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„Establishment of Serum ELISA Tests

against Putative Lung Adenocarcinoma Antigens“

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1. Abstract

This project is based on preliminary work by an interdisciplinary team of scientists. It was possible to identify tumor-specific proteins of lung adenocarcinoma by means of chirurgical gained material through proteome-analysis.

After *PubMed* (http://www.ncbi.nlm.nih.gov/sites/entrez) research, the clinical findings had been limited to eight useful candidates of proteins that seem to be potential tumor markers of cancer. The biochemical selection criteria of these proteins were membrane expression and extracellular secretion. Under these criteria the chance is high to find the proteins in human serum. There were five commercially antibodies available against the antigens of our interest.

The aim of this diploma thesis was to test whether the antigens as specific serum markers are detectable in the patients' blood samples. We tried to develop a fine-tuned ELISA for these proteins and to test the ELISA for its clinical suitability. 40 serum samples were collected and analyzed via D-ELISA and S-ELISA. Patients suffering from lung adenocarcinoma and a healthy control group were compared.

This successfully established ELISA system would provide a valuable clinical tool for lung cancer diagnosis. It will be possible to measure the protein concentration within two days after blood taking from the patient. That could become a basic standard procedure for early diagnosis of lung tumors, in which curative resection is possible.

2. Zusammenfassung

Dieses Projekt baut auf Vorarbeiten eines interdisziplinären Teams von Wissenschaftern auf. Aus chirurgisch gewonnenem Material wurden mittels Proteom-Analyse tumor-spezifische Proteine aus Lungenadenokarzinomen identifiziert.

Nach eingehender Literaturrecherche in *PubMed* (http://www.ncbi.nlm.nih.gov/sites/entrez), wurden nach einer ersten Auswahl acht interessante Proteinkandidaten gefunden, die potentielle Tumormarker zu sein scheinen.

Die Auswahl erfolgte nach den biochemischen Kriterien der Membranständigkeit und Sezernierbarkeit dieser Proteine, sodaß die Wahrscheinlichkeit sehr hoch ist, diese auch im humanen Serum zu finden. Gegen fünf dieser Antigene gibt es kommerzielle Antikörper.

Zielsetzung dieser Diplomarbeit war es zu testen, ob die Antigene als spezifische Serummarker im Blut von Patienten zu finden sind. Wir planten einen fein abgestimmten ELISA für jene Proteine zu entwickeln, sowie diesen ELISA für seine klinische Tauglichkeit zu prüfen. 40 Blutproben wurden gesammelt und mittels D-ELISA und S-ELISA analysiert. Patienten, die unter dem Adenokarzinom litten, wurden mit der gesunden Kontrollgruppe verglichen.

Der erfolgreich etablierte ELISA könnte die Grundlage eines Standardtests zur Diagnose von frühen Tumoren oder die Monitorisierung von bereits diagnostizierten Lungentumoren durch eine einfache Blutabnahme sein. Diese Methode könnte ein Standardprozedere für frühzeitige Diagnose des Lungenkrebses sein, in welcher eine kurative Resektion ermöglicht wird.

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“…Daß ich erkenne, was die Welt

Im Innersten zusammenhält,…“

(Faust I – J.W.Goethe)

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3. Scientific Background

3.1 Introduction

For each patient, the diagnosis “lung cancer” is a very hard situation to cope with. This type of cancer represents an enormous problem in public health. The five-year survival rate has not turned better during the last 20 years. Fact is that during therapeutically indicated surgical resections of lung carcinomas, a high amount of interesting lung cancer tissue accumulates. This material gives the chance to identify new risk, respectively cancer markers, by using the incoming tissue material for recognizing novel cancer cell specifics. Via proteome-analysis, this lung material was used to screen for tumor-specific proteins in adenocarcinomas. Here, we aimed to prove whether these proteins are clinically capable in serum-test and, if yes, to develop a serum-test for these proteins.

3.2 Lung cancer

3.2.1 Lung cancer occurrence, development and classification

Lung cancer is a common malignant disease, which is the leading cause of cancer deaths in the world [1]. Typically, bronchogenic cancers exist without clinical symptoms over many years and become clinically evident at a very late stage of the disease. This is the reason, why about two thirds of all cases of bronchogenic cancer are diagnosed at an incurable stage. Only one third of patients with this malignoma is seen by the surgeon and unfortunately just another third of these has got a realistic chance of getting cured. Therefore, the overall five-year survival of patients suffering from lung cancer is as poor as 15 % [2]. The lack of progress in this area reflects the limited knowledge available concerning the factors that promote oncogenic transformation and proliferation of carcinoma cells in the lung [3]. It is a disappointing fact that almost no advance in this field could be achieved over the past 30 years. Besides a few external factors, the main cause of lung cancer is surely the consumption of cigarettes. Almost 90% of lung cancer is caused by cigarette smoking. Although nicotine is one of the major ingredients of cigarette smoke and the causative agent for addiction, it is not a carcinogen by itself. Nonetheless, several investigators have shown that nicotine can induce

cell proliferation and angiogenesis. To mention the genetic analyses of Puliyappadamba et al., showed that the proliferative index of nicotine is different in the lung cancer cell lines H1299 (p53-/-) and A549 (p53+/+), which indicates that the mode of up-regulation of survival signals by nicotine might be different in cells in the presence or absence of p53 [4]. It is now generally agreed that lung carcinogenesis is a multi-step process and that molecular alterations precede morphological, cytological and histological changes. On an epigenetic level, for instance it has been shown that the gene RASSF1A (3p21.3) is frequently inactivated by aberrant methylation in lung tumors [5]. Aberrant methylation of certain genes commences early during the multistage development of lung cancer. Further, the methylation of p16, this is already found in bronchial epithelium with mildly abnormal changes (hyperplasia/squamous metaplasia), has been detected [6] [7].

Lung carcinomas can be divided into two major histopathologic groups:

1.) **Non-small cell lung cancers (NSCLC)**
2.) **Small cell lung cancers (SCLC)**

Non-small cell lung cancers can be subdivided predominantly into adenocarcinomas and squamous cell carcinomas, and both account for 85% of lung cancer cases [8]. The rest are large cell carcinomas and other rare tumor types. The tumor size, lymph node status and possible presence of metastases determine the actual clinical stage of the disease, and is described in the **TNM** (**T**=primary tumor, **N**=regional lymph nodes, **M**=distant metastasis) staging system for lung cancer [9]. This tumor staging system is explained more detailed in the *6.1.1 Results* section, where we list our patient cohort.

In this study, we turned our attention to adenocarcinomas, because this type has replaced squamous cell carcinomas as the most frequent histological subtype of lung cancer [10]. The WHO defines adenocarcinomas as "a malignant epithelial tumor with tubular, acinar, or papillary growth pattern, and/or mucus production by the tumor cell." **Fig. 1** shows a cross-section of healthy human respiratory epithelium., showing regular cellular organization of the goblet cells, which are growing on a layer of basal cells that form the basal membrane by excreting extracellular matrix components. On the other hand, in **Fig. 2** we see the disturbed morphology of a lung adenocarcinoma, where the goblet cells have undergone malignant transformation and high amounts of tumor stroma have developed.

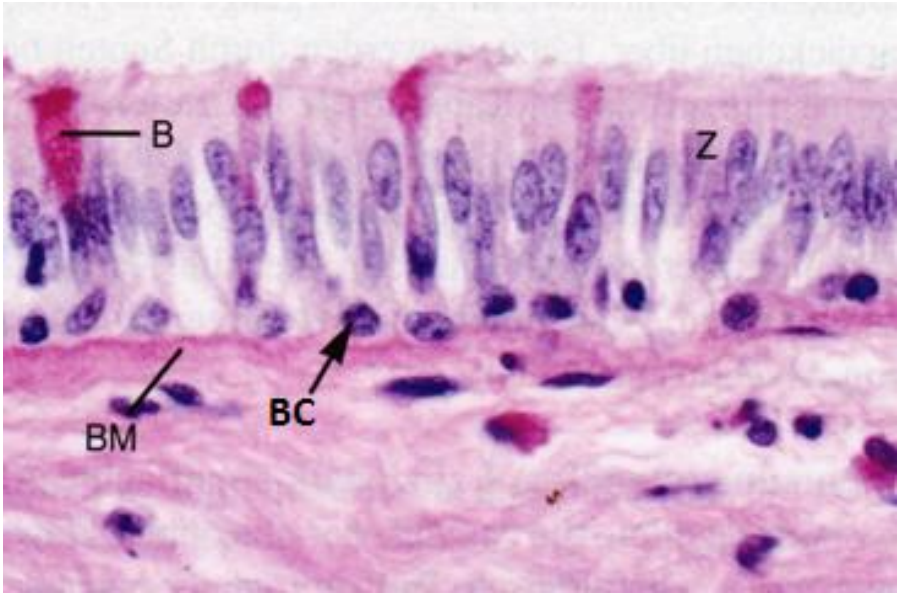

Fig. 1: Visualization of respiratory epithelium of a normal lung with PAS-staining [11]

Z: Respiratory epithelium with cilia containing cylinder epithelial cells

B: Caliciform cells

BM: Basal membrane

BC: Basal cells

3.2.2 Lung Adenocarcinoma

The WHO-classification differentiates between papillary, solid and mucous forming adenocarcinomas [12]. Primary lung-adenocarcinomas are mostly peripherally localized near the pleura [11]. The bronchoalveolar carcinoma, or malign lung-adenomatosis, represents a special form of carcinoma. Lung adenocarcinomas are mostly recognized in an advanced stage of medical condition, because of their occurrence without symptoms over a long time.

Fig. 2 shows the histopathological exposure of the typical adenous and papillary structure of a lung adenocarcinoma.

Characteristics for this cancer type are expanded central fibrotic lesions. Problematic is the development of primary pulmonary adenocarcinomas to lung metastasis in many ways. Immunohistochemical examinations allow a distinction compared to metastases of thyroid-, prostate-, or hepatic carcinomas as well as other primary tumors [12].

The SEER population database contains information on the median survival times of patients with distant non–small-cell lung cancer. The database was also inspecting potential changes in the survival of all patients with NSCLC.

According to Breathnach et al. the median survival time for all patients with NSCLC in the SEER database showed an increase from 9.1 months in the 1973-to-1974 time period to only 10 months in the 1993-to-1994 time period [13], indicating that an earlier detection time of the tumors would be essential to increase life span.

