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IL-22 in lavage as a biomarker in patients with lung cancer

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1. Introduction

1.1. Lung Cancer

Lung cancer is not only a common disease, but remains, despite advances in our understanding of its' biology and novel therapeutic options, an exceedingly deadly disease. A predicted 186 970 men and 82 640 women will die of lung cancer in the European Union in 2013 [1]. Although the majority of lung cancer patients are men, the incidence of lung cancer in women is climbing. Since 2009 there has been a 7% increase in the incidence of lung cancer in European women, likely in part due to changes in smoking behaviour. In Great Britain more women now die of lung cancer than breast cancer (standardised death rate 21.2 per 100 000). Lung cancer is also an important cause of mortality worldwide[2]. In 2010 respiratory cancers caused 1.5 million deaths, and accounted for 19% of all deaths from cancer[3]. Although smoking is the predominant cause of most lung cancers, other environmental factors such as radon and asbestos are relevant in some patients[4], and genetic and epigenetic factors which may contribute to the disease are under investigation[5].

Primary lung cancers are categorized histologically as small cell lung cancer or non small cell lung cancer. In Germany small cell lung cancer accounts for approximately 20 % of lung cancer cases[6]. Non small cell lung cancer (NSCLC) describes a clinically and biologically heterogeneous group of histologies including squamous cell lung cancer, adenocarcinoma and large cell carcinoma. The adenocarcinomas can be further classified histologically, and these subtypes are of increasing clinical relevance due to their association with tumour biology and clinical prognosis[7]. Mutations in subsets of NSCLC, especially in adenocarcinomas, can now be targeted by specific drugs. These so-called driver mutations are also of increasing interest in squamous cell tumours. Currently the most clinically relevant driver mutations are activating mutations of the epidermal growth factor receptor (EGFR)

gene and fusion of the echinoderm microtubule-associated protein-like 4 (EML4) gene with the gene for anaplastic lymphoma kinase (ALK)[8].

1.2. The challenge of treating lung cancer

Most patients with lung cancer are diagnosed at an advanced stage of disease [9]. This is because lung cancer often remains asymptomatic until late in the course of disease; and when it does cause symptoms these are often non-specific and overlap significantly with the symptoms of chronic bronchitis experienced by many smokers [10]. Likely due at least in part to the non-specific nature of lung cancer symptoms, there is often a clinically relevant delay between the onset of symptoms, the suspicion of lung cancer and, finally, the initiation of appropriate treatment[11].

The treatment of lung cancer, both in early stages and advanced disease, is often associated with significant morbidity and even mortality. Therefore, clinicians must weigh the chances of benefit against the risk of treatment complications when counselling patients. Treatment decisions are often further complicated by advanced patient age, multiple comorbidities and limitations in lung function[12]. The following section presents the main treatments currently available to patients with non small cell lung cancer. The same principles of therapy can also be applied to patients with small cell lung cancer; however, small cell lung cancer metastasises much earlier in the course of disease, and is initially more responsive to systemic chemotherapy[13].

1.2.1 Treatment modalities in early stage and locally advanced lung cancer:

Surgical resection is a curative option for some patients with early stage lung cancer[14]. Whenever technically possible, lobectomy is preferred over pneumonectomy, as it is associated with a significantly lower loss of lung function and exercise capacity[15], as well as lower postoperative mortality. A Swiss study described mortality rates of 7.9 % following pneumonectomy compared with only

1.2% after lobectomy[16]. A large retrospective analysis of surgical resections in Rotterdam also showed higher mortality after pneumonectomy, at 5.7%, compared with other types of resection[17].

Unfortunately, even early stage, localised NSCLC often recurs after complete surgical resection. In addition to local recurrences, which occur in 10-15% of patients treated with surgery, many patients develop systemic metastases. The rates of systemic recurrence vary significantly with preoperative stage, with 15-30% of patients with resected stage I lung cancer, 40% of those with resected stage II, and 60% with resected stage III disease going on to develop systemic tumour spread[18]. These recurrence rates result in a limited prognosis even for those patients diagnosed with early stage, localised disease.

Additional systemic therapy in the form of adjuvant chemotherapy can slightly reduce the chance of recurrence after surgical resection. The Lung Adjuvant Cisplatin Evaluation (LACE) study[19] investigated the effect of adjuvant cisplatin-based chemotherapy in pooled data from 4 584 patients with resected stage I to stage III NSCLC, and found that those treated with adjuvant chemotherapy experienced an absolute increase in 5-year survival of 5.4% compared with patients who did not receive adjuvant chemotherapy. In the LACE study cancer related mortality fell more dramatically than overall survival, with a 6.9% decrease in cancer related mortality at 5 years. However, chemotherapy itself also causes significant toxicity, which may have been responsible for the 1.4% increase in non-cancer related mortality observed in those patients treated with adjuvant chemotherapy in the LACE study. It remains difficult to predict which patients will benefit from adjuvant chemotherapy. Unfortunately, many of those treated with adjuvant chemotherapy do not benefit from treatment, either because their tumour would not have recurred even without additional therapy, or because their tumour recurs despite therapy. Both of these groups continue to suffer the toxicity of chemotherapy unnecessarily.

The current guidelines recommend that adjuvant chemotherapy be offered to all fit patients with fully resected stage II-IIIa2 NSCLC. Because the benefit of adjuvant chemotherapy is less clear in patients with stage I disease, the guideline

recommends that patients with stage Ib be offered adjuvant chemotherapy only after individual consideration of patient fitness and comorbidities [6].

In contrast to patient with stage I and II lung cancer, patients with locally advanced stage III lung tumours are often not candidates for surgical resection. In general, surgery should generally only be offered to these patients as part of a multimodal treatment plan[6]. While some patients with stage IIIa disease benefit from surgery, patients with bulky or multi-level N2 lymph node metastases have been shown to have poor post-operative survival[20], and should therefore not usually be offered surgery. Fit patients with inoperable stage IIIa and those with stage IIIb disease can be treated with combined radiochemotherapy. Currently, cisplatin and etoposide or cisplatin and vinorelbine are often combined with radiation; however, the optimal amount, timing and type of chemotherapy have not yet been determined. Although simultaneous radiochemotherapy is more effective than sequential radiation and chemotherapy[21]; it is also associated with significant toxicity and may not be suitable for patients with large tumours, limited lung function or poor performance status [22]. Simultaneous radiochemotherapy leads to long term survival in over 20% of patients, and is therefore a potentially curative therapy for locally advanced lung cancer[23]. As is the case with adjuvant chemotherapy following curative resection, it is not yet possible to predict which patients will benefit from which type of radiochemotherapy.

1.2.2. Treatment of advanced lung cancer

Patients with stage IV lung cancer are generally treated palliatively with platinum doublet chemotherapy[24]. Chemotherapy targets cell proliferation relatively non-specifically; and many of the side-effects of chemotherapy are a result of these drugs' effects on normal cell division. In advanced NSCLC chemotherapy results in a clear survival benefit when compared to best supportive care[25]. However, not all patients who are treated with chemotherapy respond, and those who do respond almost invariably go on to progress within several months of treatment. The most frequently used platinum-based chemotherapy doublets have been shown to be equally effective in unselected patients[26].

Currently, the efficacy of a specific chemotherapeutic regimen in an individual patient and tumour can only be judged during or after treatment. Several recent studies have investigated the use of early imaging, in some cases only days after the first dose of therapy, to predict response to a course of systemic treatment[27-29]. In addition, many studies point to an association between specific biomarkers and response to particular chemotherapeutic agents. The use of prognostic and predictive biomarkers is described in section 1.2.5 below. Although studies are ongoing, predictive biomarkers for the efficacy of chemotherapy are not yet established in routine clinical use.

Not all patients can be treated with platinum doublets, either due to comorbidities such as renal insufficiency or congestive heart failure, or due to poor performance status. These patients are generally treated with single agent chemotherapy or with palliative care alone. In some cases a non-platinum based chemotherapy doublet may be administered. Recurrences following first-line chemotherapy may be treated with second-line therapy, generally single-agent chemotherapy[24].

1.2.3. Targeted therapies

Over the past several years new treatments for lung cancer have become available. Many of these differ from traditional chemotherapeutics in that they target a specific molecule or molecular change, which is often part of a cell proliferation signalling pathway. The EGFR receptor was the first molecule to be inhibited by a specific 'targeted' therapy in the treatment of lung cancer. Oncogenic mutations in EGFR which activate the receptor's tyrosine kinase domain are found in approximately 15% of Caucasian patients with NSCLC[30]. Inhibition of the intracellular tyrosine kinase domain of the EGFR receptor by the tyrosine kinase inhibitors (TKIs) gefitinib

(marketed as Iressa) and erlotinib (marketed as Tarceva) results in a reduction in EGFR signalling and, therefore, a reduction in tumour growth[31]. Early trials of EGFR-TKIs included unselected patients with NSCLC, that is, patients both with and without EGFR mutations[32]. With the exception of erlotinib in second line, these trials failed to show an overall benefit with EGFR-TKIs, despite impressive responses in some patients[31]. It was not until subgroup analyses and subsequent prospective trials in molecularly defined cohorts showed a benefit in patients whose tumours harbour EGFR mutations, particularly mutations in exons 19 and 21, that EGFR-TKIs found a clear clinical niche[33 34].

Another clinically relevant molecular change in NSCLC is the fusion of the EML4 gene with the ALK gene, which occurs in approximately 3-5% of Caucasians with NSCLC, particularly in patients with adenocarcinoma[35]. As with EGFR mutations, the incidence of EML4-ALK fusion is higher in East-Asian populations[36]. Patients with EML4-ALK fusion benefit from treatment with ALK-TKIs. Crizotinib (marketed as Xalkori) is currently the only ALK-TKIs licensed in North America and Europe. Because of the design of the trials leading to licensing, this drug is presently only licensed for the treatment of patients previously treated with chemotherapy[37]. As is the case with EGFR-TKIs, treatment with ALK-TKIs often results in a relatively good partial response or disease stabilisation; however, this is almost invariably followed by TKI-resistance and disease progression[38]. While some genetic changes responsible for this secondary resistance have been described, such as the T790M mutation in EGFR-mutated NSCLC[39], the search for additional mechanisms of resistance and treatments to overcome them is ongoing.

1.2.4 Immunologic Therapies for Lung Cancer:

The immune system plays a critical role in recognising and eliminating tumour cells. Micrometastases, for instance in the bone marrow[40], exist in many patients with otherwise early stage disease, and the observation that many of these patients do not go on to develop true clinically relevant metastatic disease despite having received only local therapy suggests that the immune system is capable of clearing some tumour cells without additional systemic therapy. Several attempts to harness the immune system's anti-tumour capabilities have been investigated in clinical trials. There are nonspecific approaches to stimulate the immune defence against lung cancer as well as more specific approaches [41,42]. A number of therapeutic cancer vaccines are under investigation for the treatment of NSCLC. These include an allogeneic vaccine derived from irradiated NSCLC cell lines (belagenpumatucel-L), protein-specific vaccines (for instance, human recombinant EGF, purified MAGE-A3 recombinant protein, telomerase) and antigen-specific cancer immunotherapies targeting mucin-1. Each immunotherapy differs in its mechanism of antigenic stimulus, and many incorporate the use of an immunologic adjuvant to potentiate the immune response[43 44].

1.2.5. Prognostic and predictive biomarkers

The Oxford dictionary defines a biomarker as "a naturally occurring molecule, gene, or characteristic by which a particular pathological or physiological process, disease, etc. can be identified" [45].

In medical practice, biomarkers can be either prognostic, predictive or both. Prognostic biomarkers correlate with the clinical course of disease, often quantified as overall survival, independent of which type of therapy is given. In contrast, predictive biomarkers correlate with the response to a specific treatment, and can be used to select a treatment likely to be effective against a particular tumour [46].

1.2.5.1. Prognostic factors

Prognostic markers are clinically useful in the care of patients with lung cancer, as they allow clinicians to estimate the likelihood of a particular outcome or course of disease. Many clinical attributes and biomarkers have been examined for their prognostic value in lung cancer. The stage of disease, the performance status of the patient and the histology (in particular the distinction between small cell and non small cell lung cancer) are widely accepted prognostic markers[47]. The prognostic relevance of the subgroups of NSCLC is under investigation. Especially in early lung cancer the histological subtypes of adenocarcinoma seem to be prognostically relevant [7]. With the advent of the EGFR-TKIs in advanced NSCLC it was observed that the presence of an activating EGFR mutation is itself of prognostic relevance[48]. In the setting of early stage lung cancer the question of whether the mutation itself, or rather the clinical characteristics associated with the mutation, such as the absence of smoking behaviour, are prognostic remains the subject of debate[49 50]. Although numerous other potentially prognostic biomarkers have been described, the most important prognostic factors in clinical practice are still clinical patient characteristics such as performance status, generally described using the ECOG or Karnofsky scales, and stage of disease.

Prognostic biomarkers are of scientific value because they suggest a strong link, and may imply a causal relationship between a characteristic of tumour growth, for instance invasiveness or the potential to metastasize, and a specific molecule. For many molecules investigated primarily in in vitro models of disease this represents an important first step from the bench to the bedside.

Clinically, prognostic markers are not able to directly predict the response to specific therapies; however, they may be of use in deciding how, or how often, to monitor patients for signs of disease progression, or in weighing the risks and benefits of a treatment. Biomarkers which correlate with the individual prognosis – especially after potentially curative treatment for local disease – may also help determine the need for additional systemic treatment. Similarly, for patients with advanced disease, prognostic markers may be of value in adapting the amount and intensity of systemic therapy, determining the type and frequency of monitoring during and after treatment, as well as in discussing palliative treatment decisions with patients and families.

1.2.5.2 Predictive factors

Several predictive markers are relevant for the choice of systemic therapy in advanced NSCLC. Histology itself, in particular the distinction between squamous cell and non-squamous tumours, is predictive of response to pemetrexed. While patients with non squamous NSCLC benefit from pemetrexed, those with squamous cell cancer do not [51]. Histology is also predictive of the risk of serious side effects during treatment with bevacizumab. EGFR mutations are also valuable predictors of response to some systemic therapies. An activating EGFR mutation predicts response and progression-free survival in patients treated with the EGFR-TKIs erlotinib and gefitinib [52,53]; and EML4-ALK fusion[54] is predictive of response to the ALK-TKI crizotinib. Potentially predictive molecular biomarkers for the efficacy of specific chemotherapeutic agents have also been described, for example thymidilate synthase (TS) for response to pemetrexed and ERCC1 (excision repair cross-complementing rodent repair deficiency, complementation group 1) for response to cisplatin, but these markers have not yet been universally accepted or validated[55,56]. Expression of ERCC1, which plays a role in DNA-repair, was found to be predictive of benefit from adjuvant chemotherapy in the Bio-IALT trial [56]. However, this finding was not reproducible[57], and for this reason ERCC1 has not yet found a place in routine clinical decision making. Predictive markers for the efficacy of various systemic agents in NSCLC would likely help to reduce the rate of primary progression under therapy and reduce the risk of unnecessary treatment side effects.

1.3. Early diagnosis and screening of lung cancer

While early lung cancer is a potentially curable disease, it is often clinically silent and therefore goes unrecognised. Even after patients recognise the presence of new or changing symptoms, there is often a substantial delay in seeking medical advice, and then an additional delay in making the correct diagnosis and in referral to a lung cancer specialist [10].

