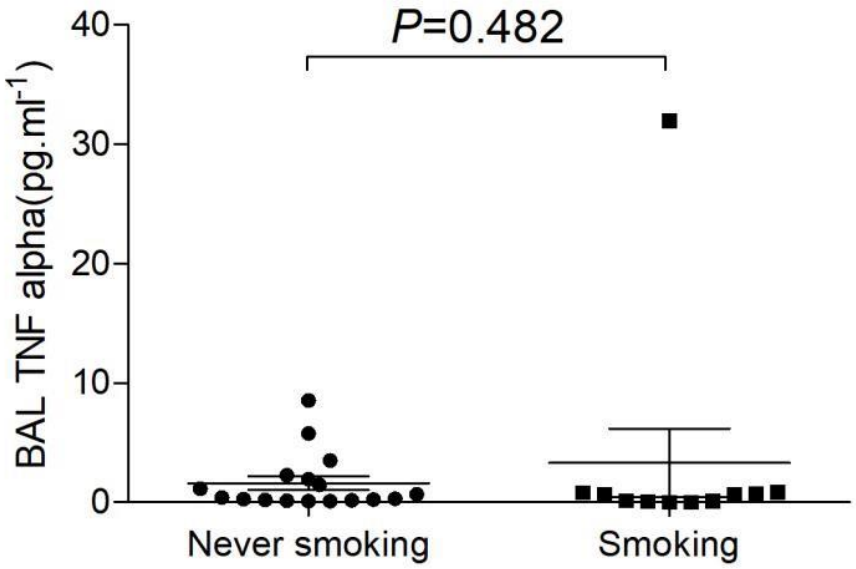

Figure 3: No significant difference of TNF alpha between patients with and without smoking history.

3.3 Impact of PPI treatment

As described previously, PPI therapy e.g. pantoprazole and omeprazole can be prescribed against GERD. However, figure 4-6 imply that PPI treatment introduced no evident impact on BAL pepsin, BAL CRP and BAL TNFa.

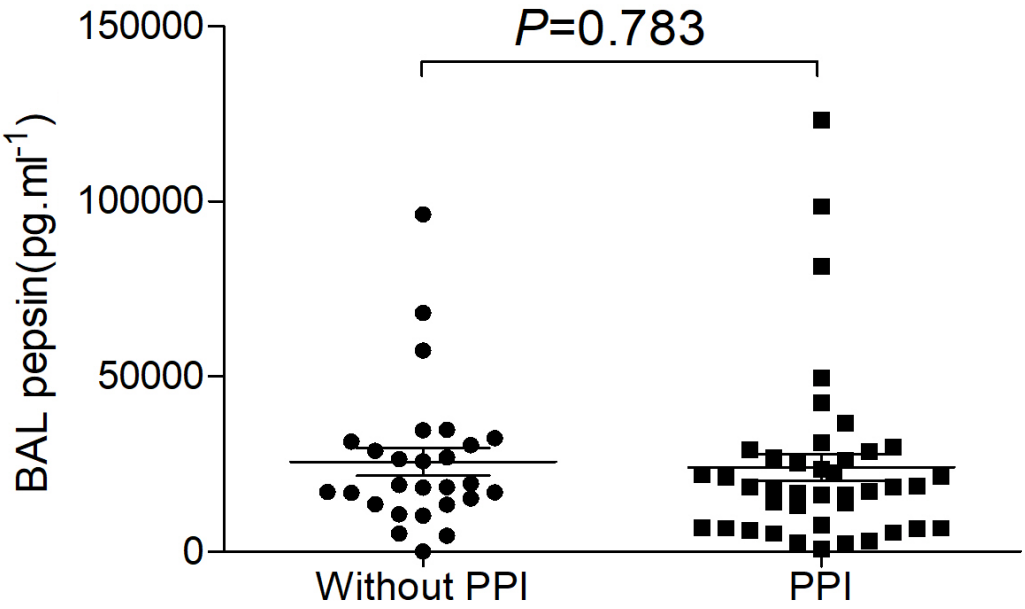

Figure 4: No significant difference of BAL pepsin between the patients with and without PPI treatment.