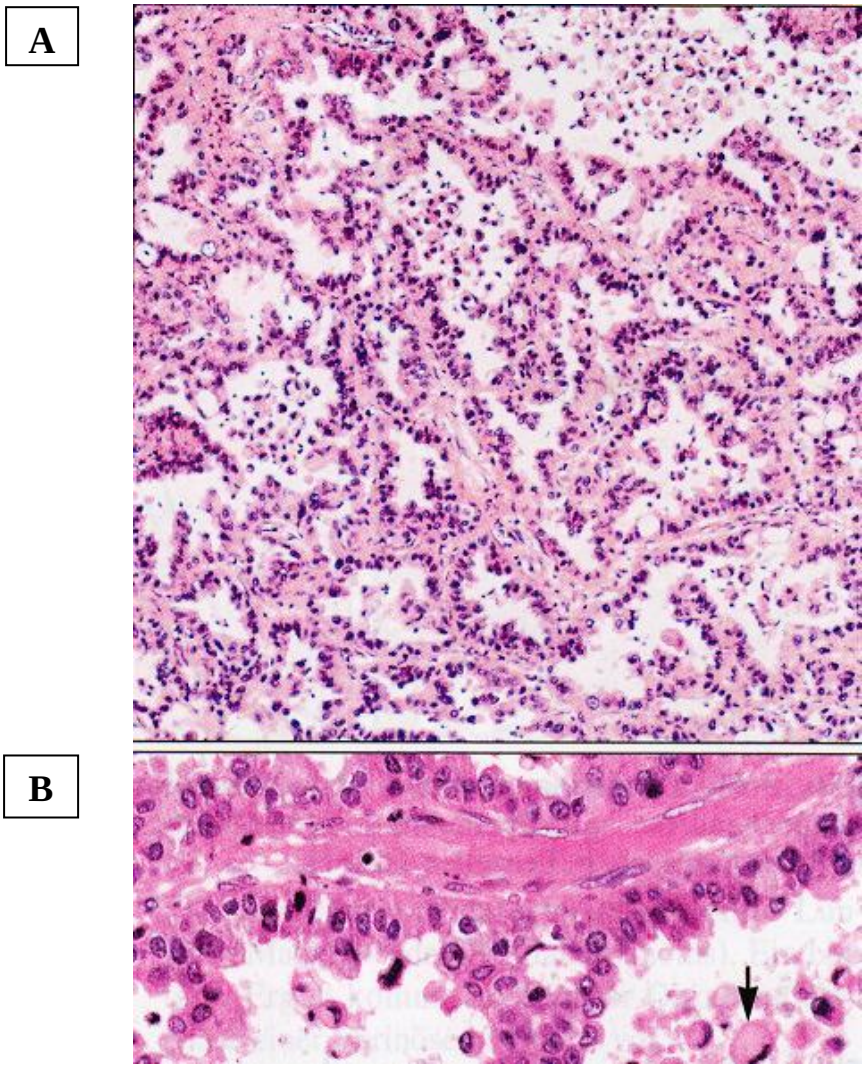

Fig. 2: Histological section and HE-staining of lung-adenocarcinoma tissue [11]

Picture **A** shows a mucous forming lung carcinoma with high amounts of tumor stroma (*purple* staining). The dark dots represent cell nuclei, which occur when abnormal cell division takes place.

Picture **B** shows a tumor papilla, which is a typical feature of adenocarcinomas. The arrow represents a signet-ring cell.

3.2.3 Diagnosis and surgical treatment of lung carcinoma

Mostly, the detection of lung cancer occurs accidentally. In the early stage, lung cancer grows without any symptoms. Later, when the tumor invades and touches the chest wall or upper parts like the plexus of the upper extremities pain can follow. Therefore, if shoulder pain is resistant to pain management, a simple chest X-ray is mandatory. Haemoptysis can also occur, when the tumor spreads out into the bronchial system. General symptoms as weight loss in short time period, fatigueness and actually reduction of life quality can come along with tumor growth.

Primary histological verification is usually achieved by chest X-ray and CT (*Computer-Tomography*), and if a lesion is detected, a bronchoscopy should be done for further histological analysis. Histological verification is usually achieved endoscopically in centrally located tumors and by CT-guided needle biopsy in peripheral lesions. Modern spiral CT enables a very reliable judgment concerning malignant infiltration of surrounding anatomical structures and enlarged mediastinal lymph node stations. In selected cases, a PET (*Positron Emission Tomography*) scan of the chest is helpful. Different therapeutical options for treatment are available, depending on the stage of the cancer. In early stage surgical resection is obligatory. If the tumor invasion exceeds the organ's margin, a combined therapy with surgical resection, chemo- and radiation therapy is necessary. If surgical resection for anatomical reasons is not possible, a chemo- and/or radiation therapy should be done. The best survival rate is succeeded with surgical resection, but also conservative management can account for an extension of life with good quality.

3.2.4 Clinical screening of cancer patients

The earlier lung cancer is detected, the better are a patient's chances to be cured. Thus, new methods are needed to detect lung cancer at an earlier stage when it is amenable to curative treatment, in contrast to its poor overall prognosis when clinical tissue alterations have developed. All tumor screening studies have demonstrated a significant shift towards earlier diagnosis and distinctly increased rates of resectability. However, no screening strategy has been unequivocally demonstrated to reduce lung cancer mortality [14].

In recent years, considerable effort has led to the discovery of a number of relevant protooncogenes, DNA repair genes and suppressor genes which are aberrantly expressed in lung cancer. The pressing issue is to establish the correlation between molecular genetic

events and cyto-histological findings, which remain the gold standard of cancer diagnosis, and to identify novel biomarkers of cancer. Candidate biomarkers should have high sensitivity as well as specificity and appear early enough in the course of disease for medical intervention and to improve prognosis. Finally, the markers must be present in a biological fluid that can be obtained noninvasively, making its collection achievable for population-based screening [15]. Such molecular markers must be capable to identify individuals at the earliest stage of lung cancer, in which curative resection is possible.

Nevertheless it is often quite difficult to identify proteins secreted from epithelial cells because the proteins in bodily fluids, such as bronchial lavage fluid and bronchial secretions or in cancer tissue specimens include also a lot of proteins derived from circulating blood and mixed cell types, as well as stromal and inflammatory cells [16].

3.2.5 The interest in bio-markers

According to Hanash et al., the expected outcome of the development of panels of biomarkers, allows firstly early detection of cancer and secondly prediction of the probable response to therapy. The general goal of the cancer biomarker field is to develop simple non-invasive tests. These tests indicate cancer risk, allow early cancer detection and classify tumors. This basic approach is useful for the patient to receive the most appropriate therapy and monitor disease progression, regression and recurrence [17].

By detecting cancer at an early stage, it would become possible to harness the immune response directed against tumor antigens. It has been demonstrated that a number of intracellular and cell-surface antigens are detectable in sera from patients with different cancer types. The identification of a panel of antigenic biomarkers that are tumor-specific could be an effective strategy for cancer screening.

The immune response for identifying cancer biomarkers is remarkable, because the immune system carries out a biological amplification (= *antibodies*) that generates a detectable response to antigenic tumor proteins. Many strategies have been pursued for determining the repertoire of antibodies in the sera of patients with cancer. The most relied-on approach for validation remains the **sandwich enzyme-linked immunosorbent assay (S-ELISA)**, which is also the outcome for the experiments of this diploma thesis.

Because of its highly specificity of using a pair of antibodies against the targeted molecule, it seems to be a very attractive strategy. Anyway, the use of antibodies directed against a serum

containing lung tumor proteins of interest would have utility for elucidating those proteins with the greatest diagnostic advantage [18].
In compliance with Hanash et al. it is quite unrealistic to expect that a single biomarker will reflect all information about tissue type and malignant transformation throughout the various stages of tumor development and progression. After all, reliable panels of biomarkers are urgently needed, whose identification will be true a challenge [17].

3.3 Proteomic characterization of lung cancer tissue

It was attempted to identify tissue-specific differences between healthy and lung tumor tissue. The collaborating pathologists transferred fresh surgically removed tumor and lung tissues from the clinics to a biochemist laboratory, where proteome analysis achievement was performed. More specifically, protein samples from 5 tumors and 5 control tissues were digested, protein was extracted and subjected to 2D gel analysis and a comparative spot analysis was performed. Candidate protein markers for tumor specificity (i.e. differently expressed proteins) were identified by mass spectroscopy and compared with a tissue proteome databank available at the biochemists´ laboratory. Additionally, the worldwide "*Swiss-Prot*" database (http://expasy.org/) was screened with the protein candidates. This work package reveals functions of protein markers and contributed valuable information about their plausibility as specific tumor antigens. The criteria for picking potential tumor marker candidates were (1) lung tissue specificity and/or (2) tumor specificity.

3.4 Tumor Antigens

The analysis of five tumors and corresponding controls, as well as the comparison with proteome databanks revealed the following eight proteins with the according information as **promising candidates** for serum analysis:

- **Cytoglobin** (***CYGB***) (Stellate cell activation-associated protein) - Homo sapiens (Human), expressed in endothelial, stellate, melanoma cells and fibroblasts, known to be overexpressed in tumors; may have a protective function during conditions of oxidative stress, may be involved in intracellular oxygen storage or transfer.
- **Gametogenetin** (***GGN***) - Homo sapiens (Human), few information.

- **Progastricsin** (***PGC***) - Homo sapiens (Human), secreted enzyme.
- **Interleukin-18** precursor (***IL-18***) (Interferon-gamma-inducing factor) (IFN-gamma-inducing factor) (Interleukin-1 gamma) (IL-1 gamma) (Iboctadekin) - Homo sapiens (Human); not tumorspecific, however a promising marker for inflammatory reaction.
- **Mesothelin** precursor (***MSLN***) (Pre-pro-megakaryocyte potentiating factor) (CAK1 antigen) [Contains: Megakaryocyte-potentiating factor (MPF); Mesothelin, cleaved form] - Homo sapiens (Human), known to be quite tumor specific.
- **Mucin-5B** precursor (***MUC5B***) (Mucin-5 subtype B, tracheobronchial) (High molecular weight salivary mucin MG1) (Sublingual gland mucin) - Homo sapiens (Human), typically not tumor-specific, but unexpected in lung tissue.
- **Neudesin** precursor (***NENF***) (Neuron-derived neurotrophic factor) (Secreted protein of unknown function) (SPUF protein) - Homo sapiens (Human).
- **Proapoptotic caspase adapter protein** (*PACAP)*, Homo sapiens.

After the definition of tissue-specific differences, it was the aim to explore whether or not these eight candidate proteins were (1) assessable and (2) quantifiable in the sera of lung tumor patients.