Lung cancer screening

The early detection of lung cancer, that is, the detection of tumours at a potentially curable stage, is a clinically important goal. The recently published results of large trials of computed tomography (CT)-screening for lung cancer have shown that CT-screening can find lung cancer at an earlier and thus surgically curable stage. In the National Lung Cancer Screening Trial, the risk of lung cancer death was significantly lower in those screened on a yearly basis using CT[58]. However, in addition to lung cancer, CT-screening also finds a multitude of non-cancerous lung nodules. The expense and risk associated with following and correctly diagnosing these benign nodules is enormous[59]. Screening all smokers for lung cancer using CT alone is not feasible in Germany at this time. The widespread implementation of lung cancer screening will likely depend on the development of multimodal models of lung cancer risk (including lung function, biomarkers, imaging results) to select individuals for screening, coupled with a risk-adapted approach to patient follow-up[60]. A biomarker found in easily accessible biomaterials such as sputum, exhaled air or peripheral blood could help risk-stratify the population and focus screening on high risk individuals.

1.4. Biomaterial sampling

A detailed pathological and molecular workup of a tumour specimen, including analysis of possible prognostic and predictive markers, requires a sufficient number of tumour cells. While resected specimens are almost always large enough for these analyses, bronchoscopic biopsies and fine needle aspirations are often much smaller, in some cases capturing only a few tumour cells, and therefore present a challenge to the pathologist and molecular biologist[61]. Techniques to optimise the yield of small biopsies and aspirates, such as image guidance with endobronchial ultrasound or CT, the use of several passes with the aspiration needle, and the cell block technique for processing cytological material, are of clinical importance.

1.4.1. Non-invasive and minimally invasive biomaterial sampling

Their potential to limit the morbidity and mortality associated with invasive procedures makes non-invasive and minimally invasive techniques appealing in a wide range of clinical situations. Minimally invasive biomaterial sampling is especially well suited to improve patient care in the setting of lung cancer screening. Because 97% of CT-detected nodules are non-malignant[58] and many of the patients screened are heavy smokers with pulmonary comorbidities, the risks and benefits of invasive diagnostic procedures have to be weighed carefully when advising this group of patients. It is neither feasible nor clinically sensible to biopsy every lesion detected by CT. The measurement of biomarkers in minimally invasive samples such as blood, lavage and sputum may help assess the risk of cancer in patients with lesions detected by screening, or may even help pre-select patients at particularly high risk of cancer before screening.

In addition, minimally invasive biomaterial sampling is well-suited to improve the care of patients with recurrent tumours or progression after first-line systemic therapy. It is difficult to get enough tissue to reach an exact diagnosis and select an individualised treatment at the time of first diagnosis, and getting enough tissue to perform repeat

molecular analyses as treatment resistance develops and the tumour progresses poses an additional challenge. The advent of new molecular targets has taught us to look for and treat driver mutations, and when the tumour progresses, to look for new changes in the tumour's biology which may suggest a specific second line treatment[62]. A tumour sample is now not only required at the time of diagnosis, but also during the course of disease or at the time of progression[63]. This shift in our approach to lung cancer therapy demands that minimally invasive sampling and alternative biomaterials such as serum, bronchoalveolar lavage, and pleural fluid be developed to their fullest potential.

1.4.2. Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) describes a diagnostic medical procedure in which a flexible bronchoscope is used to collect a sample of the epithelial lining fluid (ELF) from the small airways and alveoli. The procedure is safe and well established in both adult and pediatric patient populations [64], and can be performed using local anesthesia and light sedation. During the procedure the bronchoscope is introduced into the segment of interest and advanced until a wedge position is reached. Aliquots of normal saline are then introduced through the working channel and aspirated under negative pressure into a sterile collection vessel. In generalized diseases of the lung the right middle lobe is generally chosen as the site of bronchoalveolar lavage due to the higher volume and higher total cell count of fluid recovered from this lobe [65]. The analysis of BAL fluid depends on the clinical indication and situation and may include a determination of total and differential cell counts, cell sub-

populations, identification of cell phenotypes associated with specific exposures and disease states and the identification of airway pathogens.

Various patterns of cell differentiation detected in bronchoalveolar lavage samples have been shown to correlate with disease states in the lung. This is summarized in an American Thoracic Society Clinical Practice Guideline published in 2012 [66]. Normal BAL fluid contains over 85 % macrophages, between 10% and 15% lymphocytes, fewer than 3% neutrophils, fewer than 1% eosinophils and fewer than 5% squamous epithelial or ciliated columnar epithelial cells. Elevated BAL eosinophils (>1% eosinophils) correlate with the presence of eosinophilic pneumonia, drug-induced pneumonitis, asthma, Churg-Strauss syndrome, allergic bronchopulmonary aspergillosis and Hodgkin's disease, as well as with bacterial, fungal, parasite and pneumocystis infections. In contrast, elevated BAL neutrophils (>3%) suggest a diagnosis of collagen vascular disease, idiopathic pulmonary fibrosis, aspiration pneumonia, bacterial or fungal infection, bronchitis, asbestosis, acute respiratory distress syndrome or diffuse alveolar damage; and elevated BAL lymphocytes (>15%) suggest a diagnosis of sarcoidosis, non-specific interstitial pneumonia, hypersensitivity pneumonitis, collagen vascular diseases, radiation pneumonitis, cryptogenic organizing pneumonia or lymphoproliferative disorder. The lymphocyte subtypes may also be of diagnostic relevance. For instance, an elevated CD4/CD8 ratio suggests a diagnosis of pulmonary sarcoidosis[66].

In addition to changes in the pattern of nucleated immune cells in BAL fluid, the direct microscopic identification of infectious organisms or malignant cells in BAL samples may lead to a specific diagnosis; the presence of hemorrhagic fluid may suggest

pulmonary hemorrhage; and the presence of cloudy or milky fluid may suggest pulmonary alveolar proteinosis [66].

In clinical and translational research BAL may also be used to measure the concentration of substances thought to have a causal link with pulmonary disease states. For instance, gastrooesophageal reflux disease has been clinically linked with idiopathic pulmonary fibrosis. This clinical association was corroborated by the measurement of pepsin in bronchoalveolar lavage fluid from patients with pulmonary fibrosis as recently described by Lee and colleagues[67]. These investigators found significantly elevated pepsin levels in bronchoalveolar lavage from patients with acute exacerbations of idiopathic pulmonary fibrosis compared with lavage pepsin measurements in samples from patients with stable disease.

1.5. IL-22 – discovery, molecular and biological characteristics

IL-22 was identified in 2000 by Belgian investigators searching for genes induced by IL-9 in murine T-cells [68]. The group identified a gene encoding a novel protein which seemed to be structurally related to the class II cytokine IL-10. This protein was initially named IL-10-related T-Cell Derived Inducible Factor (IL-TIF). Shortly following its' discovery, IL-TIF was shown to be present in T-cells, mast cells, thymus and brain,[68] and to activate signal transducer and activator of transcription -3 (STAT-3). The human homolog of IL-TIF was found to activate STAT-3 and STAT-1 in hepatoma cell lines as well as in several melanoma cell lines [69], and to upregulate

expression of acute phase reactants in the liver. Mice injected with E. coli lipopolysaccharide showed an acute increase in IL-TIF expression in numerous organs including gut, lung, thymus, liver and kidney, which suggested that IL-TIF plays a role in the response to inflammation[69].

The same year, an American group identified a novel cytokine produced by activated T-cells[70]. This cytokine, which they named IL-22, was found to be identical to the IL-TIF cytokine described by the Belgian group. These investigators also identified the IL-22 receptor IL-22R, which they found forms a receptor complex with cytokine receptor family proteins (CRF), in particular CRF2-4[70]. An IL-22 receptor complex consisting of a CRF2-9 chain and an IL-10R2 chain was described in 2001[71].

Since its' discovery, IL-22 has been identified in a variety of tissues and cell types [72] and has been shown to play a role in a wide range of human diseases. IL-22 appears to act predominantly on IL22-receptor-1 (IL-22-R1) positive epithelial and endothelial cells [73]. While, in some settings, IL-22 appears to protect against damage due to inflammation, in other settings it seems to contribute to the development and persistence of disease[74].

In addition to its' role in the lung, IL-22 has been found in a number of other tissues and has been shown to play a role in a number of organ systems. For instance, IL-22 has been shown to promote wound healing in both the skin and the gastrointestinal tract. Boniface and colleagues[75] showed that epidermal keratinocytes express a functional IL-22 receptor made up of IL-22RA1 and IL-10RB chains. Keratinocytes exposed to IL-22 were found to express proinflammatory proteins, and stimulation

with IL-22 resulted in keratinocyte migration in an in vitro model. Further evidence for the role of IL-22 in tissue repair and healing was published by a group investigating the role of STAT3 in epithelial wound healing in the colon[76]. The investigators examined colitis and intestinal wound repair in wild type mice, IL-22 knock-out mice, and mice in which the intestinal epithelial cells had been modified to knock out STAT3. They found that IL-22 knock- out mice developed more severe colitis than their wild type counterparts, and that mucosal healing was also delayed in the IL-22 deficient animals. In contrast to wild type animals, IL-22 knock-out mice showed very little STAT3 activation in response to colitis; however, STAT3 could be induced by exposing ex-vivo colon to IL-22. The investigators concluded that IL-22 protects intestinal epithelium in the setting of colitis and promotes wound healing through the activation of STAT3[76].

Protective effects of IL-22 have been also been demonstrated in the setting of liver injury and inflammation. Radaeva and colleagues showed that IL-22 protects hepatocytes in a mouse model of induced liver injury by inducing the expression of antiapoptotic substances [77]. Zenewicz and colleagues also investigated the role of IL-22 in liver injury [78]. Using an IL-22 deficient mouse model, the investigators showed that IL-22 is protective in acute liver inflammation. In contrast to the wild-type animals, many of the homozygous and heterozygous IL-22 deficient mice died shortly after induction of liver injury. Th17 cells expressing IL-22 were able to reduce hepatocyte damage in the IL-22 deficient mice[78]. An additional study recently showed the administration of IL-22 to be hepatoprotective in a mouse model of ischemic reperfusion injury[79].

In contrast to its' protective effects during acute inflammation and injury, IL-22 has also been shown to promote the development of various disease states, in some cases through the promotion of pathological, chronic states of inflammation. Transgenic mice engineered to aberrantly express the IL-22 receptor chain IL-22R1 on lymphocytes showed increased serum levels of IL-22, possibly due to positive autoregulation, and died prematurely as a result of multiorgan inflammation [80]. The investigators were able to show that this increase in IL-22 expression led to systemic neutrophilia as well as high levels of inflammatory cytokines in both serum and bronchoalveolar lavage samples.

In the skin, IL-22 has been implicated in the development of psoriasis[81 82].

IL-22 has been also shown to have a proinflammatory role in a mouse model of rheumatoid arthritis[83]. Levels of IL-22 were found to be higher in mice immunized to induce arthritis; and IL-22 knock-out mice developed less severe arthritis than their wild-type counterparts.

1.5.1. IL-22 in the lung

In the lung, IL-22 has been shown to be expressed by a variety of immune cells including T-cell subsets[84], monocytes and alveolar macrophages, as well as in primary lung alveolar epithelial cells [85] and cultured human bronchial epithelial cells [86]. Whittington and colleagues measured IL-22 in a variety of pulmonary samples including bronchoalveolar lavage samples from patients with acute respiratory distress syndrome (ARDS), idiopathic pulmonary fibrosis (IPF), pulmonary sarcoidosis and post-operative ventilated patients with no known lung pathology, as

well as in paraffin-embedded macroscopically normal lung tissue from patients undergoing lung cancer resection. These investigators were able to show that alveolar macrophages and alveolar epithelial cells in normal lung tissue express IL-22, and that levels of IL-22 in lavage are lower in patients with pulmonary sarcoidosis than in both normal controls and patients with idiopathic pulmonary fibrosis. They also found that IL-22 binding protein is expressed in alveolar macrophages, alveolar epithelial cells, monocytes and neutrophils; and that only alveolar epithelial cells express the IL-22 receptor chain IL-22R1[85].

IL-22 in non-malignant lung diseases

IL-22 seems to have a dual role in lung diseases, acting to protect the lung in the setting of some types of acute lung inflammation or injury while promoting inflammation and pulmonary disease in other settings.

In a model of ventilator-induced lung injury, inhaled IL-22 was shown to protect rats exposed to barotrauma against lung injury[87].

In the setting of allergic asthma there is evidence that IL-22 reduces airway inflammation and bronchoconstriction. Schnyder and colleagues studied the role of IL-22 in a mouse model of the allergic response. They found that IL-22 is produced in the lungs of mice exposed to ovalbumin, where it inhibits dendritic cell function and eosinophil recruitment thus negatively regulating the allergic response [88].

Further evidence for the anti-allergic properties of IL-22 was published by Taube and colleagues, who studied airway hyperresponsiveness in IL-22 deficient mice. The authors found increased inflammation and increased airway hyperresponsiveness in

animals deficient in IL-22, and decreased inflammation in those treated with intranasal IL-22 before antigen challenge. They conclude that IL-22 protects against inflammation and allergy-related airway constriction[89].

In contrast, work from Besnard and colleagues suggested that IL-22 may have a dual role in allergic asthma, and may not always protect the lung [90]. These investigators studied both patient samples and a mouse model of induced allergic asthma and found elevated levels of serum IL-22 in both asthmatic patients and mice. Mice deficient in IL-22 showed fewer measurable signs of asthma, including less airway hyperreactivity and mucus production and lower levels of Th2 cytokines. However, mice in which IL-22 was downregulated at the time of antigen challenge seemed to show increased allergic inflammation. The authors conclude that "IL-22 is required for the onset of allergic asthma, but functions as a negative regulator of established allergic inflammation" [90]. A recently published review summarizes the current understanding of the role of IL-22 in allergic asthma, concluding that the function of IL-22 may vary with the stage of asthma pathogenesis.[91]

The role of IL-22 in the setting of fungal, bacterial and viral pulmonary infections has also been investigated by a number of groups. Gessner and colleagues examined the role of IL-22 in a mouse model of pulmonary Aspergillus fumigatus infection and found that A. fumigatus exposure induces Il-22 expression in the lung in wild type mice, with a reduction of this effect in Dectin-1 deficient mice. IL-22 deficient mice showed higher levels of A. fumigatus in the lung than those without IL-22 deficiency. Both Dectin-1 deficiency and IL-22 deficiency also appeared to result in a lack of soluble anti-fungal molecules in the lung, suggesting that IL-22 contributes significantly to the lung's innate defense against Aspergillus infection[92]. In addition,

there is evidence that IL-22 contributes to the immune defence against candidiasis[93]. The possible role of IL-22 in the immune response to fungal infections was recently reviewed by Zelante and colleagues[94].

IL-22 also appears to have a role in the immune response to bacterial pneumonia. Aujla and colleagues used a mouse model of Klebsiella pneumoniae pneumonia to investigate the role of IL-22 in gram negative pneumonia [86]. IL-22 was elevated in the lung tissue of Klebsiella pneumoniae infected mice compared to control mice, and this IL-22 was found to originate from T-cells in the lung. Administration of an IL-22 antibody resulted in infection-related mortality. IL-22 was also shown to have antimicrobial properties against K. pneumoniae in vitro [86]. Additional evidence for the role of IL-22 in bacterial infection was provided by Zheng and colleagues, who used a mouse model of intestinal bacterial infection to show that IL-22 is induced during infection. In comparison to wild-type animals, IL-22 knockout mice were not able to clear infection with Citrobacter rodentium and suffered high rates of systemic bacterial spread and infection related mortality [95].

The role of IL-22 in viral pneumonia was recently investigated by Pociask and colleagues in a mouse model of influenza infection[96]. Both IL-22 knockout and wild-type mice were able to clear the influenza infection; however, IL-22 knockout mice showed signs of more severe lung injury, decreases in lung compliance and increased collagen deposition in the lung. The authors conclude that IL-22 mediates airway repair processes following influenza pneumonia.