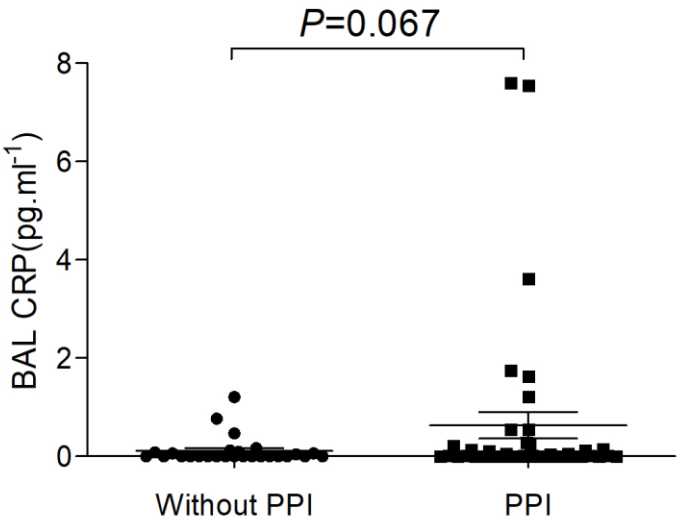

Figure 5: No significant difference of BAL CRP between the patients with and without PPI treatment.

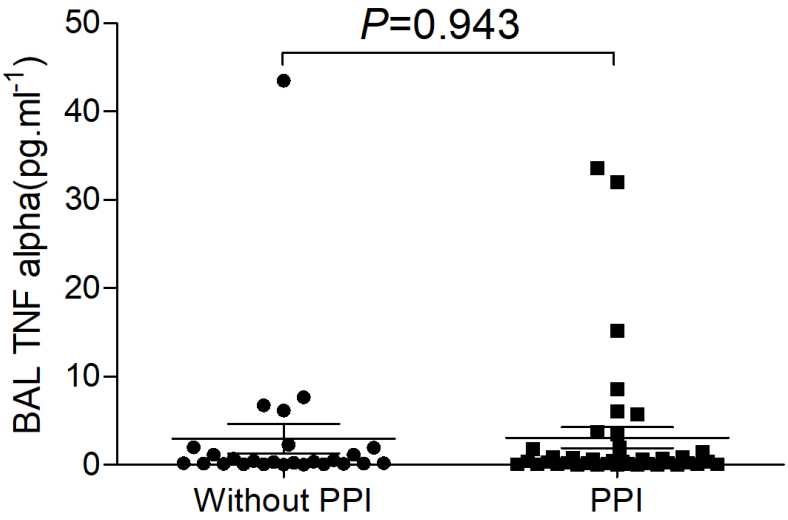

Figure 6: No significant difference of TNF alpha between the patients with and without PPI treatment.

3.4 Correlation of BAL pepsin with lung function

The calculation of Spearman correlation based on different clinical indication in table 3 reveals that BAL pepsin correlates in chronic lung disease group with BAL CRP, TNF alpha and TLC% respectively (correlation coefficients were 0.577, 0.469 and -0.430 with $P<0.05$). In the scope of all patients the correlation of BAL pepsin with all other parameters can be identified. The BAL pepsin correlates positively with other contents of BAL fluid (correlation coefficients were 0.408, 0.346 with $P<0.05$), whereas it correlates negatively with lung function parameters such as FEV_1%, VC% and TLC% (correlation coefficients were -0.386, -0.355 and -0.402 with $P<0.05$).

Table 3: Correlation of BAL pepsin with BAL CRP, BAL TNFa and lung function parameters.