Therefore, the availability of antibodies against the suggested potential tumor markers was examined by an internet search via the "*biocompare*" platform (http://www.biocompare.com/), a product directory linked with a search engine. The retrieved list of antibodies commercially available was checked regarding antibody specificity, performance in ELISA, and pricing. Concerning antigens lacking availability of commercial antibodies, academia will be searched through via the "*PubMed*" publication browser (http://www.ncbi.nlm.nih.gov/sites/entrez). We identified five commercially antibodies. To start the development and evaluation of serum tests, we decided to work with the four bold marked proteins:

Pos:	Protein	Antibodies from Producer
1	**Cytoglobin**	Antibodies by *Santa Cruz*
2	Gametogenetin	no commercially available antibody found
3	Gastricsin	no commercially available antibody found
4	**Interleukin 18**	Antibodies by *Acris, Gene Tex, GenWay*
5	**Mesothelin**	Antibodies by *Abbiotec, LifeSpan, Novus, Rockland*
6	**Mucin 5B**	Antibodies by *LifeSpan, Millipore*
7	Neudesin	no commercially available antibody found
8	PACAP	Antibodies by *Abbiotec, Abcam, Acris, Invitrogen, Proteintech, Novus Biologicals*

Table 1: Table showing commercially available antibodies against tumor antigens

The bold marked proteins in the table represent the tumor markers, which were aimed to be tested in the ELISA experiments.

In the following chapters, the four tumor antigens, which were the basis of the immunological experiments of this thesis, are shortly presented. The tables for each antigen show the accurate commercially acquired antibodies for the antigens and can be seen in *Materials and Methods*.

3.4.1 Cytoglobin (CYGB)

CYGB is part of the globins, which are a family of heme-containing proteins, which reversibly bind oxygen. They have been described in animals, plants, protists, fungi and bacteria [19] [20].

The following four mammalian globins have been identified already [21] [22] [23] [19]:

- Hemoglobin
- Myoglobin
- Neuroglobin
- Cytoglobin

CYGB is a recently discovered vertebrate globin and is distantly related to myoglobin. Unfortunately, Neuroglobin and Cytoglobin have functions that are not completely

understood [21] [23]. CYGB is assigned to the chromosomal region 17q25, that is frequently lost in multiple malignancies.

There exist recent studies, which provide preliminary evidence for increased methylation of the gene in lung cancer. Shivapurkar et al. investigated the role of CYGB as a tumor suppressor gene. They detected the tumor specificity of CYGB methylation in patients with and without lung cancer. Furthermore, they could show in their assays the tumor specificity of CYGB gene methylation [24] in multiple other epithelial and hematologic malignancies.

3.4.2 Interleukin 18 (IL-18)

IL-18 is known as a pro-inflammatory cytokine, essential for the host defense against intracellular infection [25]. It is produced by activated macrophages and dendritic cells, showing potent interferon IFN-γ inducing activity [26] [27] [28]. It's biological role is the increase of the activity of cytotoxic T-lymphocytes and natural killer cells [26] [29].

IL-18 also induces gene expression and synthesis of TNF, IL-1 and a number of chemokines [25]. Shi et al. demonstrated that IL-18 promotes autoreactive Th1-cell development in part via induction of IFN-γ by NK cells [27]. IL-18 shows similarities with proteins of the IL-1 family [25] in structure and function and shares some biological activities with IL-12 [27] [30]. Comparing IL-18 and IL-1beta, they share significant primary amino acid sequences and are related folded as all-beta sheet molecules. At first, IL-18 is synthesized as a biologically inactive precursor molecule with a lacking signal peptide. As a result of studies, the IL-18 precursor requires cleavage into an active, mature molecule by the intracellular cysteine protease. This cystein protease is called *IL-1beta-converting enzyme* (ICE), which is also known as *caspase 1* [25].

Farjadfar et al. explored in their study the association between IL-18 polymorphism and lung cancer. They suggested that IL-18 polymorphism contributes to lung cancer risk, particularly among patients suffering from squamous cell carcinoma. Farjadfar described significant differences in the IL-18-607 allele and genotype distributions between 73 lung cancer patients and controls. Further studies with larger numbers of patients are necessary to determine the possible association between IL-18 polymorphisms and different histological types of lung cancer [31].

3.4.3 Mesothelin (MSLN)

Mesothelin seems to be a promising predictive marker for early evidence of malignant mesothelioma, as several authors have proposed already [32] [33] [34] [35] [36].
MSLN, which is a differentiation antigen that was first described as the antigenic target of the monoclonal antibody K1 [37], is a cell surface protein present on normal mesothelial cells lining the body cavities, including the pleura, peritoneum, and pericardium [38]. It is highly expressed in many cancers, including mesotheliomas, ovarian and pancreatic cancers, and some squamous cell carcinomas [39] [40]. Cristaudo et al. stated that the human mesothelin gene codes for several proteins and is consistently expressed in normal mesothelial cells. 71-kDa precursor protein is the primary product of the gene which undergoes physiologic cleavage, which is resulting in two main proteins. One is the 31-kDa NH2-terminal megakaryocyte potentiation factor, which is normally secreted into the blood [32]. The COOH-terminal product of the cleavage is anchored to the cell membrane and supplies epitopes detected in immunohistochemistry. After further proximate cleavages, this cell surface protein releases *soluble mesothelin-related peptides* (SMRP). These peptides are the principal mesothelin family proteins [32] [33] [34]. Hassan et al. mentioned that cell-bound MSLN is a promising target for antibody based treatment of cancers overexpressing MSLN. It is also an immunogenic protein and anti-MSLN antibodies are frequently detected in patients with mesotheliomas as well as ovarian cancer [39] [41].

3.4.4 Mucin 5B (MUC5B)

Mucins are complex glycoproteins the major macromolecular constituents of the bronchial mucous layer conferring its viscoelastic characteristics by covering the airway epithelia. They have a leading to high carbohydrate content (50–90% by weight) due to heavy protein glycosylation, high molecular weight ($2–20*10^5$ Da), and therefore they are difficult to isolate and purify [42] [43]. Mucins play an essential role in protecting and lubricating the epithelial surface of various tissues [44]. The *tandem repeats* (TR) characterize a mucin protein. Mucins differ in the amount of these domains, especially from membrane-bound receptors. Furthermore, mucins are classified by their MUC protein backbone, disposing of 377 to
≥ 11.000 amino acids in their protein backbones. The backbone is encoded by the *MUC gene* [43].

MUC5B is one of the major airway mucins and has got TR domains that are repeated four or five times in their protein backbones. They are separated by cysteine-rich domains (**Fig. 3**).

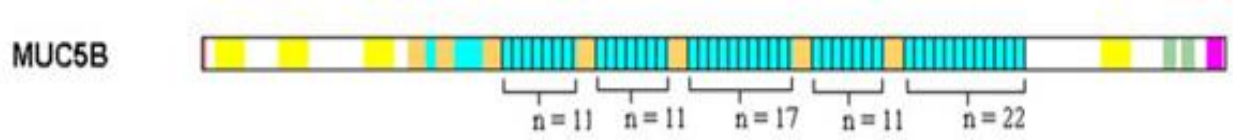

Fig. 3: Schematic drawing of a MUC5B protein backbone [43]. (Models adapted primarily from Dekker et al.)

The region marked with *n* shows the number of TR of MUC5B. The pink colored ends represent the C-terminal domain.

Historically, Mucins have been implicated since long in health and disease, especially in the fields of cancer, lung diseases [45], gastrointestinal diseases, as well as concerning mucins expressed in the eye and ear. Mucus and mucins are overproduced in the airways of patients with chronic airway diseases like COPD or CF. The characteristics of these diseases are inflammatory and immune response mediators, which are released into airway tissue and airway secretions. It is now possible to identify specific mucins in airway samples, because MUC-specific antibodies are available [43].

3.5 Antibodies against Antigens

Antibodies are glycoproteins, also named immunoglobulins (Ig). They are secreted into blood and extracellular liquids or are fixed on the surface of B-lymphocytes.

The most common antibodies in human serum are the so called IgGs (*Immunoglobulin G*) [46]. Antibodies bind their accurate antigen and recruit other cells and molecules of the immune system, which finally eliminate the antigen of the organism [47]. The molecular structure is “Y”-shaped. The compound elements of all polypeptide chains are basically covalent disulphide bridges. When immunoglobulins get disassembled, four subunits can be detected, of which two are always identical [48]:

- Two heavy chains (**H-chains**)
- Two light chains (**L-chains**)

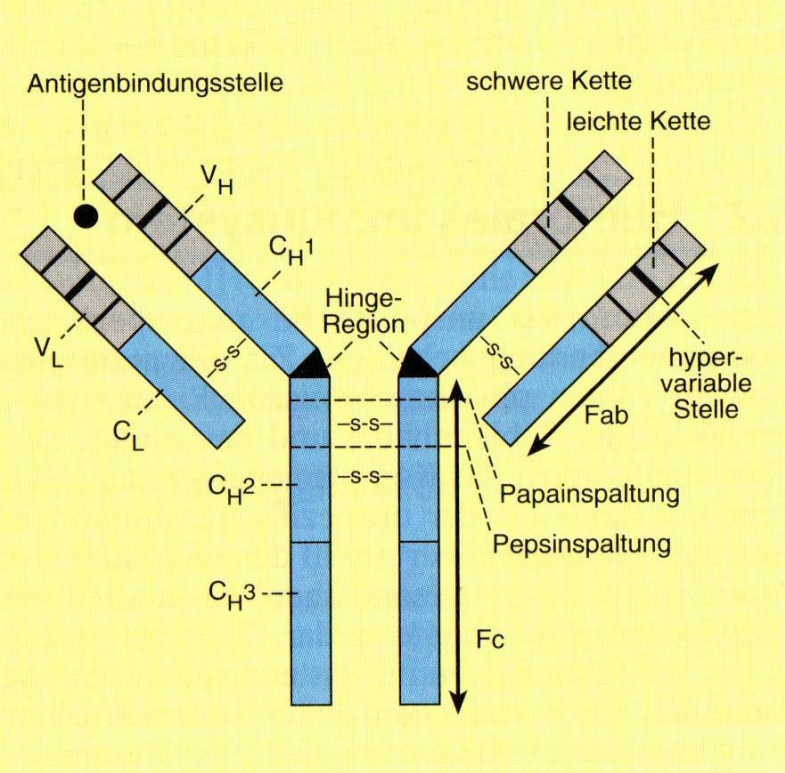

Fig. 4: Graphical representation of an immunoglobulin molecule [12]

The picture above shows the typical antibody structure with the *H-chain* and *L-chain*, which are binding with disulphide bridges. Both chains are labeled through variable and constant regions. The variable regions defined the antigen binding place. The so called *hinge-region* is located between the *Fc* and *Fab* regions that confer mobility on the two Fab arms of the antibody molecule, which allows it to combine better with the two epitopes.