IL-22 appears to play a role in the response to a range of pulmonary pathogens, as well as in numerous non-malignant pulmonary disorders. The effects of IL-22 in the lung, as in many other organs, seem to be both immunoregulatory and proinflammatory depending on the type and stage of disease[74].

1.5.2. IL-22 in lung cancer

To date, the literature concerning IL-22 in cancer has remained somewhat contradictory, with some evidence suggesting that IL-22 protects against tumour growth, and other studies suggesting that IL-22 contributes to or supports the malignant potential of tumour cells.

Xing and colleagues were able to show that IL-22 protects hepatocytes from apoptosis in a model of alcohol-induced hepatotoxicity[97]. In the setting of malignancy, the anti-apoptotic effects of IL-22 may protect tumour cells. Radaeva and colleagues described the anti-apoptotic effects of IL-22 in the hepatocellular carcinoma cell line HepG2[77]. HepG2 cells engineered to over-express IL-22 were resistant to apoptosis in vitro. The overexpression of IL-22 also resulted in increased tumour formation in a subcutaneous tumour model in nude mice. The anti-apoptotic effects of IL-22 appeared to be related to upregulation of the antiapoptotic proteins Bcl-xL, Bcl-2 and Mcl-1 and the mitogenic proteins c-myc, cyclin D1, Rb2 and CDK4[77].

There is also evidence that IL-22 may be relevant to the development of human cancers. Thompson and colleagues found an association between the single

nucleotide polymorphism rs1179251 in IL-22 and risk of colon cancer in a mostly Caucasian population [98].

Contradictory evidence, suggesting that IL-22 may inhibit cancer cells, was published by Weber and colleagues, who examined the role of IL-22 in breast cancer cell lines [99]. These investigators found that while EMT6 murine breast cancer cells express IL-22R, exposure to IL-22 inhibits cancer cell proliferation and leads to cell cycle arrest. In a subcutaneous tumour model in nude mice, they found that treatment with IL-22 decreased tumour growth.

IL-22 has been shown to be expressed in lung cancer cell lines and primary tissues, and seems to promote tumour growth in this disease. A recent project at our centre showed that IL-22R1 is expressed by the lung cancer cell lines A549, HCC827, H1339, H187 and LOU-NH91. Although the lung cancer cell lines themselves were not found to express IL-22, exposure to IL-22 induced proliferation of the cell lines in vitro[100]. Cells selected for resistance to cisplatin showed upregulation of IL-22R1. Upon exposure to IL-22, these cisplatin-resistant cells showed increased proliferation compared with cisplatin naïve cells, suggesting that IL-22 signalling may correlate with a more aggressive tumour phenotype [101].

These findings correlate well with previously published evidence from Zhang and colleagues, who described expression of IL-22 in lung adenocarcinoma and squamous cell carcinoma tissue, as well as higher IL-22 concentrations in the serum and pleural effusion of lung cancer patients compared to control patients without lung cancer[102]. These investigators also found expression of IL-22 receptors in the lung cancer cell lines A549 and PG, and expression of IL-22 in A549 cells. Cell lines

overexpressing IL-22 were resistant to chemotherapy-induced apoptosis. IL-22 silencing with small interfering RNA resulted in increased tumour cell apoptosis, and decreased tumour growth in a subcutaneous xenograft model in nude mice [102].

Immunohistochemical staining of tissue microarray samples from over 2000 patients has shown that IL-22 is expressed in both small cell and non-small cell lung cancers[103]. However, IL-22 expression in the tumours tested by Kobold and colleagues did not correlate with tumour stage or prognosis. And so, although IL-22 promotes tumour cell proliferation in vitro, the clinical impact of IL-22 in lung cancer patients remains unclear.

Despite uncertainty as to the precise nature of IL-22s' role in malignancy, it seems clear that IL-22 is relevant not only to primary lung cancer, but also to non-malignant lung diseases. For this reason, we sought to quantify IL-22 in the pulmonary compartment, as sampled by bronchoalveolar lavage and bronchial washings, in patients with lung cancer compared with patients with non-malignant pulmonary disorders and infections. In order to characterize the concentration of IL-22 in these groups, we analysed specimens from 56 patients undergoing clinically indicated lavage at our hospital and correlated IL-22 concentration in the lavage samples with both local and systemic cell counts and with serum markers of inflammation.

1.6. Aim of the dissertation

The aim of this dissertation is to investigate the feasibility of IL-22 measurements in bronchial washings and bronchoalveolar lavage in patients with lung cancer and patients with non-malignant lung illnesses, and to describe the correlation of IL-22 concentration with local and systemic cell differentiation and systemic inflammation. We hypothesize that IL-22 in lavage reflects the lung compartment, and may therefore serve as a biomarker for physiological and pathological processes localized to the lung.

2. PATIENTS AND METHODS

Patients underwent routine diagnostic or therapeutic flexible bronchoscopy with lavage in the Respiratory Medicine and Thoracic Oncology Section of the Internal Medicine Department V (Medizinische Klinik V, Sektion Pneumologie Innenstadt und Thorakale Onkologie), Ludwig Maximilians University of Munich, Germany. Bronchoscopy and lavage were carried out as clinically indicated and following written informed consent. Excess lavage material was used for IL-22 analysis. The study and its protocol were approved by the local ethics board (Ethikkommission der Universität München, decision number EK 376-11).

2.1. Patients

Adult men and women undergoing clinically indicated diagnostic or therapeutic flexible bronchoscopy on an outpatient or inpatient basis in the Department of Pneumology were included in the study. Patients were not excluded or selected based on suspected diagnosis, stage of disease or concurrent medication. The routine diagnostic evaluation included appropriate imaging, blood work, lung function analyses, biopsies and cultures as clinically indicated. Patient information was gathered retrospectively from the chart and electronic patient records. The diagnosis of the treating physician following routine analysis of the bronchoscopic samples was recorded. For the purpose of analysis diagnosis categories were then created, namely lung cancer, other thoracic malignancies (including primary intra-thoracic

cancers and metastases from other sites), pneumonia, other lung diseases, and a reference cohort made up of patients who underwent bronchoscopy due to suspected lung disease or malignancy which were not confirmed by the results of the bronchoscopy and clinical work-up.

2.2. Bronchoscopic Procedures and Sample Collection

2.2.1. Bronchoscopic procedures

Bronchoalveolar lavage (BAL) and bronchial washings were performed as clinically indicated and according to standard clinical practice at our centre, which is based on peer-reviewed international recommendations [66]. During bronchoscopy blood oxygen saturation and heart rate were monitored continually, and non-invasive blood pressure was measured at regular intervals. Following local anesthesia of the nares and pharynx, patients underwent conscious sedation with titrated midazolam with or without the additional administration of intravenous propofol, and were intubated nasally with a flexible bronchoscope. Following inspection of the laryngeal anatomy the vocal cords were anesthetised with a topical local anesthetic. The bronchoscope was then advanced into the trachea.

<u>Bronchoalveolar lavage</u>

For bronchoalveolar lavage (BAL) the bronchoscope was advanced into wedge position, preferentially in the segment of clinical interest or, in the case of diffuse

pulmonary pathology, in the right middle lobe. Normal saline was instilled in 20 ml aliquots to a total volume of 120 to 160 ml and was retrieved into a sterile container using suction.

For bronchial washings the bronchoscope was introduced into the area of clinical interest, which in most cases was the segment thought to be affected by infection or tumour, and normal saline (generally 40 to 80 ml) was instilled and retrieved using suction. A standard morphological and immunologic analysis of BAL cellular components was performed and included total cell count, differential count of macrophages, lymphocytes and neutrophils as well as flow cytometry analysis of the lymphocyte subsets, including BAL CD4/CD8 T-cell ratio. Differential cell count (leukocytes, lymphocytes, neutrophils, macrophages and CD4/CD8 ratio) subgroups were based on standard cut-off values used for the interpretation of BAL fluid[66].

2.2.2. Sample processing

The volume of lavage fluid retrieved was recorded and samples were then divided in the bronchoscopy suite according to clinical indication. Aliquots for microbiological and pathological analyses were labelled and set aside. The remaining lavage fluid was transported without delay at room temperature to the on-site pneumology laboratory and processed immediately. The fluid was filtered through a triple layer of sterile gauze to remove mucous and large particles. Fifteen millilitres of filtered BAL fluid were then set aside for fluorescence activated cell sorting (FACS) analyses and two or more 500 microlitre aliquots were frozen at -20 degrees Celsius for later

enzyme-linked immunosorbent assay (ELISA) analysis. The remaining BAL fluid was used to determine cell count and differential cell count.

2.2.2.1 Cell count

One hundred microliters of the filtered BAL fluid was combined with one hundred microliters of sterile water and one hundred microlitres of crystal violet and mixed briefly with a vortex mixer. Ten microliters of this suspension was then pipetted into a Neubauer cell counting chamber and cells were counted manually under light microscopy.

2.2.2.2 Differential cell count

Cytospin samples were prepared by cytocentrifugation using between one hundred and three hundred microliters of filtered BAL fluid, depending on the cell count of the sample. From each patient sample, two cytospin slides were stained with Giemsa solution and May-Grünwald stain, and two further unstained cytospin slides were fixed with methanol and archived. The percentage of macrophages, neutrophils, lymphocytes and eosinophils was determined by manually counting at least 300 cells using light microscopy.

2.2.2.3. CD4/CD8 Ratio

Fluorescence activated cell sorting was used to determine the proportion of CD4 and CD8 positive lymphocytes in the BAL samples. For FACS analyses, fifteen millilitres of filtered BAL fluid was centrifuged and the supernatant was poured off. Twenty

millilitres of red cell lysis solution was added to the cell pellet, the cells were resuspended using a vortex mixer, and the solution was allowed to stand at room temperature for ten minutes before being centrifuged once again. The supernatant was poured off, and the cell pellet was resuspended in twenty millilitres of phosphate buffered saline (PBS), centrifuged, and the resulting cell pellet resuspended in 500 microliters of PBS. Three aliquots were prepared for FACS analysis, each with one hundred microliters of cell suspension: one unstained, one with twenty microliters of Simultest reagent, and one with twenty microliters of MultiTest reagent. One millilitre of PBS was then added to the Simultest and MultiTest aliquots, the samples were centrifuged, and a few drops of PBS were added to the cell pellets. FACS analysis was then carried out using a FACSCalibur flow cytometer.

2.2.2.4. IL-22 ELISA

Enyzme-linked immunosorbent assays (ELISA) for IL-22 detection were obtained from R&D, Abington, UK. ELISA analyses were performed in the Department of Clinical Pharmacology (Prof. S. Endres and Dr. S. Kobold). In brief, 50 µl of diluted samples (in triplicates) were loaded and incubated for 2 h at room temperature (RT). Detection antibody was applied for 2 h at RT and streptavidin-bound horseradish peroxidase (HRP) was added for 20 min at RT. Absorption was measured at 450 nm using a Mithras reader (Berthold Technologies, Bad Wildbad, Germany). The detection limit of the ELISA was 15 pg/ml.

2.3. Statistical Methods

For comparative analysis between two groups (paired comparisons) only data from samples above the detection limit of the ELISA (> 15 pg/ml) were used. For the IL-22 levels, mean values of three independent experiments each performed in triplicates were calculated and used for subsequent analysis. GraphPad Prism Software, version 5.0b (GraphPad Software) was used. All t-tests were unpaired and 2-sided and p values < 0.05 were considered significant.

3. RESULTS

We analysed lavage specimens from 56 patients undergoing clinically indicated bronchoscopic lavage and correlated IL-22 expression with local and systemic cell counts and with serum markers of inflammation.

3.1. Cohort demographics

Samples from 56 adult in-patients and out-patients who underwent routine bronchoscopy in the Respiratory Medicine and Thoracic Oncology Section of the Internal Medicine Department V (Medizinische Klinik V, Sektion Pneumologie Innenstadt und Thorakale Onkologie), Ludwig Maximilians University of Munich, Germany were included in the analysis. Samples (43 bronchoalveolar lavages and 13 bronchial washings) were collected from twenty women and thirty-six men. The patients ranged in age from 18 to 84 years, with a mean age of 58.3 years. Most patients were adults aged sixty-one to eighty years (thirty-one patients), or forty-one to sixty years (fourteen patients). Nine patients were between twenty-one and forty years of age, one patient was younger than twenty years of age, and one was older than eighty.

The diagnostic evaluation included bronchoscopy and appropriate imaging, blood work, biopsies and cultures as indicated, and revealed sixteen patients (29%) with infection. Three of these patients had tuberculosis and thirteen had other bacterial pneumonias. Fourteen patients (25%) had a diagnosis of lung cancer, with thirteen

cases of non-small-cell lung cancer and one case of small cell lung cancer. Seven patients (13%) had other thoracic malignancies or pulmonary metastases from extrathoracic tumours. The diagnostic work-up revealed eleven patients with other lung diseases, including one patient with Wegener's granulomatosis, one patient with chronic graft rejection following lung transplantation, two patients with acute respiratory distress syndrome (ARDS), four patients with interstitial lung disease or fibrosis and three patients with sarcoidosis. Nine patients underwent bronchoscopy due to a suspected lung disease or malignancy which was not confirmed by the results of the bronchoscopy and clinical work-up. These patients were therefore included in the reference cohort.

3.2. IL-22 concentration in BAL and bronchial washings

IL-22 is found in significant amounts in lavage samples

In thirty-five of the fifty-six samples analysed, the measured level of IL-22 was above the limit of detection of the ELISA (Figure 1A). In the remaining twenty-one samples no IL-22 was detected, or the measured level of IL-22 was below the limit of detection (Figure 1A). The average IL-22 level in all samples with detectable IL-22 was 44 pg/ml (range 15 -121 pg/ml). The average IL-22 concentrations in the BAL samples and the bronchial washings were similar at 47 pg/ml and 33 pg/ml, respectively (difference not statistically significant).

IL-22 concentration does not correlate with gender or age

The average IL-22 concentration in samples from male (45 pg/ml) and female (48 pg/ml) patients was not significantly different, with an average IL-22 concentration of 45 pg/ml in samples from men, and an average IL-22 concentration of 48 pg/ml in samples from women. The average IL-22 concentration in samples from patients older than the mean cohort age (58.3 years) did not differ significantly from the concentration in younger patients. Patients above the mean age had an average IL-22 concentration of 41 pg/ml, while those below the mean age had an average IL-22 concentration of 54 pg/ml. There was also no clear correlation with age in subgroups of patients aged under twenty years, twenty-one to forty years, forty-one to sixty years, sixty-one to eighty years and over eighty years.

3.3. Correlation of IL-22 with diagnosis

Patients with lung diseases have higher IL-22 concentrations in lavage samples.

The 29 patients with a lavage IL-22 concentration above the detection limit included twenty-three patients with confirmed lung disease and six patients without lung disease. IL-22 levels were significantly higher in patients with lung disease (50 pg/ml) compared to those of patients without lung disease (26 pg/ml). This is shown in figure 1B. Subgroup analyses for a relationship between specific diagnosis and IL-22 concentration revealed a trend towards higher IL-22 levels in patients with bacterial pneumonia (53 pg/ml), patients with NSCLC (44 pg/ml) and patients with pulmonary manifestations of other malignancies (57 pg/ml) compared to patients without lung disease (26 pg/ml). Table 1 provides a summary of the demographic characteristics and diagnoses of the patients included in this study, as well as the IL-22 concentration in subgroups based on gender, age and diagnosis.