Characteristics	Chronic		Acute		Normal		All Patients	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
BAL CRP	0.577	0.001	0.242	0.224	-0.036	0.911	0.408	0.000
FEV_1%	-0.368	0.077	-0.427	0.088	-0.050	0.898	-0.386	0.006
VC%	-0.287	0.174	-0.167	0.668	-0.164	0.668	-0.355	0.011
TNF alpha	0.469	0.010	0.149	0.458	0.204	0.505	0.346	0.004
TLC%	-0.430	0.041	-0.123	0.627	-0.200	0.580	-0.402	0.003

CRP: C-reactive protein; FEV_1: forced expiratory volume in 1 second;
VC%: vital capacity; TNF alpha: tumour necrosis factor alpha;
TLC %: total long capacity.

3.5 Contents in BAL fluid in different lung disease groups

The contents of BAL fluid, namely BAL CRP, BAL TNFa and BAL pepsin among three groups were compared via Kruskal Wallis H test in Table 4. As a result, there is a significant difference in BAL CRP and BAL TNFa among groups of different lung diseases, whereas the BAL pepsin level does not vary significantly.

Table 4: Comparison of the contents in BAL fluid among three groups - acute, chronic and normal groups.

Group	Acute	Chronic	Normal	H	P
BAL CRP	0.403 ± 1.416	0.594 ± 1.546	0.008 ± 0.018	7.818	0.020
BAL TNFa	4.398 ± 10.268	2.656 ± 6.191	0.190 ± 0.208	8.621	0.013
BAL pepsin	23662.15 ± 17243.75	28751.41 ± 30242.81	16772.67 ± 10837.13	1.955	0.376

A further pairwise comparison via Post-Hoc test confirmed that the BAL CPR and BAL TNFa are different between the normal/acute groups (P=0.038) as well as the normal/chronic groups (P=0.010). There is however no significant difference between the acute/chronic groups.

3.6 Comparison of BAL pepsin with lung cancer

The further comparison in figure 7 between subgroups with different types of lung cancer PLECA (squamous cell carcinoma) and non-PLECA such as adenocarcinoma, SCLC (small cell lung carcinoma) and LCC (large-cell carcinoma) shows that BAL pepsin level of patients with PLECA was significantly higher than those with non-PLECA.

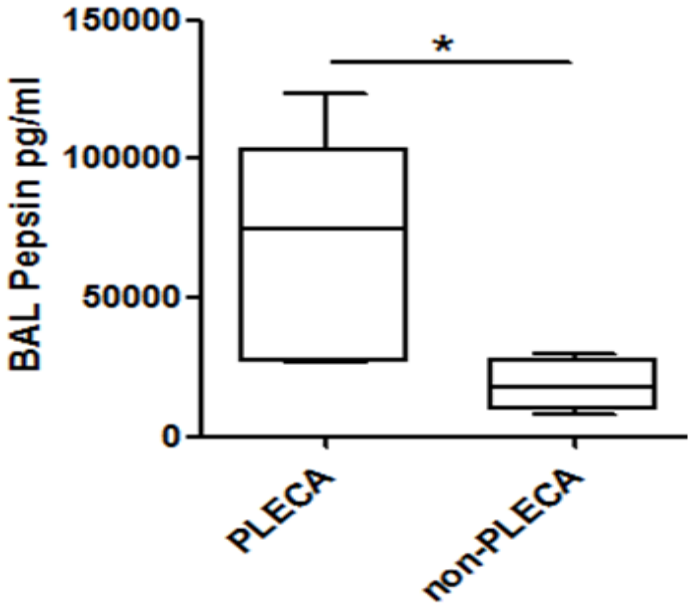

Figure 7: Significant difference of BAL pepsin between patients with PLECA and non-PLECA.

4. Discussion

4.1 Discussion of the methods

This paper studies pepsin, which exists in the airways of patients with various lung diseases including lung cancer. The pepsin concentration can be measured in bronchoalveolar lavage fluid using ELISA. Compared with individuals without lung disease, patients with lung cancer or pneumonia have higher levels of lavage pepsin. The pepsin concentration in BAL fluid correlates with pulmonary function restriction TLC%, VC% and FEV_1% in the scope of all participants, suggesting that lavage pepsin represents processes localized to the lung. Pepsin may therefore be proved to be a valuable biomarker in patients with lung cancer or chronic lung diseases.