There are only two types of L-chains which are either of *lambda* or *kappa* type, but there exist five main classes (IgM, IgG, IgD, IgE, IgA) of the H-chains.

Leucocytes play an important role in the specific and unspecific immune defense. Especially two subtypes, *B cells* and *T cells*, are essential for the formation of antibody-antigen complex. B cells and T cells generate an antigen receptor and express it on the surface to finally bind the antigen [49]. The *paratope*, or antigen binding place of the antibody, connects reversible to the fitting structure of the antigen [47]. The part of the antigen which binds to the paratope, is called *epitope*. Although antibodies are highly selective, they nevertheless show cross reactivity to similar epitopes of other antigens [47].

The general production of antibodies is done by immunizing animals, like rabbits, goats, mice, etc. and afterwards the extraction of the immunized animal's sera. Polyclonal Antibodies are a mixture of different antibodies which can recognize a specific epitope or different epitopes (f. ex.: an antigen with multiple epitopes).

Monoclonal Antibodies result through fusion of antibody-producing B-cells, from the spleen of an immunized mouse, with cells from hybridoma cell lines.

In this study we discuss especially the four antibodies **anti-Cytoglobin, anti-Interleukin 18**, **anti-Mesothelin** and **anti-Mucin 5B**, which have been generated in mice and/or rabbits against the four correspondent tumor antigens in our lung adenocarcinoma patients.

3.6 The ELISA

ELISA stands for "**Enzyme Linked Immuno-Sorbent Assay**". The ELISA-system is one of the most prominent used methods in immunochemistry and it is applied in fields like clinical diagnostics, veterinary diagnostics, pharmaceutical diagnostics, as well as food- and environmental analysis.

The basic of this technique is the unique specificity of the antibody-antigen-binding, the recognition of a specific antigen surface structure, the epitope. The reactant (antibody or antigen) is bound on a *fixed phase* (f. ex.: microtiter plate) and interacts with the antigen or antibody. The formation of the immune complex is then detected by an enzymatic color reaction. Normally, the antibody (mostly Secondary Antibody) is covalently linked to an enzyme. The color intensity is measured with a spectrometer. Finally, the optical density (= **OD-value**) of the colored solution can be directly correlated with the concentration of the coated (= Direct ELISA) or captured (= Sandwich ELISA) antigen. In order to control pipetting and handling errors, at least double values of the same sample concentrations are performed and the mean OD-values and standard deviations are calculated.

The following picture (**Fig. 5**) shows the principle of the color reaction of an ELISA.

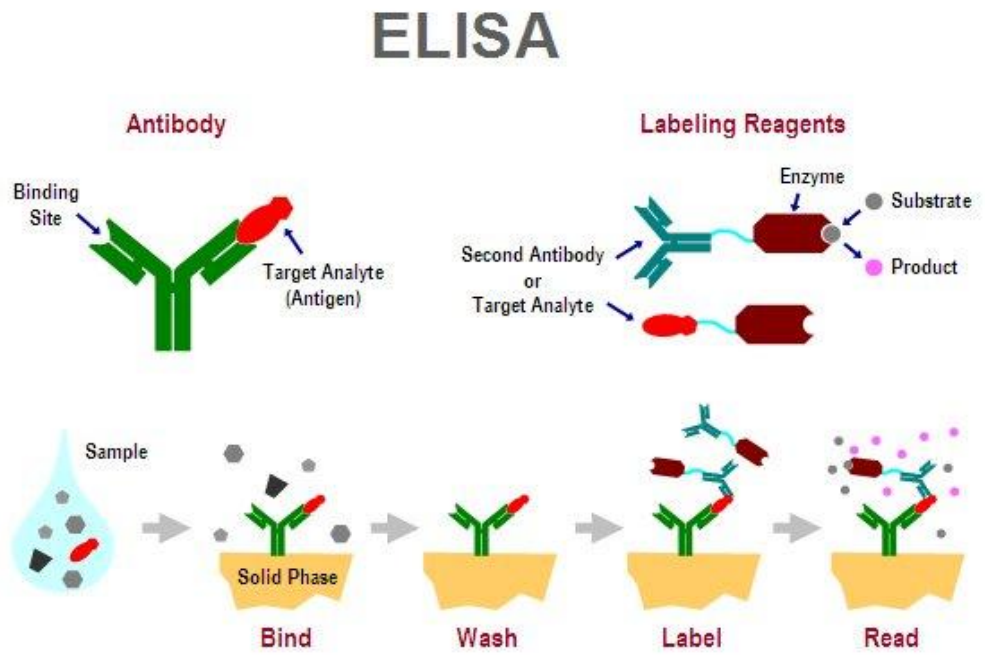

Fig. 5: Enzymatic color reaction of an ELISA

The enzyme catalyzes the reaction and changes a colorless substrate into a dyed one. The more immune complexes are present, the higher is the so-called optical density OD-value. This effect makes the amount of antigen-antibody- reaction measureable. (adapted from http://virology-microbiology-b.blogspot.com/)

Fig. 6: Typical format and outcome of an ELISA experiment

(adapted from http://www.med4you.at/laborbefunde/lbef_anca.htm)

The ELISA establishment itself and certain application/conditions is a procedure that differs from lab to lab, because of the high variety of the interactions. Many factors influence the outcome of an ELISA experiment. These include the choice of the correct antibody- and antigen- dilutions, the reagents, incubation times, the coated amount of antibody and/or

antigen, as well as the choice of enzymatic color reaction and subsequent measurement of photometry data. Important is the selection of the correct microtiter plate. In this study I worked with *Nunc MaxiSorp® 96 well plates*, because of their surface quality to bind all types of molecules (**Fig. 7**).

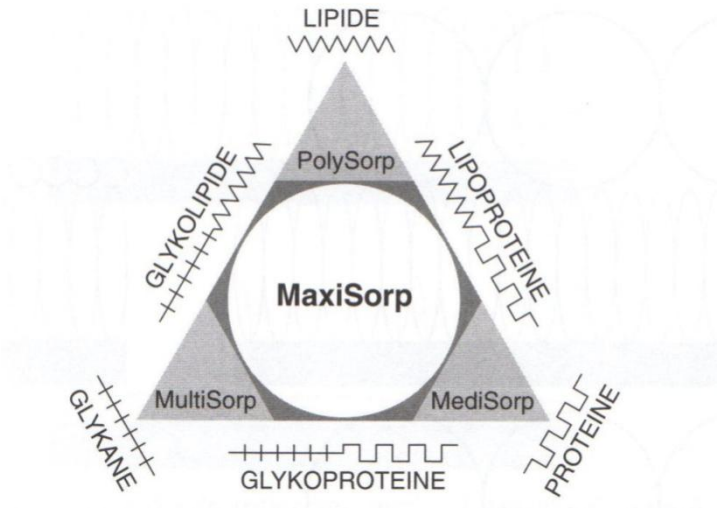

Fig. 7: Schematic drawing of a *MaxiSorp*® well plate [47]

The picture shows that all macromolecular types can bind on the surface modification.

MaxiSorp® is a polystyrol surface with a high bond capacity for proteins and other molecules with hydrophobic and hydrophilic areas. This allows the immobilization of antibodies. *PolySorp*®, *MultiSorp*® and *MediSorp*® have got different characteristics to accomplish experimental designs.

My experiments were accomplished with two main test formats:

- Sandwich-ELISA (**S-ELISA**)
- Direct-ELISA (**D-ELISA**)

The principle of the **Sandwich ELISA** system is to measure the amount of antigen brought between two layers of antibodies *(→ Capture-* and *Detection Antibody*). The importance is that the antigen to be measured must contain at least two antigenic sites capable of binding to antibodies. It is possible to use either *Monoclonal* or *Polyclonal Antibodies* acting as Capture- and Detection Antibodies in Sandwich ELISA systems. The difference between the two antibody types is that Monoclonal Antibodies recognize a single epitope which allows fine detection and quantification of small differences of the antigen amounts, whereas Polyclonal Antibodies are often used as the Capture Antibody to pull down as much of the antigen as possible on the plastic surface. The advantage of Sandwich ELISA is that it is up to 2 to 5 times more sensitive than direct ELISAs. S-ELISAs are preferred when targeting bigger antigen-structures like antibodies, viruses, proteins, etc.

The advantage of D-ELISAs is the strong signal-amplification, because of the ability of enzyme-linked Secondary Antibodies (*Tracer-Antibodies*), to bind tracers per Primary Antibody [47]. The graphics below (**Fig. 8**) shows the principle of work flow of an S-ELISA versus a D-ELISA:

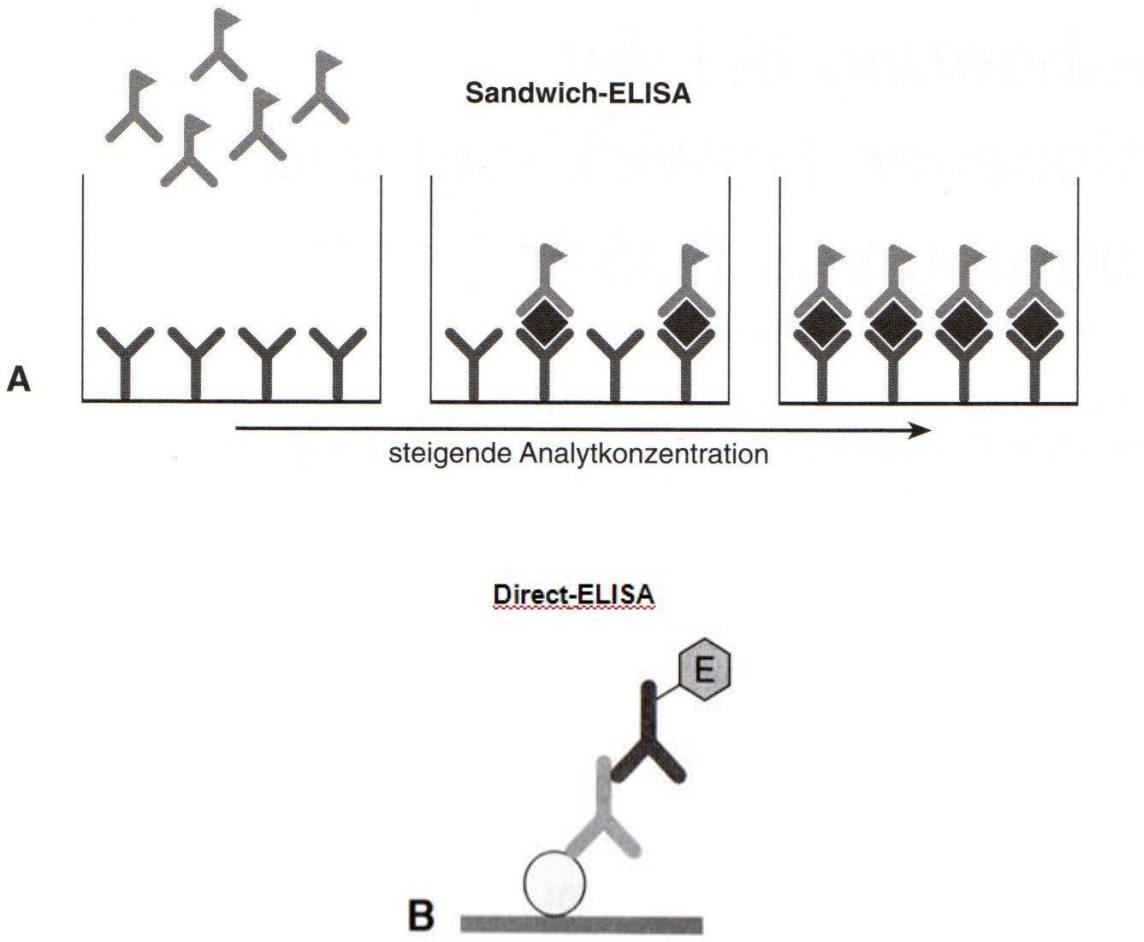

Fig. 8: Schematic comparison of an S-ELISA and an D-ELISA [47]