FIGURE 1:

IL-22 concentrations in lavage are higher in patients with lung diseases than in controls.

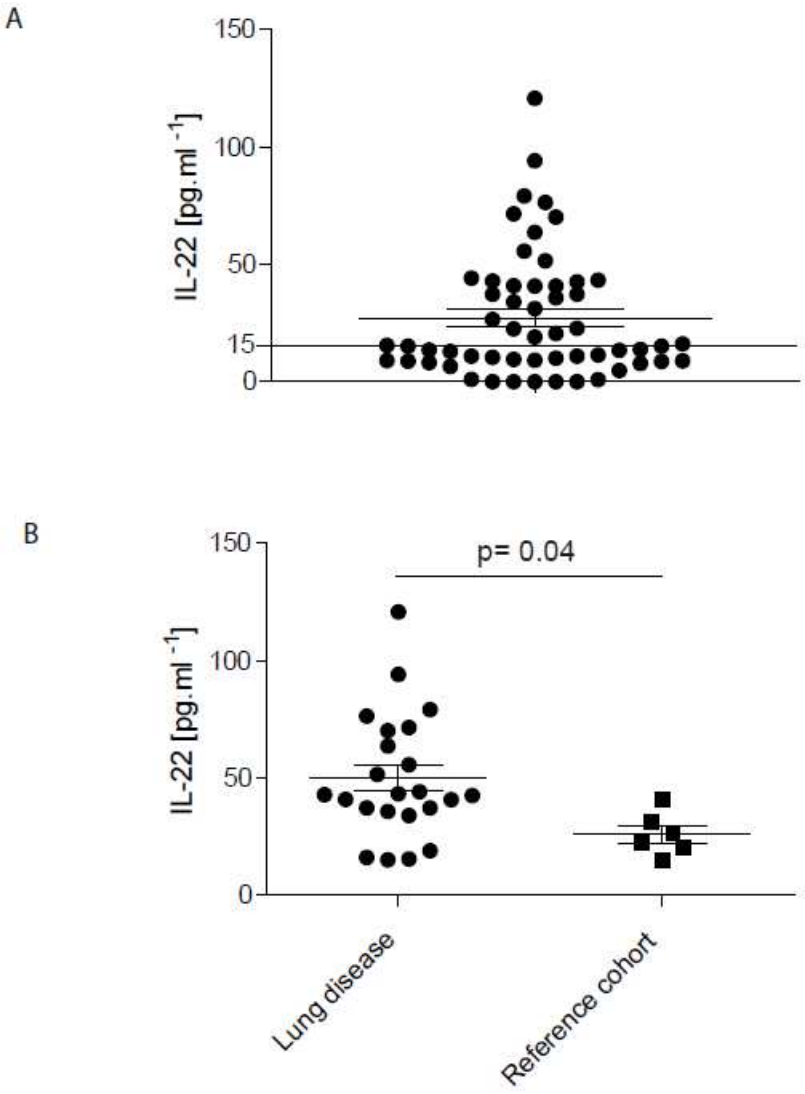

Figure 1A: Dot plot analysis of IL-22 concentrations found in n = 56 lavage samples. The detection limit is 15 pg/ml (continuous black line). The mean ± SEM for all patients is indicated in the graph. **Figure 1B:** Comparison between BAL IL-22 concentrations for samples above the detection limit of patients with and without lung disease. Each dot represents the mean value of three independent measurements by ELISA, each performed in triplicates. Means ± SEM are indicated for both groups.

TABLE 1

Correlation of Patient Characteristics with IL-22 in the Lavage

Characteristics	Number of patients (% of study cohort)	IL-22 [pg/ml] (mean of all values)	IL-22 [pg/ml] (mean of values above DL)	Number of samples below DL (%)
Gender				
Male	36 (64.3%)	29	45	14 (38.8)
Female	20 (35.7%)	24	48	12 (60)
Age (years)				
< 20	1 (2%)	11	n.a.	1 (100)
21-40	9 (16%)	41	56	3 (33.3)
41-60	14 (25%)	21	63	10 (71.4)
61-80	31 (55%)	25	39	13 (41.9)
> 80	1 (2%)	43	43	0 (0)
Diagnosis				
Pneumonia	13 (23.6%)	32	53	6 (46.2)
NSCLC	13 (23.6%)	28	44	6 (46.2)
SCLC	1 (1.8%)	9	n.a.	1 (100)
Sarcoidosis	3 (5.4%)	27	40	1 (33.3)
Tuberculosis	3 (5.4%)	23	37	1 (33.3)
Cancer (non lung)	7 (12.7%)	29	57	4 (57.1)
Wegener granulomatosis	1 (1.8%)	70	70	0 (0)
ILD	4 (7.3%)	11	41	3 (75)
ARDS	2 (3.6%)	11	14	2 (100)
GVHD	1 (1.8%)	64	64	0 (0)
Control (no lung disease)	9 (16.4%)	19	26	3 (33.3)

n.a. stands for not applicable.

3.4. Correlation of IL-22 with cellular components of BAL

IL-22 does not correlate with the cell count in BAL

IL22 concentration did not show a significant association with the overall cell counts in the BAL fluid. These results are summarized in table 2, along with the mean IL-22 concentrations in subgroups based on differential cell counts in the BAL and systemic markers of inflammation.

TABLE 2

Correlation of BAL Parameters and Systemic Parameters with IL-22 in the Lavage

Characteristics	Number of patients (% of study cohort)	IL-22 [pg/ml] (mean of all values)	IL-22 [pg/ml] (mean of values above DL)	Number of samples below DL (%)
BAL parameters				
Cells/ml				
< 100,000/ml	14 (25.4%)	33	56	6
≥ 100,000/ml	16 (29.1%)	25	45	8
% Lymphocytes				
< 10%	15 (27.3%)	26	39	6
≥ 10%	13 (23.6%)	30	72	8
CD4/CD8 ratio				
CD4/CD8 < 1,3	8 (14.5%)	16	33	6
CD4/CD8 > 1,3	14 (25.4%)	34	66	7
% Neutrophils				
< 5%	14 (25.4%)	44	58	8
≥ 5%	13 (23.6%)	16	32	4
% Macrophages				
< 80%	10 (18.2%)	37	48	2
≥ 80%	18 (32.7%)	23	55	12
Peripheral blood parameters				
CRP (mg/dl)				
< 0,5	13 (23.6%)	27	42	5
≥ 0,5	40 (72,7%)	27	46	19
Leukocytes (G/l)				
< 11	42 (76.4%)	24	41	20
≥ 11	12 (21.8%)	32	46	5
Lymphocytes (cells/µl)				
< 1500	21 (38.2%)	25	47	11
≥ 1500	12 (21.8%)	26	42	5

Samples were analyzed according to clinical indication, therefore some categories describe fewer than 56 samples; n.a. stands for not applicable.

IL-22 correlates with a higher proportion of lymphocytes in BAL

IL-22 concentration in BAL samples was compared with the cell differentiation in the BAL. IL-22 concentrations were significantly higher in patients with an elevated proportion of lymphocytes in the BAL fluid (IL-22 72 pg/ml for ≥ 10% lymphocytes vs. 39 pg/ml for < 10% lymphocytes, p=0.029. This is shown in figure 2A.

IL-22 correlates with a higher CD4/CD8 lymphocyte ratio in BAL

IL-22 was higher in the subgroup of patients with an elevated CD4/CD8 ratio compared to those with a lower CD4/CD8 ratio. Patients with a CD4/CD8 ratio equal to or greater than 1.3 had an average IL-22 concentration of 66 pg/ml, whereas patients with a CD4/CD8 ratio below 1.3 had an average IL-22 concentration of 33 pg/ml, p=0.033. These results are summarized in figure 2B.

IL-22 does not significantly correlate with the proportion of neutrophils in BAL

There was a numerical trend towards lower IL-22 concentrations in patients with higher BAL neutrophils; however, this did not reach statistical significance. The average IL-22 concentration was 58 pg/ml for patients with less than 5 % neutrophils in the BAL, compared to an average IL-22 concentration of 32 pg/ml for patients with greater than or equal to 5% neutrophils in the BAL. These results are summarized in figure 2C.

IL-22 does not correlate with the proportion of macrophages in BAL

There was no significant correlation between IL-22 concentration and the proportion of macrophages in the BAL. The average IL-22 concentration in the BAL was 55 pg/ml for samples with greater than 80 % macrophages, compared to an average IL-22 concentration of 48 pg/ml for BAL samples with less than 80% macrophages. These results are summarized in figure 2D.

Figure 2:

IL-22 concentrations in BAL are higher in patients with a higher proportion of BAL CD4+ lymphocytes.

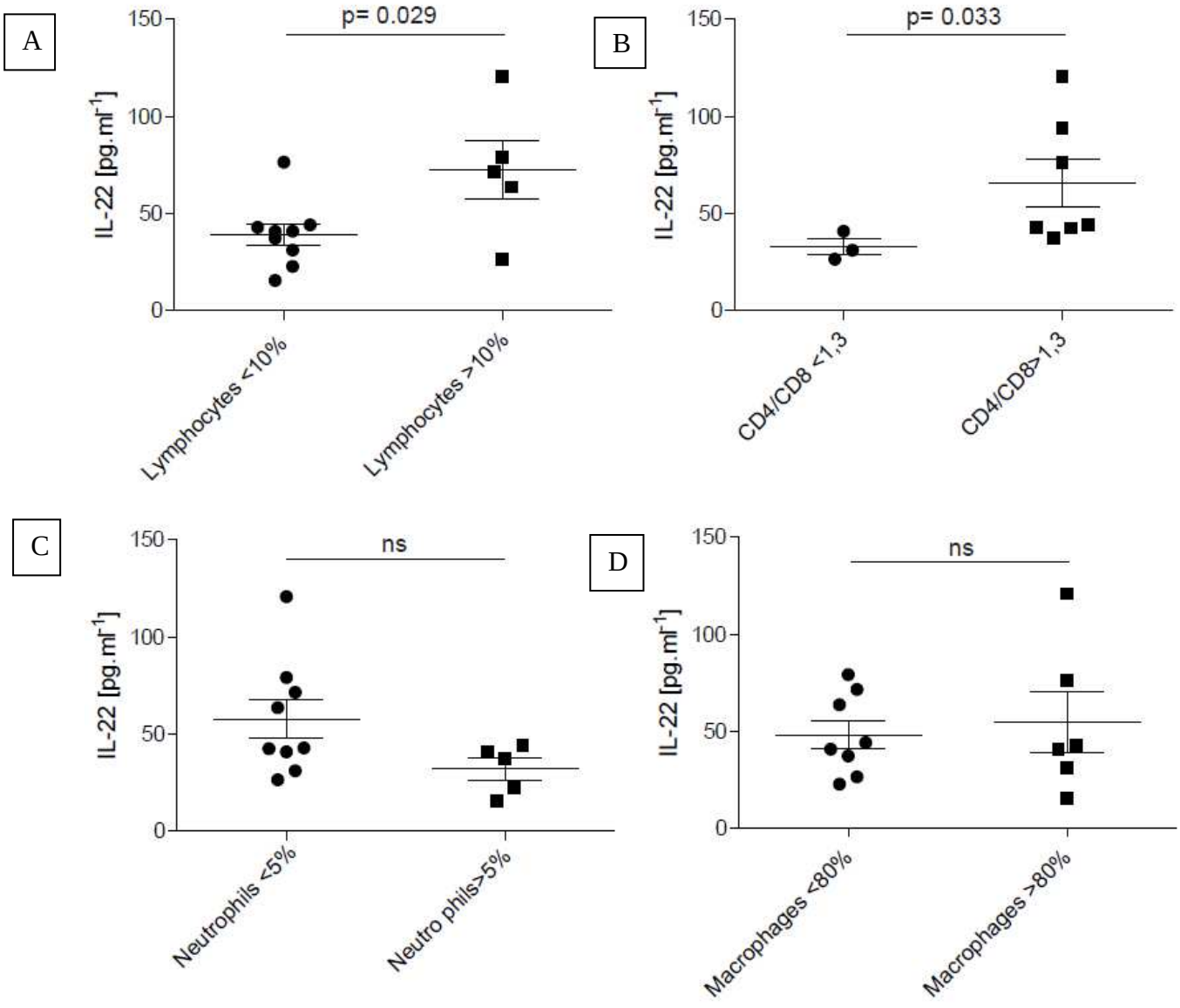

Figure 2A: Comparison of IL-22 concentrations found in BAL samples with a normal (<10%) and a high proportion of lymphocytes (>10%). **Figure 2B:** Comparison of IL-22 concentrations found in BAL samples with a normal (<1.3) and a high CD4/CD8 ratio (>1.3). **Figure 2C:** Comparison of IL-22 concentrations found in BAL samples with a normal (<5%) and a high proportion of neutrophils (>5%). **Figure 2D:**

Comparison of IL-22 concentrations found in BAL samples with a normal (<80%) and a high proportion of macrophages (>80%). One dot represents the mean value of three independent ELISA measurements. Means ± SEM are indicated for all groups.

3.5. Correlation of IL-22 with systemic markers of inflammation

In order to assess the possibility that IL-22 concentrations measured in the lung are a product of systemic inflammation, IL-22 concentration in lavage was compared with standard clinically relevant indicators of systemic inflammation and infection, namely systemic levels of C-reactive protein (CRP) as well as systemic leukocyte and lymphocyte counts.

IL-22 in lavage does not correlate with systemic leukocyte counts

There was no significant correlation between IL-22 concentration in the lung, as sampled by lavage, and leukocyte count in the peripheral blood. Lavage IL-22 levels in patients with systemic leukocytes below 11 G/l did not differ significantly from those from patients with higher systemic leukocyte counts. Patients with leukocyte counts below 11 G/l showed an average lavage IL-22 concentration of 41 pg/ml; and patients with elevated leukocyte counts (above 11 G/l) showed an average IL-22 concentration of 46 pg/ml. These results are summarized graphically in figure 3A.

IL-22 in lavage does not correlate with systemic lymphocyte counts

As with systemic leukocyte counts, absolute lymphocyte counts in peripheral blood did not show any significant association with IL-22 concentration in lavage. The

average IL-22 concentration in lavage from patients with low systemic lymphocyte counts (lymphocytes < 1500/µl) was 47 pg/ml. Patients with high systemic lymphocyte counts (lymphocytes > 1500/µl) had an average IL-22 concentration in lavage of 42 pg/ml. The lavage IL-22 concentration in these patients is shown in figure 3B.

IL-22 in lavage does not correlate with systemic CRP-levels

Serum CRP levels showed no correlation with IL-22 concentration in lavage. Patients with a clinically elevated CRP level (≥ 0.5 mg/dl) had an average pulmonary IL-22 concentration in lavage of 46 pg/ml. This was similar to the IL-22 concentration in lavage from patients with non-elevated systemic CRP levels (CRP < 0.5 mg/dl), which was measured to be 42 pg/ml. This data is summarized in figure 3C.

Figure 3

IL-22 concentrations in lavage do not correlate with systemic parameters of inflammation.