The sample size in this study is relatively small. Especially in the chronic lung disease group the cases were dispersed among individual diseases included. In order to confirm or complement the results obtained it is highly recommended to increase the population. Further, the pepsin in BAL fluid was assumed theoretically in association with GERD as a clinical survey on GERD symptoms was not carried out in the enrolled population. Moreover, a contamination of gastric protein in the upper respiratory tract was possible in absence of laryngeal mask in course of bronchoscopy.

4.2 Discussion of the results

The relatively homogeneous distribution of the remaining 70 patients provided a representative basis for the study with 29 cases of acute lung disease and

equally 29 cases of chronic lung disease as well as 12 cases in a normal group for control. Acute lung diseases include tuberculosis and pneumonia, whereas pulmonary nodular lesions, interstitial lung disease, squamous cell carcinoma, and non-squamous cancer refer to chronic lung diseases. Patients in the normal group were clinically symptomatic (cough, cough, shortness of breath) but for whom it finally came out with no specific diagnosis. Various types of lung diseases could be involved in this study; however, the case number of each type was relatively small. Research on specific diseases with sufficient cases can be carried out detailed in future studies.

Animal experimental[140 -141] results showed that acute acid inhalation could cause lung injury. As the pH of the acidic liquid dropped into the trachea decreased, the degree of lung tissue damage in rabbits and dogs gradually aggravated, with pulmonary haemorrhage, pulmonary oedema and neutrophil inflammatory infiltration. After inhaling gastric juice, it could quickly distribute to the lungs of dogs and reached the outer zone of the lungs within 12 to 18 seconds, causing chemical pneumonia, pulmonary oedema, decreased partial pressure of oxygen in arteries, epithelial cell damage and pulmonary interstitial fibrosis. Hydrochloric acid was injected into the right main bronchus of mice. After 12-24 hours, arterial oxygen partial pressure decreased, lung compliance decreased, lung weight increased, and myeloperoxidase activity increased to a peak. Although these abnormal changes improved in 2 weeks, pulmonary compliance was still reduced, and fibrous scars appeared in lung tissues. Perfusion of gastric juice into the right main bronchus of pigs also caused alveolar injury and interstitial fibrosis. Gastric juice was repeatedly injected into the left lung of rats to make them models of chronic inhalation. The pathological features of the lung tissue were peri bronchiolitis and pulmonary interstitial fibrosis, lymphocytic bronchiolitis and obliterative bronchiolitis. The

increase of macrophages and T cells in bronchoalveolar lavage fluid was accompanied by the increase of CD4/CD8 ratio and the increase of IL-1alpha and beta, IL-2, TNF-alpha and TNF-beta levels1[142].

In our study, we have found that bronchoalveolar lavage pepsin correlated positively with TNF alpha and CRP, and negatively with FEV_1%, VC% and TLC% as well. This is similar to the research results at home and abroad, that is, gastroesophageal reflux is closely related to respiratory diseases, and it is one of the main causes of chronic cough. 60% of asthmatic patients had gastroesophageal reflux symptoms, which was significantly higher than the control group (38.1%). The incidence of abnormal oesophageal pH, esophagitis and hiatal hernia in asthmatic patients were 50.9%, 37.3% and 51.2%, respectively. Gastroesophageal reflux is an important factor leading to asthma attack[143]. Gastroesophageal reflux is closely related to recurrent aspiration pneumonia. Recessive inhalation often occurs in patients with neurological diseases (such as stroke and Parkinson's disease) and oesophageal diseases (such as cardiac arrhythmia). In patients with neurological disorders or oesophageal diseases, autopsy revealed that they had nodular granuloma with foreign body reaction[144].