A: S-ELISA: The well is coated with a Capture Antibody, before the antigen (*analyte*) is added. The antigen will bind to the Capture Antibody. A Detecting Antibody (Secondary-Antibody) is added and binds to the antigen. After adding the POX- (*peroxidase*) antibody, the ELISA-reader evaluates the absorbency of the plate wells to determine the presence and quantity of the antigens.

B: D-ELISA: The D-ELISA is the same procedure just without using a Capture Antibody.

The POX-antibody will bind again to the Secondary Antibody.

4. Aim of the Thesis

Due to preliminary work by an interdisciplinary team of scientists, it was possible to identify tumor-specific proteins of lung adenocarcinoma by means of chirurgical gained material through proteome-analysis. After *PubMed* research, the clinical findings had been limited to eight useful candidates of proteins that seem to be potential biomarkers of cancer. The biochemical selection for these, were membrane expression and secretion criteria of these proteins. Under these criteria the chance is high to find the proteins in human serum, because they should have high sensitivity and specificity to belong to lung cancer, especially when they appear early enough in the course of disease for medical intervention to improve prognosis.

There were five commercially available antibodies against the antigens of our interest. The aim of this diploma thesis was to test, whether the antigens as specific serum markers are detectable in the patients' blood samples.

The aim of my thesis was to develop a fine-tuned ELISA for these proteins and to test the ELISA for its clinical suitability. 40 serum samples were collected and analyzed via D-ELISA and S-ELISA. Patients carrying tumor markers and a healthy control group were compared. This successfully established ELISA system would provide a valuable clinical tool for lung cancer diagnosis. Provided that the methodology (antibody quality) can be successfully established, it will be possible to measure the protein concentration within two days after blood taking from the patient. That could become a basic standard procedure for early diagnosis of lung tumors, in which curative resection is possible.

5. Material and Methods

5.1 Reagents – Buffers and Solutions

10 x PBS (1 liter)

5.244 g $NaH_2PO_4 * H_2O$

28.83 g $Na_2HPO_4 * 2H_2O$

Distilled water was added to a volume of 1000 ml and pH was adjusted to 7.4

1 x PBS (1 liter)

100 ml 10 x PBS

Distilled water was added to a volume of 1000 ml

0.2 % PBST (1 liter)

100 ml 10 x PBS

2 ml Tween 20

Distilled water was added to a volume of 1000 ml

Carbonate Buffer (1 liter)

1.59 g Na_2CO_3

2.93 g $NaHCO_3$

0.20 g NaN_3

Distilled water was added to a volume of 1000 ml and pH was adjusted to 9.0-9.6

Wash Buffer (1 liter)

1000 ml 1 x PBS

0.5 ml Tween 20

10 x TBS (1 liter)

24.5 g Tris

80.0 g NaCl

Distilled water was added to a volume of 1000 ml and pH was adjusted to 7.6

Blocking Buffer (0.5 liter)

5 g (1%) BSA

0.5 ml Tween 20

Adjust 1 x PBS to 500 ml

Assay Buffer (1 liter)

5 g (0.5%) BSA

0.5 ml Tween 20

Adjust 1 x PBS to 1000 ml

TMB - Substrate Solution

Add 50 µl to each well to let start color reaction

TMB- Stop Solution

Add 50 µl to each well to stop the color reaction

ELISA "Color Buffer" (1 liter)

5.1 g $C_6 H_8 O_7 * H_2O$

9.15 g $Na_2 HPO_4 * 2H_2O$

Distilled water was added to a volume of 1000 ml

ELISA Reaction Buffer with Phenylenediamine (for 1 plate)

6 mg Phenylenediamine

15 ml "color buffer"

Add 6 µl (30%) H_2O_2 just before pipetting to the wells

Add 100 µl to each well

Stop Solution for Reaction Buffer

Add 50 µl 8N $H_2 SO_4$ quickly to each well

Additional components of IL-18 Kit from **Bender MedSystems®** without exact descriptions of the content are:

Standard Protein (S): BMS267/2MSTS (for 10 assays)
Coating Antibody (CA): BMS267/2MSTCA
Sample Diluent (SD): BMS267/2MSTSD
Biotin-Conjugate (BK): BMS267/2MSTBK
Streptavidin-HRP (PK): BMS267/2MSTPK

ELISA plate reader: Synergy HT BIO-TEC®
OD-values were evaluated and visualized with ***Microsoft Excel*** software.

5.2 Methods

5.2.1 Establishment of ELISAs using recombinant Antigens

Before we started to use the collected serum samples, we aimed to establish a suitable antigen-specific ELISA. The appropriate antigens and antibodies were subjected to preliminary checkerboard titrations in order to determine the optimum concentration ranges of antigen and sera as well as antibodies used in the assay.

The general test procedure for the establishment of the **S-ELISA** was as follows:

1) The appropriate Capture Antibody (*Primary Antibody*) was diluted in 1 x PBS. In order to establish ideal conditions, the dilutions differed between each experiment. However, always 100 µl of the coating solution was added to each well. Immediately after coating and before coating overnight, the microtiter plates were sealed with an adhesive film. The plate was stored overnight at 2°C, allowing antigen/antibody binding process to take place.
2) On the following day, the plate was washed once with 400 µl Wash Buffer. After the washing step, the plate was tapped on absorbent pad or paper towel to remove excess

Wash Buffer. 250 µl of Blocking Buffer were added to each well and incubated at room temperature for 2 hours. Washing steps were repeated two times.

3) The antigen, in our case the *recombinant proteins* (CYGB / IL-18 / MSLN / MUC5B), was diluted in Assay Buffer to start a geometric dilution series. The starting dilution differed from each experiment but always contained two blank wells for the negative control. The serum samples were commonly diluted in Sample Diluent, mostly **1:2**, but dependent on the pretests. 100 µl of the diluted antigen- or serum sample mixture was coated per well on a 96- well plate (*Nunc MaxiSorp*®) overnight at 2°C. Serial dilutions were always dispensed in duplicates.
4) On the next day, the plates were washed 4 times. Plates were blocked again with 250 µl Blocking Buffer for 2 hours at room temperature.
5) The Detection Antibody (*Secondary Antibody*) was diluted in Assay Buffer and dilutions differed between each experiment. Mostly we used a dilution factor of **1:500**. 50 µl of this dilution was added to the wells. Plates were incubated for 1 hour at room temperature. Washing steps were repeated 6 times (see above).
6) The dilution of POX- labeled antibody was prepared in Assay Buffer and the common dilution factor was **1:4000**. 100 µl were added per well and incubated for 1 hour at room temperature. Then 6 washing steps were done. 50 µl TMB-Substrate Solution were added to all wells. The microwells were incubated at room temperature for approximately 10 minutes with avoiding direct exposure to intense light.
7) 50 µl of the TMB-Stop Solution were quickly and uniformly spread to stop the enzyme reaction. The ELISA plate reader monitored the results by reading the absorbance of each microwell using 450 nm as wave length. The OD-value was used to define the results and produce statistical analyses via Excel tables and t-tests. OD-values measured in the linear range between 0.3 and 1.2 were suitable for valid antigen detection.

For **Direct ELISAs (D-ELISA)**, the protocol was the same as for Sandwich ELISAs, but without using Capture Antibodies for coating.

5.2.2 Human blood samples and serum preparation

Fresh serum samples were collected by the clinical cooperator, documented and transferred to our laboratory. The advantage of this method is that blood can be easily and routinely taken from the patients. All patients had not received any pharmacological treatment prior to our analyses. The serum samples are still available for further studies analyzing additional proposed tumor marker candidates.

Approximately 5-10 ml human venous blood was taken for our analysis. All blood samples were resinated **1:10** with the protease-inhibitor *trasylol* and rapidly cooled. Trasylol, also called *aprotinin,* is the bovine version of the small protein *basic pancreatic trypsin inhibitor*. This enzyme inhibits trypsin and related proteolytic enzymes. Trasylol's main effect is to slow down the fibrinolysis, which is the process that leads to the breakdown of blood clots [50]. For serum exploitation, the blood samples were coagulated under air and centrifuged for 10 min/3000 rpm. The serum supernatant was collected and stored in tubes at 3°C, where they can be used over a period of several months.