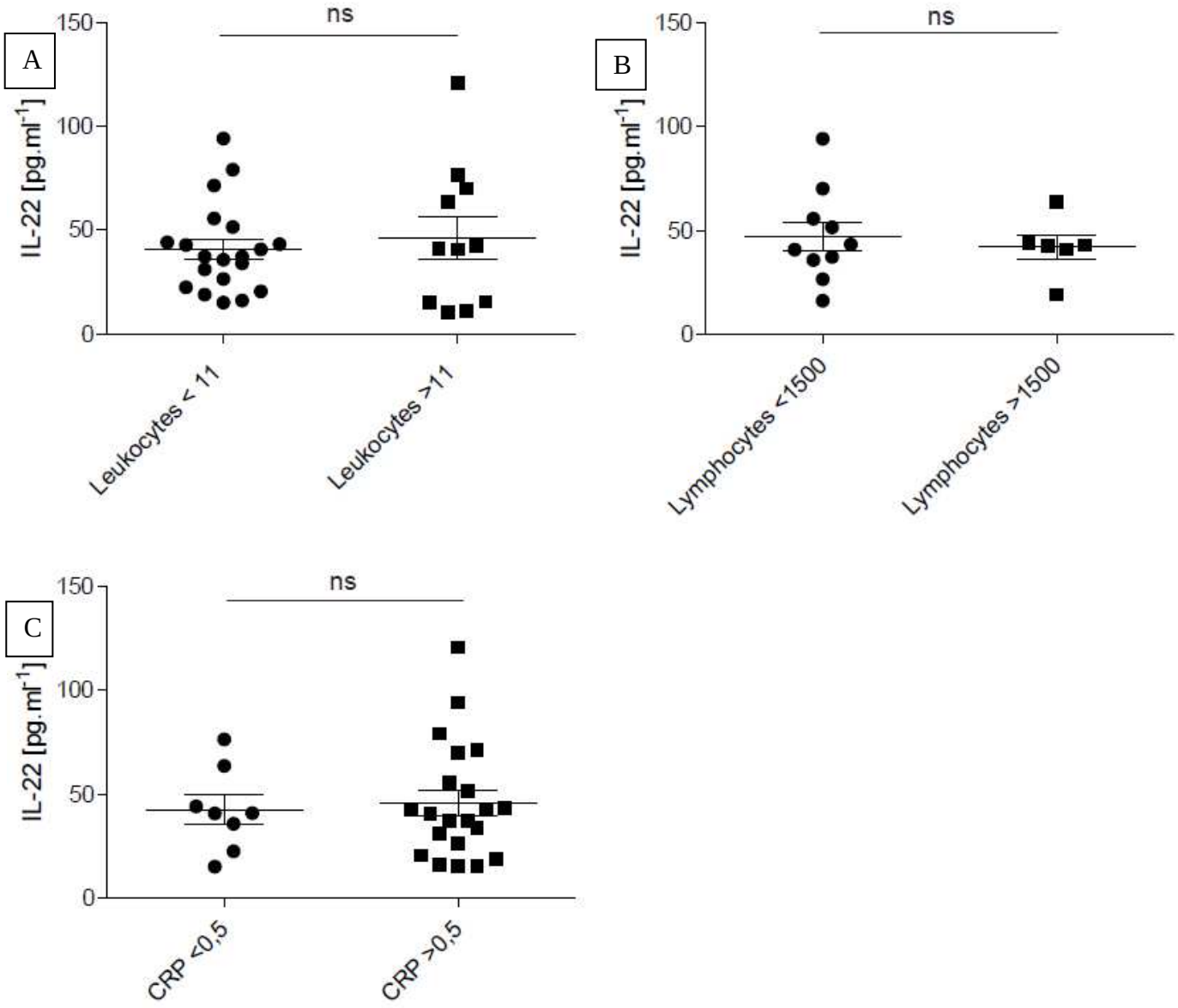

Figure 3A: Comparison of IL-22 concentrations found in lavage from patients with a normal (< 11 G/ml) and a high (> 11 G/ml leukocyte count in the peripheral blood).

Figure 3B: Comparison of IL-22 concentrations found in lavage from patients with a normal (< 1500) and a high (> 1500) lymphocyte count in the peripheral blood.

Figure 3C: Comparison of IL-22 concentrations found in lavage from patients with a normal (< 0.5 mg/dl) and a high (≥ 0.5 mg/dl) serum CRP concentrations.

4. DISCUSSION

This study established that IL-22 is found in the airways of patients with a variety of pulmonary diseases including lung cancer patients, and can be measured in lavage samples using ELISA. IL-22 in lavage is elevated in patients with lung cancer and in patients with pneumonia compared to individuals with no bronchoscopic evidence of pulmonary disease. IL-22 concentrations in lavage correlate with cell differentiation in the BAL but not with systemic cell counts and CRP, suggesting that lavage IL-22 levels represent processes localized to the lung. Lavage IL-22 may therefore prove to be a valuable biomarker in patients with lung cancer.

4.1. Discussion of the methods

4.1.1. Diversity of patients and diagnoses

This study included patients with a variety of clinical diagnoses, spanning a broad range of pathologies encountered in the practice of respiratory medicine, and ranging in age from young adults to the elderly. This diversity represents an important advantage of the study design and increases the potential to translate the results to clinical practice. Patients with lung cancer often suffer from additional pulmonary comorbidities. Although smoking-related diseases such as chronic bronchitis and emphysema are the most common, patients with other pulmonary pathologies such as idiopathic pulmonary fibrosis are also at an increased risk of developing lung cancer, as are individuals with occupational exposures to substances such as silica[104]. In addition, recent large lung cancer screening trials have shown that many of those screened for lung cancer show radiographic evidence of other lung

diseases[105,106]. It is, therefore, crucial that lung cancer biomarkers intended for diagnostic use be able to distinguish not only lung cancer cases from healthy individuals without lung disease, but also lung cancer cases from benign pulmonary pathologies.

In the current study, levels of IL-22 in lavage were highest in patients with lung cancer, patients with other pulmonary malignancies and patients with pneumonia. Levels were lower in patients with no bronchoscopic evidence of pulmonary disease, although all of these patients had clinical symptoms, which had led to the indication for bronchoscopy. Although the study also included patients with interstitial lung diseases, this subgroup was too small to allow for meaningful statistical analysis. The IL-22 concentrations measured in the current study showed a significant overlap between patients with lung cancer and patients with bacterial pneumonia. Strategies to differentiate these patient populations from another, perhaps through the use of serial measurements after antibiotic treatment, quantification of IL-22 in the systemic circulation, or the measurement of additional markers in the lavage fluid, are the subject of an ongoing project in our department.

4.1.2. Inclusion of washings and bronchoalveolar lavage

This study included patient samples collected by two distinct means, namely bronchoalveolar lavage and bronchial washing. Although similar, these two procedures do not sample identical regions of the airways. While bronchoalveolar lavage aims to collect cells from the alveoli, the clinical goal of a bronchial washing is generally to sample material from a more proximal airway. This is most often of

relevance when the pathological process to be sampled, for instance the suspected tumour, is localised centrally. In such cases a sample collected from the more distal airways is less likely to lead to a diagnosis than a sample collected proximally. Although generally safe and well tolerated, bronchoalveolar lavage may cause transient decreases in arterial oxygen saturation in patients with severe respiratory insufficiency[107]. The clinician may therefore find it prudent to limit the amount of saline used to collect a bronchoalveolar lavage sample in critically ill patients or those with significant pre-existing limitations of gas exchange. In some such cases the clinician may elect to perform a bronchial washing rather than a bronchoalveolar lavage, especially if the clinical situation does not require alveolar sampling. For instance, in patients with severe pneumonia undergoing bronchoscopy for the purpose of establishing a microbiological diagnosis, a bronchial washing may be more clinically appropriate than a bronchoalveolar lavage. Because this study aimed to examine the feasibility of IL-22 measurements in routine samples, both types of sample, that is, both bronchoalveolar lavage and bronchial washings, were included. A retrospective subgroup analysis comparing the washings with bronchoalveolar lavage showed no significant difference in mean IL-22 concentration; however, the subgroups may have been too small to detect such a difference.

The anatomy, functionality and cellular composition of the airways vary from the trachea through the large central airways and into the alveoli. It is therefore not only possible but also quite likely that, rather than representing a single compartment, the airways represent several interrelated compartments which may be sampled differently depending on the precise position of the bronchoscope. Whether this is of relevance for the measurement of IL-22 concentration and characterisation of IL-22 signalling in the lung has yet to be established.

This study analysed samples collected by experienced clinicians in routine practice, without seeking to influence the collection method or type of sample collected. Because a bronchoalveolar lavage is more often clinically indicated in patients suspected of having interstitial lung diseases, and a washing may be more often indicated when the clinical suspicion is tumour, it is not possible to rule out that systematic bias in the type of sample collected from different patient subgroups may have somewhat influenced the results.

4.1.3. Dilution of Samples

Bronchoalveolar lavage seeks to sample the lung's epithelial lining fluid (ELF). However, because lavage involves the introduction and then aspiration of saline into the airways, there is an unavoidable dilution of the ELF sample, and the degree of dilution may vary between procedures and between patients. It is, therefore, difficult to measure the exact concentration of substances in the epithelial lining fluid itself. There have been numerous attempts to correct for this dilution in previous studies. While some of these approaches have sought to correlate the serum and lavage concentrations of molecules such as albumin or urea, others have used a dilutional approach, measuring the concentration of markers in the fluid introduced into and the fluid removed from the airways. However, the precision of each of these techniques is limited by the complex physiology of the diffusion of molecules across the blood-air barrier, which has also been shown to vary with different disease states[108,109].

Based on the assumption that urea diffuses freely from the blood into the epithelial lining fluid of the lung, Rennard and colleagues[110] compared blood urea levels with urea concentrations in bronchoalveolar lavage in order to estimate the degree of dilution in lavage samples. They performed lavage with normal saline, and found that the concentration of urea in the recovered samples was approximately 1% of the serum urea concentration.

Jones and colleagues compared the use of urea and albumin as measures of dilution in bronchoalveolar lavage in sixty-seven patients with a variety of pulmonary diseases, and found significant variability in lavage albumin concentrations between patients[109]. The authors concluded that urea is a superior marker of dilution in lavage samples compared with albumin. However, the use of urea as a marker is also not unproblematic, as urea likely does not diffuse as freely into the lung as previously thought. There is now evidence that multiple factors, including the timing of cigarette smoking before bronchoscopy, may influence the urea permeability of the blood-air barrier in the small airways[108].

Complicating both the use of urea and albumin as markers of dilution is the observation that dwell time, that is, the time which saline is allowed to remain in the airways before being aspirated, affects the concentration of urea, glucose and albumin in lavage samples. This is likely due to diffusion of these molecules from the blood into the lavage fluid[110].

Ward and colleagues examined the use of albumin as a marker of dilution in lavage[108]. These investigators used injections of radioactive albumin in the systemic circulation to estimate the contribution of the diffusion of albumin from the systemic

circulation to the total albumin measured in lavage samples. There was a trend toward higher flux of albumin from the systemic circulation in asthmatic compared to healthy subjects undergoing lavage.

Baughman and colleagues described the use of methylene blue introduced in the lavage fluid as a marker of dilution[111]. Interestingly, these investigators found that the albumin concentration in BAL fluid varied with the diagnosis, suggesting that corrections for the dilution of samples based on albumin may not be reliable in all groups of patients.

Another technique based on the dilution of a marker introduced into the lavage fluid was described by Restrick and colleagues, who performed bronchoalveolar lavage in mild asthmatics as well as healthy controls using the inert polysaccharide inulin at a concentration of 0.1mM in the instilled fluid as a marker of dilution. These investigators found that the use of inulin as a marker in lavage was well tolerated by both the asthmatic and healthy subjects. They calculated that epithelial lining fluid made up an average of 7.0% of the fluid recovered from asthmatics and 9.4% of the fluid recovered from healthy subjects. However, there was significant variability between subjects, with epithelial lining fluid accounting for between 2% and 23% of the fluid recovered from the airways[112].

Although at our hospital bronchoalveolar lavage is performed in a standardized fashion, the amount of normal saline instilled during the procedure may vary slightly from clinician to clinician. In this study we did not correct for the effect of dilution in

the samples collected. Therefore, the IL-22 concentrations which were measured in the present study can not directly reflect the absolute IL-22 concentration in the epithelial lining fluid of the lung. The true concentration of IL-22 in the epithelial lining fluid is likely higher than the concentration measured in the uncorrected lavage samples. In addition, the variability observed between patient samples may in part have been due to varying degrees of dilution during sample collection. Absolute cell count may correlate with the degree of dilution in lavage samples; however, we did not find a significant correlation between absolute cell count in the lavage and lavage IL-22 concentration.

4.1.4. Correlation with Systemic Parameters

This study compared IL-22 concentrations measured in lavage with markers of systemic inflammation, namely with systemic leukocyte counts and with serum CRP levels. We were able to show that IL-22 concentration in the lung seems to be independent of these makers of systemic inflammation. However, as the study protocol did not include the collection of additional blood samples for the purpose of systemic IL-22 measurements, we were not able to directly assess possible differences in systemic and local IL-22 concentration. Previous studies have examined the differential expression of various substances including microRNAs[113], [114] in the systemic circulation and in the lung and have often found differences in the concentration in these two compartments. It is therefore not unlikely that IL-22 concentration also differs between the systemic circulation and the lung. The concentration of IL-22 in the systemic circulation as well as in other compartments

such as the pleural fluid is currently being addressed by an ongoing project at our institution.

The expression of the systemic markers of inflammation examined in this study may be affected by medications such as corticosteroids. Because the current study did not document the type and timing of medications administered to the patients sampled, a retrospective analysis of the possible confounding effect of such medications on the assessment of systemic inflammation is not possible.

4.2. Discussion of the results

4.2.1. IL-22 in Lavage

To date, there is little published data on IL-22 in bronchial lavage fluid. To our knowledge, no previous studies have investigated the concentration of IL-22 in lavage samples from patients with lung cancer. However, there is some evidence from groups studying other patient populations that IL-22 can be reliably measured in lavage.

A study from Whittington and colleagues examined IL-22 concentration in bronchoalveolar lavage samples from patients with non-malignant lung diseases, finding IL-22 concentrations in epithelial lining fluid (ELF) in the range of 0 to 15 ng/microliter, with significant variability between subgroups of patients[85]. The present study reports on IL-22 concentration in the total lavage fluid and not in the epithelial

lining fluid, and so, as expected, the concentrations reported in the present study are lower than those reported in the Whittington study.

A recent study in bronchoalveolar lavage samples from patients suffering from bronchial asthma revealed that IL22 concentration is elevated in asthmatic subjects compared to healthy controls[115], with an IL-22 concentration in lavage of 0.159 pg/ml in controls and 0.709 pg/ml in asthmatics.

Whittington and colleagues measured IL-22 concentration in lavage in patients with acute respiratory distress syndrome (ARDS), idiopathic pulmonary fibrosis, pulmonary sarcoidosis and control patients without known lung disease who were ventilated postoperatively. The investigators found lower IL-22 concentrations in samples from patients with ARDS compared with the ventilated control patients without lung disease[85].

The finding of lower IL-22 concentrations in lavage from patients with pulmonary diseases compared to normal controls is in disagreement with the finding of the present study. Our data showed a significantly higher concentration of IL-22 in lavage from patients with pulmonary illnesses compared to controls. This discrepancy may be explained by the fact that the Whittington study used post-operative ventilated patients without lung disease as controls, whereas the control patients in our study were not intubated and ventilated, and had not undergone surgery on the day of lavage. It is well established that mechanical ventilation can cause lung injury [116], which may have contributed to higher IL-22 levels in this group of patients. Our data agree with the data from Pennino and colleagues, which also showed higher concentrations of IL-22 in lavage samples from patients with pulmonary disease compared with controls [115].

4.2.2. The correlation of IL-22 in lavage with clinical pathology

The present study found that patients with bacterial pneumonia, NSCLC or pulmonary manifestations of other tumours appear to have higher levels of IL-22 in bronchoalveolar lavage and bronchial washings compared with a reference population with no bronchoscopic evidence of lung disease. This finding is in line with published evidence supporting a role for IL-22 in both lung cancer and in pneumonia.