Barnes et al[145] reported 4 elderly patients with diffused inhaled bronchiolitis caused by chronic insidious inhalation that were confirmed pathologically. The cases have reported that chronic insidious inhalation caused by gastroesophageal reflux disease can be secondary to bronchiolitis obliterans with organizing pneumonia. Taking glucocorticoids increases the risk of gastroesophageal reflux. Patients with obstructive bronchiolitis and organic pneumonia may also have gastroesophageal reflux. Oesophageal dyskinesia

in patients with systemic sclerosis was associated with decreased lung function. HRCT showed pulmonary fibrosis in 40% of patients with systemic sclerosis. Compared with those without pulmonary fibrosis, the lower oesophageal sphincter pressure was significantly reduced, because of the long acid exposure time, the incidence of acid or non-acid reflux were significantly increased in these patients after 24 hours oesophageal pH monitoring and pressure measurement. Obliterative bronchiolitis syndrome (BOS) is a chronic rejection reaction that occurs in small airways. After a perioperative period, approximately 2/3 of patients who have lung transplant will eventually develop BOS whose main clinical manifestations are progressive dyspnoea, coughing, and recurrent infections with characteristic progressive airflow limitation. 48% to 76% of patients with lung transplantation have gastroesophageal reflux which may be an important factor in the development of BOS. Risk factors for gastroesophageal reflux-related lung disease include gastroparesis, vagal dysfunction, postoperative dysphagia, and inhibition of transplanted lung cough reflex.

It is worth noting that proton pump inhibitors and H2 receptor antagonists can inhibit gastric acid reflux, relieve symptoms of gastroesophageal reflux, but cannot inhibit and treat gastroesophageal reflux disease. Even if acid reflux is inhibited, recurrent alkali reflux and chronic micro inhalation may still be factors that trigger dynamic processes of lung parenchymal damage and abnormal repair.

4.3 Outlook

In summary, in this trial bronchoalveolar lavage pepsin is closely related to respiratory diseases. There is also a close relationship between bronchoalveolar lavage fluid pepsin and the total lung capacity. Therefore, in clinical practice, routinely detecting the content of pepsin in bronchoalveolar lavage fluid may help diagnose lung diseases, which may have a certain impact on the treatment and prognosis of lung diseases.

5. Conclusion

Bronchoalveolar lavage pepsin concentrations showed a correlation to inflammation markers BAL CRP, TNF alpha and lung function with TLC%, VC% and FEV_1%. In the chronic patients group the BAL pepsin was significant correlated with BAL CRP, TNF alpha and TLC%. There is a higher level of pepsin in lavage especially in the presence of PLECA than other lung cancers. The concentration of pepsin in lavage correlates also with TNF alpha in normal patients who reported clinical symptoms but without specific diagnosis after testing. In the study neither smoking behaviour nor PPI treatment can be proven to have a significant influence on the pepsin concentration in lavage. The study demonstrates that the pepsin measured in the lavage is associated to physiological and pathological processes localized to the lung. Therefore, pepsin may be used to distinguish lung diseases from other diseases, and it can be studied as a potential biomarker for lung cancer in the future.

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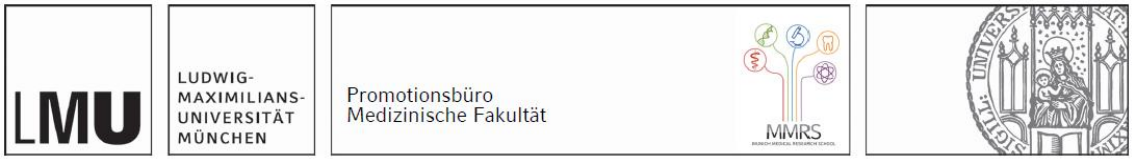
Affidavit



Affidavit

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I hereby declare, that the submitted thesis entitled:

Pepsin From Bronchoalveolar Lavage (BAL) Fluid in Lung Diseases and Tumours

is my own work. I have only used the sources indicated and have not made unauthorized use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.

I further declare that the submitted thesis or parts thereof have not been presented as part of an examination degree to any other university.

Munich, 09.05.2022 — Yue Jiang

Place, date — Signature doctoral candidate

List of publications

J Götschke, Y Jiang, K Berghof, K Kahnert, R Kiefl, M Schaule, J Behr, RM Huber, A Tufman:
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