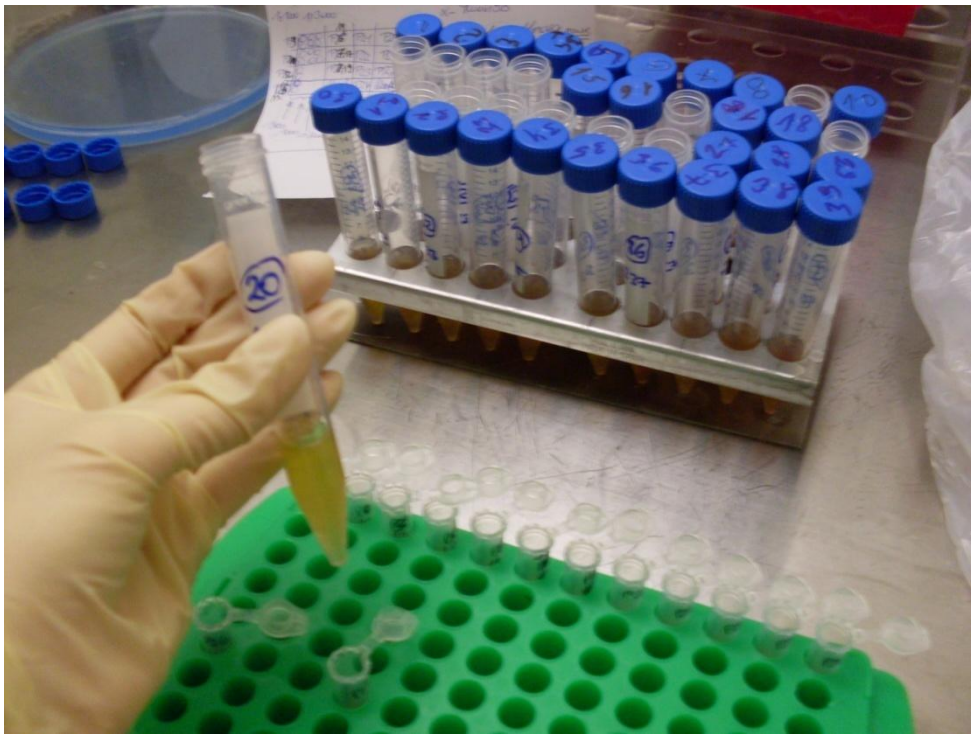

Fig. 9: Preparation of the collected serum samples

After the spin down of the blood, the serum samples were filled in tubes and marked with ascending numbers to differ between lung adenocarcinoma-patients and healthy control group. These tubes were stored in the cold room at 3°C.

5.2.3 Cytoglobin ELISA

The following table shows the commercially available recombinant protein and the two antibodies that were used to establish a *Direct ELISA* or *Sandwich ELISA*:

Recombinant Protein (Abnova) CYGB	**Anti-CYGB (Santa Cruz)** Rabbit polyclonal antibody	**Anti-CYGB (Abnova)** Mouse monoclonal antibody
Catalog ID H00114757-P01	**Catalog ID** sc-66855	**Catalog ID** H00114757-M02
Concentration 0.08 µg/µl	**Concentration** 200 µg/ml	**Concentration** 1 mg/ml
Amino acid sequence 1 a.a.-191 a.a. full-lenght with GST-tag	**Amino acid sequence** raised against amino acids 1-190	**Amino acid sequence** 1 a.a.-191 a.a. full-lenght with GST-tag

Table 2: Summary of commercially available Cytoglobin recombinant protein and antibodies

Reagents:

- Recombinant protein CYGB (Abnova; H00114757-P01)
- Anti-CYGB rabbit antibody (Santa Cruz; sc-66855)
- Anti-CYGB mouse antibody (Abnova; H00114757-M02)
- POX- anti- mouse antibody
- POX- anti- rabbit antibody
- Sera dilutions
- Assay Buffer
- Blocking Buffer
- Carbonate Buffer
- 1x PBS
- 0.2% PBST
- Wash Buffer
- ELISA Reaction Buffer with Phenylenediamine

- 8N H_2 SO_4
- TMB-Substrate Solution
- TMB-Stop Solution

The following protocol describes the general procedure for CYGB-ELISA tests. It contains descriptions for the establishment of *Direct ELISA* and *Sandwich ELISA*, as well as operating the sera. The protocol is the same, but sometimes the reagents and dilution series differed by actual need.

1) After delivery of the recombinant CYGB, it was immediately stored in aliquots at -80°C, to avoid repeated thawing of the antigen, which could lead to degradation. To establish the optimum standard curve, we diluted the stock-solution of CYGB. The dilution range of the protein was between **250 ng/ml** and **0.15 ng/ml**. The CYGB protein was mixed mostly in Carbonate Buffer or 1x PBS before we prepared the geometric dilution series. 100 µl were added to each well according to plan. Additionally, of wells one row was only filled with 1x PBS or Assay Buffer, as a negative control.
2) When we established the S-ELISA system, we commonly diluted the Capture Antibody (*Primary Antibody*) **1:250** in 1x PBS or Assay Buffer. 100 µl were added into each well according to plan. Immediately after pipetting, the microtiter plates were sealed with an adhesive film. The plates were stored overnight at 2°C, allowing the binding process to take place.
3) On the following day, the plates were washed once with 400 µl Wash Buffer. After the washing step, the plates were tapped on absorbent pad or paper towel to remove excess Wash Buffer. 250 µl of Blocking Buffer were added to each well and incubated at room temperature for 1 hour. Washing steps were repeated two times.
4) When we included the serum samples, we used a dilution range between **1:2 - 1:2000**. The sera were diluted in Sample Diluent. 100 µl of the diluted serum sample mixture was coated per well and stored overnight at 2°C. On the next day, the plates were washed 4 times. Plates were blocked again with 250 µl Blocking Buffer for 1 hour at room temperature.
5) The Detection Antibody (*Secondary Antibody*) was diluted in Assay Buffer or 1x PBS and dilutions differed between each experiment. Dilutions ranged between **1:500** and

1:32000. 50 µl were added per well for 1 hour at room temperature. Washing steps were repeated 6 times.

6) POX-anti-rabbit or POX-anti-mouse antibody was commonly prepared in Assay Buffer and the dilution factor was always **1:4000**. 100 µl were added per well and incubated for 1 hour at room temperature. Then 6 washing steps were done. We used two different methods for visualizing the enzyme reaction:

a) An ELISA Reaction Buffer with Phenylenediamine (see description in *Materials Reagents - Buffers and Solutions*) was prepared. 100 µl of this mixture was added to each well and incubated at room temperature for approximately 10 minutes. After that time, the color reaction was stopped by adding 50 µl 8N H_2SO_4. The color intensity can be read by the ELISA plate reader at 490 nm and 630 nm.
b) As an alternatively method, we also used TMB Substrate. Afterwards 6 repeats of washing steps, 50 µl of TMB-Substrate Solution was quickly added to all wells and incubated at room temperature for about 10 minutes. We avoided the direct exposure of the plate to intense light. The enzyme reaction was stopped by adding 50 µl TMB-Stop Solution to all wells. The absorbance can be read at 450 nm.

5.2.4 CYGB Dot Blot

We aimed to test whether the two commercially available antibodies react appropriately with the antigen. Therefore, a Dot Blot was performed.

Reagents:

- Anti- CYGB antibodies
  (Santa Cruz rabbit sc 66855 and Abnova mouse H00114757-M02)
- CYGB- recombinant protein (Abnova H00114757-P01)
- POX-anti-rabbit and POX-anti-mouse antibody
- TBST
- Whatman® Protan® Nitrocellulose Transfer Membrane
- 5% Nonfat dry milk
- 5% BSA

1) The CYGB antigen stock-solution was **80 ng/µl**. The **1:8** dilution was achieved by mixing 2µl of the antigen in 14 µl PBS. A geometric dilution series of recombinant CYGB protein started at **20 ng** and ended at **1.25 ng** by dropping the dilutions on the Nitrocellulose Membrane.
2) The membrane was dried on air for a few minutes. Afterwards the membrane was blocked with 5% nonfat dry milk in TBST on a microplate shaker for 1 hour at room temperature. Then it was washed 2 times in TBST.
3) Dilutions of the Primary Antibodies were prepared in TBST/5% BSA and were added for 1 hour at room temperature. Anti- CYGB antibody (*Abnova*) was diluted **1:500** and Anti- CYGB antibody (*Santa Cruz*) was diluted **1:200**.
4) Next, the membrane was washed 3 times for 5 minutes in TBST. The Secondary Antibodies (POX-anti-rabbit and POX-anti-mouse antibody) were diluted **1:3500** in 5% nonfat dry milk in TBST, added to the membranes and left on the microplate shaker for 1 hour at room temperature. The membrane was washed 3 times for 15 minutes in TBST.
5) The chemoluminescence reaction with ECL Plus reagent (*GE Healthcare Amersham*) was detected in the LUMI Imager (*Boehringer Ingelheim*).

5.2.5 CYGB Western Blot

Reagents:

- Recombinant protein CYGB (Abnova; H00114757-P01)
- Anti-CYGB rabbit antibody (Santa Cruz; sc-66855)
- POX-anti-rabbit antibody
- Serum samples P1-P13
- LSB +
- 5% milk powder
- TBST
- ECL-Plus

1) 1 µl, 2 µl and 3 µl of the recombinant CYGB protein were mixed with LSB +. This mixture was pipetted in each case to 13 tubes. Afterwards 3 µl serum (P1-P13) was added into the appropriately numbered tubes.
2) Furthermore, samples were incubated for 10 minutes at 95°C to denature the proteins and then centrifuged for approximately 2 minutes.
3) 20 µl were loaded on the polyacrylamid gel (10% PAGE) into every slot. The gel ran at 120 V for about 1 ½ hours. The proteins were transferred on nitrocellulose at 23 V overnight.
4) The blots were blocked with 5% milk powder in TBST and then washed 3 times with TBST. The Primary anti-CYGB Antibody was diluted 1:200 in 5% BSA in TBST and added to the blots and incubated for1 hour at room temperature. After that, 3 washing steps with TBST followed for 5 minutes each.
5) The POX-labeled Secondary Antibody was diluted 1:3000 in 5% milk powder in TBST, added to the blots and shook for 1 hour at room temperature. 3 washing steps with TBST followed.
6) The photoreaction with ECL Plus Westernblotting Detection System (*GE Healthcare Amersham*) was detected in the LUMI Imager (*Boehringer Ingelheim*).

5.2.6 Interleukin 18 ELISA

The following table shows the commercially available recombinant protein and the two antibodies that were used to achieve a *Direct ELISA* or *Sandwich ELISA*:

Recombinant Protein (Everest Biotech) IL-18	**α-IL-18 (Everest Biotech)** Goat polyclonal antibody	**IL-18 Module Set** (*Bender MedSystems*®) see **Table 4**
Catalog ID EBP09393	**Catalog ID** EB09393	
Concentration 100 µg of dried peptide	**Concentration** 0.5 µg/µl	
Amino acid sequence C-NPPDNIKDTKSDI	**Amino acid sequence** C-NPPDNIKDTKSDI	

Table 3: Summary of commercially available Interleukin 18 protein and antibodies

The following table (**Table 4**) shows information about the reagents of the Module Set from *Bender MedSystems*® (BMS267/2MST) for the development of the Sandwich ELISA with anti-Human IL-18 Matched Antibody Pairs. This purchased S-ELISA was done with IL-18, because no 2nd antibody from a different species was commercially available.