Studies on the host response towards bacterial or fungal pneumonia have revealed that IL-22 contributes both to the acute phase, where it seems to support clearance of the infection, and to the chronic phase, where it may prolong inflammation [117] [86]. In chronic infections such as tuberculosis, IL22 seems to play a disease-supporting role[118]. The present study detected a trend towards higher levels of IL-22 in lavage samples from patients with pneumonia compared with controls, supporting the suggestion that IL-22 plays a role in the pulmonary response to infection.

The finding that patients with lung cancer show relatively high levels of IL-22 in pulmonary lavage specimens is also in line with the results of a previous study which reported that lung cancer cells express IL-22[102]. Zhang and colleagues found high expression of IL-22 in primary tumour tissue, serum, and malignant pleural effusion from NSCLC patients, as well as expression of IL-22 and the IL-22 receptor (IL22-R1) in lung cancer cell lines. In addition, a study from our institution recently showed that IL-22 is expressed in tissue microarray samples of a large cohort of lung cancer patients, in particular in patients with large and small cell lung cancers[103]. The cohort

of lung cancer patients in the present study is too small to attempt to confirm this variability in IL-22 expression between histological subgroups; however, the finding that IL-22 is expressed in lung cancer tissue correlates with our finding of elevated lavage IL-22 in these patients.

IL-22 concentration in lavage was quite variable between the individual patients we sampled, and not all of the patients with lung cancer in our study cohort showed elevated levels of IL-22 in the lavage. IL-22 was above the limit of detection in seven out of thirteen lavage samples (53.8%) from patients with NSCLC. This finding correlates well with the previous finding from Kobold and colleagues who detected IL-22 in 23-54% of NSCLC samples in tissue microarrays, depending on histological subtype[103]. It seems likely that not all lung cancers express IL-22 to the same degree, and that the expression of IL-22 may also vary during the course of the disease. This biological variability may be one cause of the clinical variability observed in the course of disease and response to treatment of lung cancer patients.

Although, to the best of our knowledge, the present study is the first to show that IL-22 is elevated in lavage samples from lung cancer patients, a wide range of other potential biomarkers found in lavage have been previously investigated in lung cancer [119 120 121]. The minimally-invasive nature of flexible bronchoscopy and the relative safety of lavage compared with other procedures make bronchoalveolar lavage and bronchial washings an appealing means of collecting diagnostically valuable material from the local tumour environment in lung cancer patients.

4.2.3. Correlation of IL-22 with parameters in lavage and in the systemic circulation

Correlation with differential cell counts in the BAL

The present study found that IL-22 concentration in the lavage fluid correlated with a high lymphocyte count in the lavage. In addition, lavage IL-22 concentrations correlated with a high lavage CD4/CD8 ratio. There was a trend towards a negative correlation of lavage IL-22 with lavage neutrophil counts; and a correlation with lavage macrophage counts was not identified. These correlations add to the existing body of evidence that IL-22 is mainly produced by lymphoid cells, among others by CD4-positive lymphocytes [122] [123].

In a previous study which analysed BAL samples in patients with tuberculosis, T helper cells were identified as the major source of IL-22 production in BAL fluid[123]. In our current study, we show that IL-22 production, presumably by CD4-positive lymphocytes in the lung, is not restricted to tuberculosis but also detectable in other lung diseases including lung cancer and other bacterial pneumonias.

Whittington and colleagues[85] previously published evidence that a number of cell types, including alveolar macrophages, also produce IL-22 in the lung. In the current study we did not find a correlation between the percentage of alveolar macrophages in the BAL fluid and the concentration of IL-22 in lavage. However, it remains possible that some of the IL-22 we detected was produced by macrophages or other immune cell populations, but that the relative amount of IL-22 produced by these cells was too little to significantly alter the overall concentration of IL-22 in the BAL.

In addition, lung cancer cells themselves may have directly contributed to the production of IL-22 in the lung cancer patients sampled. Zhang and colleagues found that lung cancer tissue strongly expresses IL-22, and that autocrine feedback loops may contribute to IL-22 signalling in lung cancer[102]. Even peripheral lung tumours can be detected at a molecular level in BAL fluid[124]. It seems, therefore, quite possible that IL-22 produced by lung cancer cells may also be detectable in lavage.

Correlation of BAL IL-22 with markers of systemic inflammation:

Recently, IL-22 has been detected in lavage samples from patients with community-acquired pneumonia and correlated with serum levels of IL-22 [125]. Interestingly, these authors reported that serum levels of IL-22 were elevated in patients with pneumonia compared to healthy controls, but that BAL IL-22 was similar in both groups. They also found that BAL fluid from patients with pneumonia contained an increased proportion of CD4 T-cells positive for IL-17A and IL-22 compared to blood, and that the proportion of these cells in the BAL did not correlate with the proportion in the systemic circulation. However, patients admitted to the intensive care unit had a higher proportion of IL17A and IL-22 positive CD4 T-cells in their blood. Taken together, these data seem to suggest that IL-22 can be simultaneously involved in both local and systemic inflammation, but that the systemic inflammatory response and the local response in the lung do not always result in the same levels of IL-22 expression and signalling.

In the present study, we found a dissociation between local IL-22 concentrations and systemic leukocyte counts, systemic lymphocyte counts and the clinically relevant

systemic parameter of inflammation CRP. In addition, the IL-22 concentrations in lavage correlated with local cell differentiation, which seems to suggest that the IL-22 measured in the lavage reflects local processes in the lung rather than simply mirroring systemic inflammation.

The concept of the airways and the lung as a distinct biological compartment with cytokine levels differing from those in the systemic circulation is supported by previous studies: Hollander and colleagues [126] found that the concentrations of IL-8 and of other markers of inflammation were significantly higher in BAL samples compared to serum samples in patients with bronchial asthma and COPD.

Because lung cancer is very often a systemic illness, it is possible that systemic levels of IL-22, and not only local levels in the lung as measured by lavage, may be elevated in this disease. Other investigators have reported elevated serum levels of IL-22 in non-malignant systemic illnesses. For instance, IL-22 was detected in the systemic circulation of 83.5% of patients with Crohn's disease, compared with only 16.1% of healthy controls [127]. Similarly, Paats and colleagues[128] also recently reported that the cytokine profiles in CD4+ and CD8+ T-cells in the systemic circulation correlate with the severity of chronic obstructive lung disease and, in part, also with smoking status. Currently there is a project underway in our department to collect simultaneous BAL and serum samples from patients with lung cancer in order to directly compare the expression of IL-22 in the systemic and pulmonary environments in these patients.

Differential cytokine expression in various biological compartments has been found to be of relevance in other types of cancer. In the setting of ovarian cancer, there is a growing interest in measurements of cytokine levels in ascites fluid as a prognostic marker for disease progression in this biological compartment. Giuntoli and colleagues[129] examined matched ascites and serum samples from thirty-seven patients with ovarian, fallopian tube or primary peritoneal cancer and used a multiplex assay to measure a number of cytokines. They found significant differences in serum and ascites concentrations of many of the cytokines measured. In addition, the authors describe a significant correlation between the CD4/CD8 ratio in ascites and overall survival. Patients with an ascites CD4/CD8 ratio under 1.6 had a median survival of over three years, whereas patients with higher CD4/CD8 ratios (CD4/CD8 over 1.6) in their ascites fluid lived only an average of 1.3 years.

Although these investigators did not directly measure IL-22 concentration in the ascites fluid, their finding that elevated CD4/CD8 ratios also correlate with a poor tumour-related prognosis seems to suggest that similar immunregulatory cell populations may be involved.

4.2.4. Correlation with patient demographics

The current study did not find any significant correlation between IL-22 concentration in the lavage and patient characteristics such as age and gender. Interestingly, there is some recent evidence that levels of serum IL-22 are higher in very elderly individuals. Basile and colleagues[130] compared IL-22 concentrations in serum in healthy centenarians to a group of elderly controls aged sixty to ninety-five years, and found an average serum IL-22 concentration of 45.7pg/ml in the centenarians compared to 11.1 pg/ml in the control population ($p=0.031$). Our study was not designed to detect such differences in age-based subgroups, and included only one patient over eighty years of age.

4.2.5 Future Directions

The diagnostic and therapeutic options available to clinicians treating patients with lung cancer are rapidly expanding. However, with an increasing number of options comes an increasing complexity. The challenge of choosing not only the most appropriate therapy for a particular patient, but also of identifying the most appropriate time to change to another therapeutic approach, will continue to increase. Biomarkers which can guide this clinical decision making will likely be of importance; and IL-22 measurements not only at one point in time, but also over the course of disease and treatment, may prove helpful. However, additional studies to describe the longitudinal course of IL-22 expression during disease and its' correlation with the response or progression of a tumour during therapy are needed before the possible clinical uses of IL-22 measurement can be assessed.

IL-22 is an especially appealing but also potentially complex biomarker in lung cancer because it seems to have a regulatory role in both infections of the lung and in lung cancer itself. In clinical practice these entities often coexist in the same patient, for instance in the case of pneumonia caused by the retention of secretions distal to a malignant stenosis, or pneumonia in an immunosuppressed tumour patient following chemotherapy. In addition, non-infectious pulmonary inflammatory reactions following radiation therapy or chemotherapy are not uncommon, and whether these reactions are associated with changes in IL-22 signalling is not yet known. Although distinguishing these entities from another is often critical to patient assessment,

radiological imaging studies alone are often unable to clearly differentiate tumour from atelectasis or pneumonia. And, because these entities are all associated with increased uptake of glucose, standard FDG-PET is not always helpful. A better understanding of the differential role of IL-22 in infection, inflammation and in tumour may lead to the development of biomarkers or biomarker panels capable of distinguishing and assessing these entities.

The possibility of manipulating IL-22 or its' receptor as a therapy for lung cancer or a means of improving the response to existing therapies may also be of future interest. Our previous collaborative projects have suggested that cisplatin resistant cells respond to IL-22 differently than non-resistant cells[101]. Alterations in the expression or signalling of IL-22 may, therefore, prove to alter the susceptibility of lung cancer cells to specific treatments.

5. CONCLUSION

IL-22 can be measured in pulmonary lavage samples using ELISA, and the concentration of IL-22 in lavage correlates with the presence and type of underlying lung disease. Lavage IL-22 concentrations are highest in patients with pneumonia and in patients with lung cancer, and lowest in patients without bronchoscopic evidence of lung disease. IL-22 concentration in BAL is associated with high BAL lymphocyte counts and with the CD4/CD8 ratio in BAL but not with overall cell counts in BAL. This finding corroborates previous evidence that IL-22 is produced by CD4-positive lymphocytes. IL-22 concentration in lavage was not found to correlate with serum CRP levels or blood leukocyte and lymphocyte counts. This suggests that the IL-22 measured in the lavage is produced locally and is not simply the result of systemic inflammation. Therefore, lavage may represent a distinct compartment for disease processes in the lung, where IL-22 can be studied as a potential lung cancer biomarker.

6. REFERENCES

1. Malvezzi,M., Bertuccio,P., Levi,F., La Vecchia,C. & Negri,E. European cancer mortality predictions for the year 2013. *Ann. Oncol.* **24**, 792-800 (2013).

2. Ferlay,J. *et al.* Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int. J. Cancer* **127**, 2893-2917 (2010).

3. Lozano,R. *et al.* Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* **380**, 2095-2128 (2012).

4. Häußinger,K. & Gesierich,W. MANUAL Tumoren der Lungen und des Mediastinums. Huber,R.M. (ed.), pp. 1-4 (W. Zu8ckschwerdt Verlag, Munich,2011).

5. Piperi,C., Vlastos,F., Farmaki,E., Martinet,N. & Papavassiliou,A.G. Epigenetic effects of lung cancer predisposing factors impact on clinical diagnosis and prognosis. *J. Cell Mol. Med.* **12**, 1495-1501 (2008).

6. Goeckenjan,G. *et al.* Prevention, diagnosis, therapy, and follow-up of lung cancer: interdisciplinary guideline of the German Respiratory Society and the German Cancer Society. *Pneumologie* **65**, 39-59 (2011).

7. Travis,W.D. *et al.* International association for the study of lung cancer/american thoracic society/european respiratory society international multidisciplinary classification of lung adenocarcinoma. *J. Thorac. Oncol.* **6**, 244-285 (2011).

8. Gaughan,E.M. & Costa,D.B. Genotype-driven therapies for non-small cell lung cancer: focus on EGFR, KRAS and ALK gene abnormalities. *Ther. Adv. Med. Oncol.* **3**, 113-125 (2011).

9. Mountain,C.F. The international system for staging lung cancer. *Semin. Surg. Oncol.* **18**, 106-115 (2000).

10. Buccheri,G. & Ferrigno,D. Lung cancer: clinical presentation and specialist referral time. *Eur. Respir. J.* **24**, 898-904 (2004).

11. Salomaa,E.R., Sallinen,S., Hiekkanen,H. & Liippo,K. Delays in the diagnosis and treatment of lung cancer. *Chest* **128**, 2282-2288 (2005).

12. Janssen-Heijnen,M.L. *et al.* Effect of comorbidity on the treatment and prognosis of elderly patients with non-small cell lung cancer. *Thorax* **59**, 602-607 (2004).

13. Sorensen,M., Pijls-Johannesma,M. & Felip,E. Small-cell lung cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann. Oncol.* **21 Suppl 5**, v120-v125 (2010).

14. Crino,L., Weder,W., van Meerbeeck,J. & Felip,E. Early stage and locally advanced (non-metastatic) non-small-cell lung cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann. Oncol.* **21 Suppl 5**, v103-v115 (2010).

15. Nezu,K., Kushibe,K., Tojo,T., Takahama,M. & Kitamura,S. Recovery and limitation of exercise capacity after lung resection for lung cancer. *Chest* **113**, 1511-1516 (1998).

16. Licker,M. *et al.* Perioperative mortality and major cardio-pulmonary complications after lung surgery for non-small cell carcinoma. *Eur. J. Cardiothorac. Surg.* **15**, 314-319 (1999).

17. Damhuis,R.A. & Schutte,P.R. Resection rates and postoperative mortality in 7,899 patients with lung cancer. *Eur. Respir. J.* **9**, 7-10 (1996).

18. Pisters,K.M. & Le Chevalier,T. Adjuvant chemotherapy in completely resected non-small-cell lung cancer. *J. Clin. Oncol.* **23**, 3270-3278 (2005).

19. Pignon,J.P. *et al.* Lung adjuvant cisplatin evaluation: a pooled analysis by the LACE Collaborative Group. *J. Clin. Oncol.* **26**, 3552-3559 (2008).

20. Andre,F. *et al.* Survival of patients with resected N2 non-small-cell lung cancer: evidence for a subclassification and implications. *J. Clin. Oncol.* **18**, 2981-2989 (2000).

21. Auperin,A. *et al.* Meta-analysis of concomitant versus sequential radiochemotherapy in locally advanced non-small-cell lung cancer. *J. Clin. Oncol.* **28**, 2181-2190 (2010).

22. De Ruysscher,D. *et al.* Eligibility for concurrent chemotherapy and radiotherapy of locally advanced lung cancer patients: a prospective, population-based study. *Ann. Oncol.* **20**, 98-102 (2009).

23. Huber,R.M. *et al.* Simultaneous chemoradiotherapy compared with radiotherapy alone after induction chemotherapy in inoperable stage IIIA or IIIB non-small-cell lung cancer: study CTRT99/97 by the Bronchial Carcinoma Therapy Group. *J. Clin. Oncol.* **24**, 4397-4404 (2006).

24. D'Addario,G. *et al.* Metastatic non-small-cell lung cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann. Oncol.* **21 Suppl 5**, v116-v119 (2010).

25. Chemotherapy in addition to supportive care improves survival in advanced non-small-cell lung cancer: a systematic review and meta-analysis of individual patient data from 16 randomized controlled trials. *J. Clin. Oncol.* **26**, 4617-4625 (2008).

26. Schiller,J.H. *et al.* Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. *N. Engl. J. Med.* **346**, 92-98 (2002).

27. Tsuchida,T. *et al.* Imaging the early response to chemotherapy in advanced lung cancer with diffusion-weighted magnetic resonance imaging compared to fluorine-18 fluorodeoxyglucose positron emission tomography and computed tomography. *J. Magn Reson. Imaging* (2012).

28. Yabuuchi,H. *et al.* Non-small cell lung cancer: detection of early response to chemotherapy by using contrast-enhanced dynamic and diffusion-weighted MR imaging. *Radiology* **261**, 598-604 (2011).

29. Dingemans,A.M. *et al.* First-line erlotinib and bevacizumab in patients with locally advanced and/or metastatic non-small-cell lung cancer: a phase II study including molecular imaging. *Ann. Oncol.* **22**, 559-566 (2011).

30. Rosell,R. *et al.* Screening for epidermal growth factor receptor mutations in lung cancer. *N. Engl. J. Med.* **361**, 958-967 (2009).

31. Janne,P.A., Engelman,J.A. & Johnson,B.E. Epidermal growth factor receptor mutations in non-small-cell lung cancer: implications for treatment and tumor biology. *J. Clin. Oncol.* **23**, 3227-3234 (2005).

32. Shepherd,F.A. *et al.* Erlotinib in previously treated non-small-cell lung cancer. *N. Engl. J. Med.* **353**, 123-132 (2005).

33. Fukuoka,M. *et al.* Biomarker analyses and final overall survival results from a phase III, randomized, open-label, first-line study of gefitinib versus carboplatin/paclitaxel in clinically selected patients with advanced non-small-cell lung cancer in Asia (IPASS). *J. Clin. Oncol.* **29**, 2866-2874 (2011).

34. Petrelli,F., Borgonovo,K., Cabiddu,M. & Barni,S. Efficacy of EGFR tyrosine kinase inhibitors in patients with EGFR-mutated non-small-cell lung cancer: a meta-analysis of 13 randomized trials. *Clin. Lung Cancer* **13**, 107-114 (2012).

35. Bang,Y.J. The potential for crizotinib in non-small cell lung cancer: a perspective review. *Ther. Adv. Med. Oncol.* **3**, 279-291 (2011).

36. Zhang,X. *et al.* Fusion of EML4 and ALK is associated with development of lung adenocarcinomas lacking EGFR and KRAS mutations and is correlated with ALK expression. *Mol. Cancer* **9**, 188 (2010).

37. Kwak,E.L. *et al.* Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N. Engl. J. Med.* **363**, 1693-1703 (2010).

38. Katayama,R. *et al.* Mechanisms of acquired crizotinib resistance in ALK-rearranged lung Cancers. *Sci. Transl. Med.* **4**, 120ra17 (2012).

39. Pao,W. *et al.* Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS. Med.* **2**, e73 (2005).

40. Osaki,T. *et al.* Prognostic impact of micrometastatic tumor cells in the lymph nodes and bone marrow of patients with completely resected stage I non-small-cell lung cancer. *J. Clin. Oncol.* **20**, 2930-2936 (2002).

41. Winter H., Hatz R., Hautmann H., Huber,R.M. & Rüttinger D. MANUAL Tumoren der Lunge und des Mediastinums. Huber,R.M. (ed.), pp. 171-179 (W. Zuckschwerdt Verlag, Munich,2011).

42. Murala,S., Alli,V., Kreisel,D., Gelman,A.E. & Krupnick,A.S. Current status of immunotherapy for the treatment of lung cancer. *J. Thorac. Dis.* **2**, 237-244 (2010).

43. Mellstedt,H., Vansteenkiste,J. & Thatcher,N. Vaccines for the treatment of non-small cell lung cancer: investigational approaches and clinical experience. *Lung Cancer* **73** , 11-17 (2011).

44. Butts,C. *et al.* Updated survival analysis in patients with stage IIIB or IV non-small-cell lung cancer receiving BLP25 liposome vaccine (L-BLP25): phase IIB randomized, multicenter, open-label trial. *J. Cancer Res. Clin. Oncol.* **137**, 1337-1342 (2011).

45. www.oxforddictionaries.com/definition/english/biomarker. Oxford Dictionary of English Accessed 25.04.2013. 2013. 25-4-2013.
Ref Type: Electronic Citation

46. Coate,L.E., John,T., Tsao,M.S. & Shepherd,F.A. Molecular predictive and prognostic markers in non-small-cell lung cancer. *Lancet Oncol.* **10**, 1001-1010 (2009).

47. Berghmans,T., Paesmans,M. & Sculier,J.P. Prognostic factors in stage III non-small cell lung cancer: a review of conventional, metabolic and new biological variables. *Ther. Adv. Med. Oncol.* **3**, 127-138 (2011).

48. Eberhard,D.A. *et al.* Mutations in the epidermal growth factor receptor and in KRAS are predictive and prognostic indicators in patients with non-small-cell lung cancer treated with chemotherapy alone and in combination with erlotinib. *J. Clin. Oncol.* **23**, 5900-5909 (2005).

49. Scoccianti,C. *et al.* Prognostic value of TP53, KRAS and EGFR mutations in nonsmall cell lung cancer: the EUELC cohort. *Eur. Respir. J.* **40**, 177-184 (2012).

50. Kim,Y.T. *et al.* The presence of mutations in epidermal growth factor receptor gene is not a prognostic factor for long-term outcome after surgical resection of non-small-cell lung cancer. *J. Thorac. Oncol.* **8**, 171-178 (2013).

51. Scagliotti,G. *et al.* The differential efficacy of pemetrexed according to NSCLC histology: a review of two Phase III studies. *Oncologist.* **14**, 253-263 (2009).

52. Mok,T.S. *et al.* Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. *N. Engl. J. Med.* **361**, 947-957 (2009).

53. Rosell,R. *et al.* Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): a multicentre, open-label, randomised phase 3 trial. *Lancet Oncol.* **13**, 239-246 (2012).

54. Kim D.W., Ahn,M.J. & Shi Y. Results of a global phase II study with crizotinib in advanced ALK-positive non-small cell lung cancer. J clin 30(Suppl). 2012. Ref Type: Abstract

55. Eismann U, Oberschmidt O & Ehnert M. Thymidylate Synthase Gene Expression in Solid Tumors Predicts for Response to Pemetrexed in vitro. *J. Clin. Oncol.* **24**, 13058 (2006).

56. Olaussen,K.A. *et al.* DNA repair by ERCC1 in non-small-cell lung cancer and cisplatin-based adjuvant chemotherapy. *N. Engl. J. Med.* **355**, 983-991 (2006).

57. Friboulet,L. *et al.* ERCC1 isoform expression and DNA repair in non-small-cell lung cancer. *N. Engl. J. Med.* **368**, 1101-1110 (2013).

58. Aberle,D.R. *et al.* Reduced lung-cancer mortality with low-dose computed tomographic screening. *N. Engl. J. Med.* **365**, 395-409 (2011).

59. Goulart,B.H., Bensink,M.E., Mummy,D.G. & Ramsey,S.D. Lung cancer screening with low-dose computed tomography: costs, national expenditures, and cost-effectiveness. *J. Natl. Compr. Canc. Netw.* **10**, 267-275 (2012).

60. Tammemagi,M.C. *et al.* Selection criteria for lung-cancer screening. *N. Engl. J. Med.* **368**, 728-736 (2013).

61. Travis,W.D. *et al.* Diagnosis of lung cancer in small biopsies and cytology: implications of the 2011 International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society classification. *Arch. Pathol. Lab Med.* **137**, 668-684 (2013).

62. Yoon,H.J. *et al.* Repeat biopsy for mutational analysis of non-small cell lung cancers resistant to previous chemotherapy: adequacy and complications. *Radiology* **265**, 939-948 (2012).

63. Sequist,L.V. *et al.* Genotypic and histological evolution of lung cancers acquiring resistance to EGFR inhibitors. *Sci. Transl. Med.* **3**, 75ra26 (2011).

64. Henderson,A.J. Bronchoalveolar lavage. *Arch. Dis. Child* **70**, 167-169 (1994).

65. Pingleton,S.K. *et al.* Effect of location, pH, and temperature of instillate in bronchoalveolar lavage in normal volunteers. *Am. Rev. Respir. Dis.* **128**, 1035-1037 (1983).

66. Meyer,K.C. *et al.* An official American Thoracic Society clinical practice guideline: the clinical utility of bronchoalveolar lavage cellular analysis in interstitial lung disease. *Am. J. Respir. Crit Care Med.* **185**, 1004-1014 (2012).

67. Lee,J.S. *et al.* Bronchoalveolar lavage pepsin in acute exacerbation of idiopathic pulmonary fibrosis. *Eur. Respir. J.* **39**, 352-358 (2012).

68. Dumoutier,L., Louahed,J. & Renauld,J.C. Cloning and characterization of IL-10-related T cell-derived inducible factor (IL-TIF), a novel cytokine structurally related to IL-10 and inducible by IL-9. *J. Immunol.* **164**, 1814-1819 (2000).

69. Dumoutier,L., Van Roost,E., Colau,D. & Renauld,J.C. Human interleukin-10-related T cell-derived inducible factor: molecular cloning and functional characterization as an hepatocyte-stimulating factor. *Proc Natl. Acad. Sci. U. S. A* **97**, 10144-10149 (2000).

70. Xie,M.H. *et al.* Interleukin (IL)-22, a novel human cytokine that signals through the interferon receptor-related proteins CRF2-4 and IL-22R. *J. Biol. Chem.* **275**, 31335-31339 (2000).

71. Kotenko,S.V. *et al.* Identification of the functional interleukin-22 (IL-22) receptor complex: the IL-10R2 chain (IL-10Rbeta) is a common chain of both the IL-10 and IL-22 (IL-10-related T cell-derived inducible factor, IL-TIF) receptor complexes. *J. Biol. Chem.* **276**, 2725-2732 (2001).

72. Aggarwal,S., Xie,M.H., Maruoka,M., Foster,J. & Gurney,A.L. Acinar cells of the pancreas are a target of interleukin-22. *J. Interferon Cytokine Res.* **21**, 1047-1053 (2001).

73. Vivier,E., Spits,H. & Cupedo,T. Interleukin-22-producing innate immune cells: new players in mucosal immunity and tissue repair? *Nat. Rev. Immunol.* **9**, 229-234 (2009).

74. Pene,J. *et al.* Chronically inflamed human tissues are infiltrated by highly differentiated Th17 lymphocytes. *J. Immunol.* **180**, 7423-7430 (2008).

75. Boniface,K. *et al.* IL-22 inhibits epidermal differentiation and induces proinflammatory gene expression and migration of human keratinocytes. *J. Immunol.* **174**, 3695-3702 (2005).

76. Pickert,G. *et al.* STAT3 links IL-22 signaling in intestinal epithelial cells to mucosal wound healing. *J. Exp. Med.* **206**, 1465-1472 (2009).

77. Radaeva,S., Sun,R., Pan,H.N., Hong,F. & Gao,B. Interleukin 22 (IL-22) plays a protective role in T cell-mediated murine hepatitis: IL-22 is a survival factor for hepatocytes via STAT3 activation. *Hepatology* **39**, 1332-1342 (2004).

78. Zenewicz,L.A. *et al.* Interleukin-22 but not interleukin-17 provides protection to hepatocytes during acute liver inflammation. *Immunity.* **27**, 647-659 (2007).

79. Chestovich,P.J. *et al.* Interleukin-22: implications for liver ischemia-reperfusion injury. *Transplantation* **93**, 485-492 (2012).

80. Savan,R. *et al.* A novel role for IL-22R1 as a driver of inflammation. *Blood* **117**, 575-584 (2011).

81. Zheng,Y. *et al.* Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. *Nature* **445**, 648-651 (2007).

82. Tohyama,M. *et al.* IFN-alpha enhances IL-22 receptor expression in keratinocytes: a possible role in the development of psoriasis. *J. Invest Dermatol.* **132**, 1933-1935 (2012).

83. Geboes,L. *et al.* Proinflammatory role of the Th17 cytokine interleukin-22 in collagen-induced arthritis in C57BL/6 mice. *Arthritis Rheum.* **60**, 390-395 (2009).

84. Simonian,P.L. *et al.* gammadelta T cells protect against lung fibrosis via IL-22. *J. Exp. Med.* **207**, 2239-2253 (2010).

85. Whittington,H.A., Armstrong,L., Uppington,K.M. & Millar,A.B. Interleukin-22: a potential immunomodulatory molecule in the lung. *Am. J. Respir. Cell Mol. Biol.* **31**, 220-226 (2004).

86. Aujla,S.J. *et al.* IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. *Nat. Med.* **14**, 275-281 (2008).

87. Hoegl,S. *et al.* Protective properties of inhaled IL-22 in a model of ventilator-induced lung injury. *Am. J. Respir. Cell Mol. Biol.* **44**, 369-376 (2011).

88. Schnyder,B., Lima,C. & Schnyder-Candrian,S. Interleukin-22 is a negative regulator of the allergic response. *Cytokine* **50**, 220-227 (2010).

89. Taube,C. *et al.* IL-22 is produced by innate lymphoid cells and limits inflammation in allergic airway disease. *PLoS. One.* **6**, e21799 (2011).

90. Besnard,A.G. *et al.* Dual Role of IL-22 in allergic airway inflammation and its cross-talk with IL-17A. *Am. J. Respir. Crit Care Med.* **183**, 1153-1163 (2011).

91. Hirose,K., Takahashi,K. & Nakajima,H. Roles of IL-22 in Allergic Airway Inflammation. *J. Allergy (Cairo.)* **2013**, 260518 (2013).

92. Gessner,M.A. *et al.* Dectin-1-dependent interleukin-22 contributes to early innate lung defense against Aspergillus fumigatus. *Infect. Immun.* **80**, 410-417 (2012).

93. De Luca,A. *et al.* IL-22 defines a novel immune pathway of antifungal resistance. *Mucosal. Immunol.* **3**, 361-373 (2010).

94. Zelante,T., Iannitti,R., De Luca,A. & Romani,L. IL-22 in antifungal immunity. *Eur. J. Immunol.* **41**, 270-275 (2011).

95. Zheng,Y. *et al.* Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nat. Med.* **14**, 282-289 (2008).

96. Pociask,D.A. *et al.* IL-22 is essential for lung epithelial repair following influenza infection. *Am. J. Pathol.* **182**, 1286-1296 (2013).

97. Xing,W.W. *et al.* Interleukin-22 protects against acute alcohol-induced hepatotoxicity in mice. *Biosci. Biotechnol. Biochem.* **75**, 1290-1294 (2011).

98. Thompson,C.L., Plummer,S.J., Tucker,T.C., Casey,G. & Li,L. Interleukin-22 genetic polymorphisms and risk of colon cancer. *Cancer Causes Control* **21**, 1165-1170 (2010).

99. Weber,G.F. *et al.* IL-22-mediated tumor growth reduction correlates with inhibition of ERK1/2 and AKT phosphorylation and induction of cell cycle arrest in the G2-M phase. *J. Immunol.* **177**, 8266-8272 (2006).