Standard Protein IL-18	**Coating Antibody** anti-human IL-18 monoclonal antibody	**Biotin-Conjugate** anti-human IL-18 monoclonal antibody	**Streptavidin-HRP**
Concentration 100 ng/ml lyophilized protein	**Concentration** Vial (1.1 ml) 100 µg/ml	**Concentration** Vial 55 µl	**Concentration** 22 µl

Table 4: Summary of reagents from *Bender MedSystems*® Module Set for human IL-18

Reagents:

- Recombinant protein IL-18 (Everest Biotech; EBP09393)
- Anti-IL-18 goat antibody (Everest Biotech; EB09393)
- Standard protein IL-18 (Module Set)
- Coating antibody (Module Set)
- POX- anti- goat antibody
- Biotin-Conjugate (Module Set)
- Streptavidin-HRP (Module Set)
- Sera dilutions
- Assay Buffer
- Blocking Buffer
- Carbonate Buffer
- 1x PBS
- 0.2% PBST
- Wash Buffer
- ELISA Reaction Buffer with Phenylenediamine
- 8N H_2 SO_4
- TMB-Substrate Solution
- TMB-Stop Solution

The following protocol describes the general procedure for IL-18-ELISA tests. It contains descriptions for the establishment of *Direct ELISA* and *Sandwich ELISA,* as well as operating the sera. The protocol is the same, but sometimes the reagents and dilution series differed by actual need.

1) After delivery of the recombinant IL-18, it was immediately stored in aliquots at -80°C, to avoid repeated thawing of the antigen, which could lead to degradation.
   To establish the optimum standard curve, we diluted the stock-solution of IL-18. The dilution range of the protein was between **250 ng/ml** and **0.07 ng/ml**.
   IL-18 was mixed mostly in Carbonate Buffer or 1x PBS before we prepared the geometric dilution series. 100 µl were added to each well according to plan. Additionally, of wells one row was only filled with 1x PBS or Assay Buffer, as a negative control.

2) When we established the S-ELISA system, we ordered the Module Set from *Bender MedSystems*®. Before starting with the experiments, we immediately aliquoted kit components and stored them at -20°C to avoid loss of bioactive human IL-18. Some Buffers and Solutions could be prepared by ourselves, following the description in the product information of *Bender MedSytems*®. Other reagents were not provided and could only be ordered.
3) Commonly the Capture Antibody (*Primary Antibody*) was diluted **1:250** in 1x PBS. 100 µl were added into each well according to plan. Immediately after pipetting, the microtiter plates were sealed with an adhesive film. The plates were stored overnight at 2°C, allowing the binding process to take place.
4) On the following day, the plates were washed once with 400 µl Wash Buffer. After the washing step, the plates were tapped on absorbent pad or paper towel to remove excess Wash Buffer. 250 µl of Blocking Buffer were added to each well and incubated at room temperature for 1 hour. Washing steps were repeated two times.
5) When we included the serum samples, we used a dilution range between **1:2 - 1:500**. The sera were diluted in Sample Diluent. 100 µl of the diluted serum sample mixture was coated per well and stored overnight at 2°C. On the next day, the plates were washed 4 times. Plates were blocked again with 250 µl Blocking Buffer for 1 hour at room temperature.
6) The Detection Antibody (*Biotin-Conjugate* used from Module Set) was diluted in Assay Buffer and dilutions differed between each experiment. Dilution ranged between **1:500** and **1:32000**. 50 µl were added per well 1 hour at room temperature. Washing steps were repeated 6 times.
7) POX-anti-goat antibody or *Streptavidin-HRP* was commonly prepared in Assay Buffer and the dilution factor was **1:4000** or **1:5000**. 100 µl were added per well and incubated for 1 hour at room temperature. Then 6 washing steps were done. We used two different methods for visualizing the enzyme reaction:

a) An ELISA Reaction Buffer with Phenylenediamine (see description in *Materials Reagents - Buffers and Solutions*) was prepared. 100 µl of this mixture was added to each well and incubated at room temperature for approximately 10 minutes. After that time, the color reaction was stopped by adding 50 µl 8N H_2SO_4. The color intensity can be read by the ELISA plate reader at 490 nm and 630 nm.

b) As an alternatively method, we also used TMB Substrate. Afterwards 6 repeats of washing steps, 50 µl of TMB-Substrate Solution was quickly added to all wells and incubated at room temperature for about 10 minutes. We avoided the direct exposure of the plate to intense light. The enzyme reaction was stopped by adding 50 µl TMB-Stop Solution to all wells. The absorbance can be read at 450 nm.

5.2.7 Mesothelin ELISA

The following table shows the commercially available recombinant proteins inclusively the two antibodies that were purchased to perform a *Direct ELISA* or *Sandwich ELISA*:

Recombinant Protein (Abnova) MSLN	**Recombinant Protein (Abnova)** MSLN	**Anti-MSLN (Abnova)** Mouse polyclonal antibody	**Anti-MSLN (Abnova)** Rabbit polyclonal antibody
Catalog ID H00010232-P01	**Catalog ID** H00010232-Q01	**Catalog ID** H00010232-A01	**Catalog ID** H00010232-D01P
Concentration 0.04 µg/µl	**Concentration** 0.24 µg/µl	**Concentration** 0.05 ml	**Concentration** 1 mg/ml
Amino acid sequence 1 a.a.-621 a.a. full-length rec. protein with GST-tag	**Amino acid sequence** 464 a.a.-563 a.a. partial rec. protein with GST-tag	**Amino acid sequence** 464 a.a.-564 a.a. partial rec. protein with GST-tag	**Amino acid sequence** 1 a.a.-621 a.a. full-length human protein

Table 5: Summary of commercially available Mesothelin proteins and antibodies

Reagents:

- Recombinant protein MSLN (Abnova; H00010232-P01)
- Recombinant protein MSLN (Abnova; H00010232-Q01)
- Anti-MSLN rabbit antibody (Abnova; H00010232-D01P)
- Anti-MSLN mouse antibody (Abnova; H00010232-A01)
- POX- anti- mouse antibody

- POX- anti- rabbit antibody
- Sera dilutions
- Assay Buffer
- Blocking Buffer
- Carbonate Buffer
- 1x PBS
- 0.2% PBST
- Wash Buffer
- ELISA Reaction Buffer with Phenylenediamine
- 8N H_2 SO_4
- TMB-Substrate Solution
- TMB-Stop Solution

The following protocol describes the general procedure for MSLN-ELISA tests. It contains descriptions for the establishment of *Direct ELISA* and *Sandwich ELISA*, as well as operating the sera. The protocol is the same, but sometimes the reagents and dilution series differed by actual need.

1) After delivery of the recombinant MSLN, it was immediately stored in aliquots at -80°C, to avoid repeated thawing of the antigen, which could lead to degradation.
   To establish the optimum standard curve, we diluted the stock-solution of MSLN. The dilution range of the protein was between **250 ng/ml** and **7.8 ng/ml**.
   MSLN was mixed mostly in Carbonate Buffer or 1x PBS before we prepared the geometric dilution series. 100 µl were added to each well according to plan. Additionally, of wells one row was only filled with 1x PBS or Assay Buffer, as a negative control.
2) When we established the S-ELISA system, we commonly diluted the Capture Antibody (*Primary Antibody*) **1:500** in 1x PBS or Assay Buffer. 100 µl were added into each well according to plan. Immediately after pipetting, the microtiter plates were sealed with an adhesive film. The plates were stored overnight at 2°C, allowing the binding process to take place.
3) On the following day, the plates were washed once with 400 µl Wash Buffer. After the washing step, the plates were tapped on absorbent pad or paper towel to remove

excess Wash Buffer. 250 µl of Blocking Buffer were added to each well and incubated at room temperature for 1 hour. Washing steps were repeated two times.

4) When we included the serum samples, we used a dilution range between **1:2** – **1:500**. The sera were diluted in Sample Diluent. 100 µl of the diluted serum sample mixture was coated per well and stored overnight at 2°C. On the next day, the plates were washed 4 times. Plates were blocked again with 250 µl Blocking Buffer for 1 hour at room temperature.
5) The Detection Antibody (*Secondary Antibody*) was diluted in Assay Buffer or 1x PBS and dilutions differed between each experiment. Dilutions ranged between **1:500** and **1:32000**. 50 µl were added per well for 1 hour at room temperature. Washing steps were repeated 6 times.
6) POX-anti-rabbit or POX-anti-mouse antibody was commonly prepared in Assay Buffer and the dilution factor was always **1:4000**. 100 µl were added per well and incubated for 1 hour at room temperature. Then 6 washing steps were done. We used two different methods for visualizing the enzyme reaction:

a) An ELISA Reaction Buffer with Phenylenediamine (see description in *Materials Reagents - Buffers and Solutions*) was prepared. 100 µl of this mixture was added to each well and incubated at room temperature for approximately 10 minutes. After that time, the color reaction was stopped by adding 50 µl 8N H_2SO_4. The color intensity can be read by the ELISA plate reader at 490 nm and 630 nm.
b) As an alternatively method, we also used TMB Substrate. Afterwards 6 repeats of washing steps, 50 µl of TMB-Substrate Solution was quickly added to all wells and incubated at room temperature for about 10 minutes. We avoided the direct exposure of the plate to intense light. The enzyme reaction was stopped by adding 50 µl TMB-Stop Solution to all wells. The absorbance can be read at 450 nm.

5.2.8 MSLN Dot Blot

We aimed to test whether the two commercially available antibodies react appropriately with the antigen. Therefore, a Dot Blot was performed.

Reagents:

- Anti- MSLN antibodies:
  (Abnova rabbit H00010232-D01P and Abnova mouse H00010232-A01)
- MSLN- recombinant protein (partial protein, Abnova H00010232-Q01)
- POX-anti-rabbit and POX-anti-mouse antibody
- TBST
- Whatman® Protan® Nitrocellulose Transfer Membrane
- 5% Nonfat dry milk
- 5% BSA

1) The MSLN antigen stock-solution was **240 ng/µl**. The **1:24** dilution was achieved by mixing 2µl of the antigen in 14 µl PBS. A geometric dilution series of recombinant CYGB protein started at **20 ng** and ended at **1.25 ng** by dropping the dilutions on the Nitrocellulose Membrane.
2) The membrane was dried on air for a few minutes. Afterwards the membrane was blocked with 5% nonfat dry milk in TBST on a microplate shaker for 1 hour at room temperature. Then it was washed 2 times in TBST.
3) Dilutions of Primary Antibodies were prepared in TBST/5% BSA and were added for 1 hour at room temperature. Anti- MSLN (*Abnova*) was diluted **1:500** and Anti-MSLN (*Santa Cruz*) was diluted **1:1000**.
4) Next, the membrane was washed 3 times for 5 minutes in TBST. The Secondary Antibodies (POX-anti-rabbit and POX-anti-mouse) were diluted **1:3500** in 5% nonfat dry milk in TBST, added to the membranes and left on the microplate shaker for 1 hour at room temperature. The membrane was washed 3 times for 15 minutes in TBST.
5) The chemoluminescence reaction with ECL Plus reagent (*GE Healthcare Amersham*) was detected in the LUMI Imager (*Boehringer Ingelheim*).