100. Kobold S., Völk S., Clauditz T. *et al.* Einfluß von Interleukin-22 auf das humane Lungenkarzinom. Jahreskongress der Deutschen Gesellschaft für Hämatologie und Onkologie Poster P466. 2012.
Ref Type: Abstract

101. Kobold S., Völk S., Clauditz T. *et al.* Interleukinß22 is frequently expressed in lung cancer and may contribute to tumor progression in chemotherapy-resistant disease. J.Thorac.Oncol. 2013.
Ref Type: In Press

102. Zhang,W. *et al.* Antiapoptotic activity of autocrine interleukin-22 and therapeutic effects of interleukin-22-small interfering RNA on human lung cancer xenografts. *Clin. Cancer Res.* **14**, 6432-6439 (2008).

103. Kobold S. *et al.* Interleukin-22 wird von Lungenkarzinomzellen exprimiert. Onkologie 34(supp 6). 2011.
Ref Type: Abstract

104. Finkelstein,M.M. Silica, silicosis, and lung cancer: a risk assessment. *Am. J. Ind. Med.* **38**, 8-18 (2000).

105. Saghir Z., Dirksen A. & Rasmussen J.F. In Lung Cancer Screening By CT Incidental Findings Are Frequent and Often Of Clinical Importance. Am.J.Respir.Crit Care Med. 185. 2012.
Ref Type: Abstract

106. Kucharczyk,M.J., Menezes,R.J., McGregor,A., Paul,N.S. & Roberts,H.C. Assessing the impact of incidental findings in a lung cancer screening study by using low-dose computed tomography. *Can. Assoc. Radiol. J.* **62**, 141-145 (2011).

107. Steinberg,K.P. *et al.* Safety of bronchoalveolar lavage in patients with adult respiratory distress syndrome. *Am. Rev. Respir. Dis.* **148**, 556-561 (1993).

108. Ward,C., Thien,F., Secombe,J., Gollant,S. & Walters,E.H. Bronchoalveolar lavage fluid urea as a measure of pulmonary permeability in healthy smokers. *Eur. Respir. J.* **15** , 285-290 (2000).

109. Jones,K.P., Edwards,J.H., Reynolds,S.P., Peters,T.J. & Davies,B.H. A comparison of albumin and urea as reference markers in bronchoalveolar lavage fluid from patients with interstitial lung disease. *Eur. Respir. J.* **3**, 152-156 (1990).

110. Rennard,S.I. *et al.* Estimation of volume of epithelial lining fluid recovered by lavage using urea as marker of dilution. *J. Appl. Physiol* **60**, 532-538 (1986).

111. Baughman,R.P., Bosken,C.H., Loudon,R.G., Hurtubise,P. & Wesseler,T. Quantitation of bronchoalveolar lavage with methylene blue. *Am. Rev. Respir. Dis.* **128**, 266-270 (1983).

112. Restrick,L.J., Sampson,A.P., Piper,P.J. & Costello,J.F. Inulin as a marker of dilution of bronchoalveolar lavage in asthmatic and normal subjects. *Am. J. Respir. Crit Care Med.* **151**, 1211-1217 (1995).

113. Weber,J.A. *et al.* The microRNA spectrum in 12 body fluids. *Clin. Chem.* **56**, 1733-1741 (2010).

114. Molina-Pinelo,S. *et al.* Association between the miRNA signatures in plasma and bronchoalveolar fluid in respiratory pathologies. *Dis. Markers* **32** , 221-230 (2012).

115. Pennino,D. *et al.* IL-22 suppresses IFN-gamma-mediated lung inflammation in asthmatic patients. *J. Allergy Clin. Immunol.* **131**, 562-570 (2013).

116. Slutsky A.S. Lung Injury Caused by Mechanical Ventilation. Chest 116(Suppl 1). 1999.
Ref Type: Abstract

117. Lilly,L.M. *et al.* The beta-glucan receptor dectin-1 promotes lung immunopathology during fungal allergy via IL-22. *J. Immunol.* **189**, 3653-3660 (2012).

118. Yao,S. *et al.* Differentiation, distribution and gammadelta T cell-driven regulation of IL-22-producing T cells in tuberculosis. *PLoS. Pathog.* **6**, e1000789 (2010).

119. Dowlati,A., Loo,M., Bury,T., Fillet,G. & Beguin,Y. Soluble and cell-associated transferrin receptor in lung cancer. *Br. J. Cancer* **75**, 1802-1806 (1997).

120. Pastor,M.D. *et al.* Identification of oxidative stress related proteins as biomarkers for lung cancer and chronic obstructive pulmonary disease in bronchoalveolar lavage. *Int. J. Mol. Sci.* **14**, 3440-3455 (2013).

121. Topaloglu,O. *et al.* Detection of promoter hypermethylation of multiple genes in the tumor and bronchoalveolar lavage of patients with lung cancer. *Clin. Cancer Res.* **10**, 2284-2288 (2004).

122. Wolk,K., Witte,E., Witte,K., Warszawska,K. & Sabat,R. Biology of interleukin-22. *Semin. Immunopathol.* **32**, 17-31 (2010).

123. Scriba,T.J. *et al.* Distinct, specific IL-17- and IL-22-producing CD4+ T cell subsets contribute to the human anti-mycobacterial immune response. *J. Immunol.* **180**, 1962-1970 (2008).

124. Ahrendt,S.A. *et al.* Molecular detection of tumor cells in bronchoalveolar lavage fluid from patients with early stage lung cancer. *J. Natl. Cancer Inst.* **91**, 332-339 (1999).

125. Paats,M.S. *et al.* T helper 17 cells are involved in the local and systemic inflammatory response in community-acquired pneumonia. *Thorax* **68**, 468-474 (2013).

126. Hollander,C., Sitkauskiene,B., Sakalauskas,R., Westin,U. & Janciauskiene,S.M. Serum and bronchial lavage fluid concentrations of IL-8, SLPI, sCD14 and sICAM-1 in patients with COPD and asthma. *Respir. Med.* **101**, 1947-1953 (2007).

127. Schmechel,S. *et al.* Linking genetic susceptibility to Crohn's disease with Th17 cell function: IL-22 serum levels are increased in Crohn's disease and correlate with disease activity and IL23R genotype status. *Inflamm. Bowel. Dis.* **14**, 204-212 (2008).

128. Paats,M.S., Bergen,I.M., Hoogsteden,H.C., van der Eerden,M.M. & Hendriks,R.W. Systemic CD4+ and CD8+ T-cell cytokine profiles correlate with GOLD stage in stable COPD. *Eur. Respir. J.* **40**, 330-337 (2012).

129. Giuntoli,R.L. *et al.* Ovarian cancer-associated ascites demonstrates altered immune environment: implications for antitumor immunity. *Anticancer Res.* **29**, 2875-2884 (2009).

130. Basile,G. *et al.* Healthy centenarians show high levels of circulating interleukin-22 (IL-22). *Arch. Gerontol. Geriatr.* **54**, 459-461 (2012).

7. Summary

Background and Aims:

Lung cancer is not only common, but also difficult to diagnose and to treat. Lung cancer screening using CT may help identify tumours in a curable stage, but screening also finds a large number of benign changes which are often difficult to distinguish from cancer. Patients treated for lung cancer, either with local or multimodal therapies in early stage disease or with systemic therapy in advanced disease, often go on to progress or relapse despite an initial response to treatment. Biomarkers are needed to identify patients at a high risk of lung cancer, to help select the most appropriate treatment for an individual patient, and to follow patients during the course of the disease. Ideally, these biomarkers should be measurable in non-invasive or minimally invasive samples, such as bronchoalveolar lavage (BAL) and bronchial washings, to avoid the need for repeated invasive, potentially harmful, procedures.

Interleukin-22 (IL-22) may prove to be an informative biomarker in lung cancer. IL-22 is expressed by T-cell subpopulations in a number of inflammatory and infectious disease states, including many pulmonary diseases. IL-22 and its' receptor have been identified in lung cancer cell lines and human lung cancer biopsies. In addition, IL-22 signalling appears to promote the growth, and perhaps also chemotherapy resistance, of lung cancer in vitro.

The aim of this dissertation is to investigate the measurement of IL-22 in BAL and bronchial washing in patients with lung cancer compared with both patients with non-malignant pulmonary illnesses and a reference cohort. By correlating IL-22 with differential cell counts in the BAL as well as with systemic cell counts and markers of inflammation, we hope to assess the suitability of IL-22 in lavage as a biomarker for the local pulmonary environment in lung cancer.

Methods:

Lavage samples were collected from patients undergoing routine flexible bronchoscopy with BAL or bronchial washings at the Respiratory Medicine and Thoracic Oncology Section of the Internal Medicine Department V (Medizinische Klinik V, Sektion Pneumologie Innenstadt und Thorakale Onkologie), Ludwig

Maximilians University of Munich, Germany. Cell counts and differential cell counts were determined in BAL samples, and IL-22 was measured using ELISA. Systemic C reactive protein (CRP) levels and differential blood counts as well as patient demographic and medical information were collected from the electronic patient record.

Results:

Lavage samples from 56 patients were analysed. These included samples from 36 men and 20 women, average age 58.3 years, with lung cancer (25%), infection (29%), other thoracic malignancies (13%), other non-malignant pulmonary illnesses (14%) and no bronchoscopic evidence of disease (16%). We found IL-22 in 53% of the 56 patient samples. IL-22 was higher in patients with lung disease than in controls, and patients with pneumonia and those with lung cancer had the highest concentrations of IL-22 in lavage. IL-22 concentrations were higher in patients with elevated BAL lymphocytes and with high BAL CD4/CD8 lymphocyte ratios. Blood leukocyte and lymphocyte counts and serum CRP levels did not correlate with lavage IL-22 concentrations.

Conclusion:

Lavage IL-22 concentrations are high in patients with lung cancer and in patients with pneumonia. IL-22 in lavage is associated with lymphocyte counts and CD4/CD8 ratio in BAL, as would be expected for a cytokine produced by CD4-positive lymphocytes. IL-22 in lavage was not associated with the systemic markers of inflammation tested. These results suggest that IL-22 in lavage represents local IL-22 expression in the lung. The measurement of IL-22 in lavage warrants further study as a biomarker in lung cancer.

8. Zusammenfassung

Hintergrund und Ziele:

Das Lungenkarzinom ist eine häufige Krankheit und stellt bei der Diagnosestellung sowie bei der Therapie eine besondere Herausforderung dar. Obwohl es durch CT-Screening möglich ist, Tumoren in einem frühen und auch heilbaren Stadium zu identifizieren, werden durch Screening-Untersuchungen auch eine hohe Anzahl an gutartige Veränderungen identifiziert, die zum Teil eine invasiven Abklärung bedürfen. In frühen Stadien steht meistens eine lokale oder multimodale Therapie im Vordergrund. Bei fortgeschrittenen Tumoren wird eine systemische Therapie verabreicht. Bei vielen Patienten kommt es nach initialer Therapie zu einem Rezidiv oder zur Progression der Erkrankung. Der Kliniker braucht prognostische und prädiktive Biomarker um Patienten mit einem hohen Lungenkrebsrisiko zu identifizieren und um die optimale Therapie für den individuellen Patienten auszusuchen. Dies gilt auch für die Erkennung des Rezidivs während der Nachsorge. Um die möglichen Komplikationen eines invasiven Eingriffs zu minimieren, sollte der ideale Biomarker auch in nicht- oder wenig-invasiven Proben, z.B. der bronchoalveolären Lavage, messbar sein.
Interleukin-22 (IL-22) könnte sich als Lungenkrebsbiomarker etablieren. IL-22 wird von Untergruppen von T-Zellen bei unterschiedlichen infektiösen sowie inflammatorischen Krankheiten, unter anderem auch Lungenkrankheiten, exprimiert. IL-22 und der IL-22-Rezeptor wurden auch in Lungenkrebszelllinien sowie in Biopsiematerial von Lungenkrebspatienten identifiziert. IL-22 scheint das Wachstum und eventuell auch die Chemotherapieresistenz von Lungenkrebszellen in vitro zu unterstützen
Ziel dieser Arbeit war es, IL-22 in Lavageproben von Patienten mit Lungenkrebs zu messen, und mit IL-22-Messungen von Patienten mit gutartigen Lungenkrankheiten und Kontrollen zu vergleichen. Wir korrelierten IL-22 mit der Zellzahl und Differenzierung in der BAL sowie mit systemischen Entzündungsparametern wie erhöhten Leukozytenzahlen und dem Differentialblutbild sowie dem CRP, um IL-22 in der Lavage als Biomarker für die Lokalverhältnisse in der Lunge beim Lungenkarzinom zu untersuchen.

Methoden:
Lavageproben wurden bei Patienten, die sich zur flexiblen Bronchoskopie in der Medizinischen Klinik V, Sektion Pneumologie Innenstadt und Thorakale Onkologie vorstellten, gesammelt. Zellzahl und Zelldifferenzierung wurden bestimmt und IL-22 wurde mittels ELISA gemessen. Die Parameter im peripheren Blut (CRP, Leukozytenzahl und Lymphozytenzahl) sowie Informationen zum Patienten und die Diagnose wurden aus dem klinischen Informationssystem erfasst.

Ergebnisse:
Lavageproben von 56 Patienten wurden analysiert, darunter 36 Männer und 20 Frauen. Das Durchschnittsalter lag bei 58,3 Jahren. Die Diagnosen waren: Lungenkarzinom (25%), Pneumonie (29%), andere thorakale Tumoren (13%), andere Lungenkrankheiten (14%) und Kontrollen ohne bronchoskopische Hinweise auf Lungenpathologie (16%). IL-22 wurde in 53% der Patientenproben detektiert. Die IL-22-Konzentration in der Lavage war in Vergleich mit der Kontrollgruppe bei Patienten mit Lungenkrankheiten höher, und besonders hoch bei Patienten mit Lungenkrebs und Patienten mit Pneumonie. Die IL-22-Konzentration in der BAL korrelierte mit erhöhten Lymphozyten und einem hohen CD4/CD8 Quotienten in der BAL. Die systemischen Entzündungsparameter inklusive Leukozytenzahl, Lymphozytenzahl und CRP korrelierten mit der IL-22-Konzentration in der Lavage nicht.

Schlussfolgerung:
Die IL-22-Konzentration ist in der Lavage von Patienten mit Lungenkrebs sowie von Patienten mit Pneumonie erhöht. IL-22 in der Lavage ist mit einer hohen Lymphozytenzahl sowie einem hohen CD4/CD8-Quotienten in der BAL assoziiert. Dieses Ergebnis unterstützt die Vermutung, dass IL-22 hauptsächlich von CD4-positiven Lymphozyten produziert wird. Die IL-22 Konzentration in der Lavage zeigte keine Korrelation mit den gemessenen systemischen Entzündungsparametern. Zusammenfassend deuten diese Ergebnisse darauf hin, dass IL-22 in Lavage mit der lokalen IL-22 Expression in der Lunge korreliert. IL-22 in der Lavage könnte ein wertvoller Lungenkrebsbiomarker werden.

9. APPENDIX

9.1. List of tables and figures

TABLES

FIGURES

9.2. Acknowledgements

Thank you to Prof. Dr. med. Rudolf Maria Huber for many cups of tea and many constructive discussions, as well as for his continuing encouragement and for the supervision of this dissertation; to Dr. med. Fernando Gamarra for his assistance in collecting the lavage samples; to Rosemarie Kiefl for her assistance in processing and storing the lavage samples; and to Prof. Dr. med. Stefan Endres, Dr. Sebastian Kobold, Stefanie Völk and the Clinical Pharmacology group at the LMU for their collaboration and support.

9.3. Curriculum Vitae