5.2.9 Mucin 5B ELISA

The following table shows the commercially available recombinant proteins inclusively the two antibodies that were needed to perform a *Direct ELISA* or *Sandwich ELISA*:

Recombinant Protein (Abnova) MUC5B	**Anti-MUC5B (Santa Cruz)** Rabbit polyclonal antibody	**Anti-MUC5B (Abnova)** Mouse polyclonal antibody
Catalog ID H00727897-Q01	**Catalog ID** sc-20119	**Catalog ID** H00727897-A01
Concentration 0.17 µg/µl	**Concentration** 200 µg/ml	**Concentration** 0.05 ml
Amino acid sequence 4186 a.a.- 4296 a.a. partial rec. protein with GST-tag	**Amino acid sequence** raised against amino acids 1201-1500	**Amino acid sequence** 4186 a.a.- 4296 a.a. partial rec. protein with GST-tag

Table 6: Summary of commercially available Mucin 5B protein and antibodies

Reagents:

- Recombinant protein MUC5B (Abnova; H00727897-Q01)
- Anti-MUC5B rabbit antibody (Santa Cruz; sc-20119)
- Anti-MUC5B mouse antibody (Abnova; H00727897-A01)
- POX- anti- mouse antibody
- POX- anti- rabbit antibody
- Sera dilutions
- Assay Buffer
- Blocking Buffer
- Carbonate Buffer
- 1x PBS
- 0.2% PBST
- Wash Buffer
- ELISA Reaction Buffer with Phenylenediamine

- 8N H_2 SO_4
- TMB-Substrate Solution
- TMB-Stop Solution

The following protocol describes the general procedure for MUC5B-ELISA tests. It contains descriptions for the establishment of *Direct ELISA* and *Sandwich ELISA*, as well as operating the sera. The protocol is the same, but sometimes the reagents and dilution series differed by actual need.

1) After delivery of the recombinant MUC5B, it was immediately stored in aliquots at -80°C, to avoid repeated thawing of the antigen, which could lead to degradation.
   To establish the optimum standard curve, we diluted the stock-solution of MUC5B. The dilution range of the protein was between **250 ng/ml** and **1.25 ng/ml**.
   MUC5B was mixed mostly in Carbonate Buffer or 1x PBS before we prepared the geometric dilution series. 100 µl were added to each well according to plan. Additionally, of wells one row was only filled with 1x PBS or Assay Buffer, as a negative control.
2) When we established the S-ELISA system, we diluted the Capture Antibody (*Primary Antibody*) between **1:100** and **1:3000** in 1x PBS or Assay Buffer. 100 µl were added into each well according to plan. Immediately after pipetting, the microtiter plates were sealed with an adhesive film. The plates were stored overnight at 2°C, allowing the binding process to take place.
3) On the following day, the plates were washed once with 400 µl Wash Buffer. After the washing step, the plates were tapped on absorbent pad or paper towel to remove excess Wash Buffer. 250 µl of Blocking Buffer were added to each well and incubated at room temperature for 1 hour. Washing steps were repeated two times.
4) When we included the serum samples, we used a dilution range between **1:2 - 1:500**. The sera were diluted in Sample Diluent. 100 µl of the diluted serum sample mixture was coated per well and stored overnight at 2°C. On the next day, the plates were washed 4 times. Plates were blocked again with 250 µl Blocking Buffer for 1 hour at room temperature.
5) The Detection Antibody (*Secondary Antibody*) was diluted in Assay Buffer or 1x PBS and dilutions differed between each experiment. Dilutions ranged between **1:500** and

1:32000. 50 µl per well were added for 1 hour at room temperature. Washing steps were repeated 6 times.

6) POX-anti-rabbit or POX-anti-mouse antibody was commonly prepared in Assay Buffer and the dilution factor was always **1:4000**. 100 µl were added per well and incubated for 1 hour at room temperature. Then 6 washing steps were done. We used two different methods for visualizing the enzyme reaction:

a) An ELISA Reaction Buffer with Phenylenediamine (see description in *Materials Reagents - Buffers and Solutions*) was prepared. 100 µl of this mixture was added to each well and incubated at room temperature for approximately 10 minutes. After that time, the color reaction was stopped by adding 50 µl 8N H_2SO_4. The color intensity can be read by the ELISA plate reader at 490 nm and 630 nm.
b) As an alternatively method, we sometimes used TMB. Afterwards 6 repeats of washing steps, 50 µl of TMB-Substrate Solution was quickly added to all wells and incubated at room temperature for about 10 minutes. We avoided the direct exposure of the plate to intense light. The enzyme reaction was stopped by adding 50 µl TMB-Stop Solution to all wells. The absorbance can be read at 450 nm.

6. Results

6.1 Patient selection

During surgical resection of central adenocarcinomas, relatively large amounts of cancer and lung tissue are resected, which are disposed by the pathologists after having taken samples for histology. This is a chance for thorax surgeons, protein-biochemists and molecular pathologists to identify new risk- and cancer-markers. So far in the unemployed tissue, one can find new cancer cell-specimens. Through interdisciplinary collaboration of clinical lung surgeons and laboratory teams, it was possible to already screen for differences between tumors, control tissue and establish proteome data banks. The scientists defined eight protein candidates, of which four can be useful for serum tests. 40 blood samples were collected and documented with serial numbers by the clinical partner. Patients invited for tissue or serum donation gave their informed consent, to ensure ethic and legal standards, like to ensure clear regulations of sample ownership, patient privacy and patient self determination. Generally, there is no way to receive information of the identity of the patients due to the way the data is presented in this diploma thesis. After the patient was examined by the physician, all blood samples were drawn before anesthesia or surgery in the clinic. For using the ELISA as a diagnostic strategy for human serum-samples, fresh blood was collected and delivered by the clinical partner. Serum was immediately prepared and stored as described in *Material and Methods section 5.2.2,* p. 31.

6.1.1 Patient table with descriptions and staging

The following table (**Table 7**) shows all patients that were used in the ELISA experiments. The list refers to sex, age and staging information. We counted more females than males that were affected by lung adenocarcinoma in our cohort. Comparing **14** female and **4** male patients, the mean age was **60,3** years. The control group consisted of **15** females and **5** males.

In order to describe the patient's tumors, we used the following parameters:

T… describes the size or direct extent of the primary tumor and whether it has invaded into the surrounding tissue. It can be specified with **T0-T4**. **TX** means no clear evidence for a primary tumor incidence.

N… describes the degree of tumor spread to regional lymph nodes. It can be specified with **N0-N3**. **N0** means, that the tumor is absent from regional lymph nodes and does not yet affect the nearby tissue. **N3** means that the tumor spread to more distant or other regional lymph nodes. **NX** means no clear evidence for lymph node affection.

G… describes the differentiation grade of the tumor tissue. It can be specified with **G1-G4**. The "low grade" **G1** is well differentiated, which means that the tumor tissue is very similar to the original tissue. The "high grade" **G4** defines poorly differentiated cells that appear very different than the original tissue.

M… is another parameter of the TNM-staging system and was not included here. All patients were **M0**, because we only had patients off the time of their first surgical treatment, so the **M** was not yet known.

patient number	sex	age	tumor staging		
			T	**N**	**G**
P1	f	58	T2	N2	/
P2	m	55	T2	N0	/
P3	f	53	T1	N0	G1
P4	f	70	T2	N0	G2
P5	f	71	T1	N2	G1
P6	f	65	T1	N2	G1
P7	f	71	T3	N0	G1
P8	m	72	T3	N0	/
P9	f	67	T1	N1	G2 + G2 - G3
P10	f	60	T2	N0	G2
P11	f	35	TX	N2	G2
P12	f	70	T4	N2	G1
P13	f	64	T1	N2	G1
P15	m	64	T1	N0	G1
P16	f	57	T1	N0	G1
P17	f	49	T2	N0	G3
P19	m	51	T1	N0	/
P20	f	54	T1	N2	/

Table 7: Table of all patients with information about sex, age and staging

The table shows the correct numbering of all used patient samples in the ELISA experiments. The numbering was specially used after the working conditions in my experiments. So the order of numbering was individually arranged. Key date of the age information was **31.12. 2010**.

According to pathologists´ examinations, all tumors were lung adenocarcinomas. During our study, there occurred two deviations in the diagnostic evaluation by the pathologists:
P8 was later identified as a lung squamous epithelium carcinoma. Although we are actually concerned with lung adenocarcinomas, we nevertheless kept this patient sample in the evaluation. We were interested to observe, which ELISA results this patient serum would deliver.
P14 contained small groups of high-grade atypical cell elements. After repeated and exact inspection of the patient preparation, the pathologists did not identify a lung adenocarcinoma. Because of that fact, we excluded patient number **P14**.

Generally, we could not observe a correlation between **T** (tumor size) and **N** (lymph node status), as well as **T** and **G**, which indicates that the metatstatic potential of lung adenocarcinoma differs from tumor size and differentiation grade.

6.2 Establishment of ELISAs against four potential lung tumor antigens

The following results sections describe the detailed execution of each single ELISA test procedure in order to establish proper detection of the proposed tumor antigens. In order to accomplish this aim, we followed a clear stepwise **experimental scheme**:

1. Establishment of specific ELISAs by using recombinant antigens
This part presents the ELISA establishment phase using the recombinant proteins and continues with the establishment of the much more sensitive Sandwich ELISA-systems. The recombinant candidate proteins were additionally visualized in Dot Blots, and various conditions were tested to optimize the detection protocols. If necessary, recombinant forms of the suggested tumor marker proteins might be helpful in clarifying or overcoming potential masking effects occurring through the serum composition.
In detail, here we aimed to:

- Define optimal coating and blocking conditions
- Define optimal antibody concentrations (First and Secondary Antibody, etc.)
- Control specificity by respective dilution series and blocking studies
- Define and optimize the sensitivity threshold

2. Testing of antigen-abundance in serum samples

After the successful ELISA establishment, we turned our attention to the main objective of this thesis: To prove whether these antigens were available in sera from patients suffering from lung adenocarcinoma. The application of control sera and the inclusion of antibody controls (non-specific Primary IgG Antibodies, Secondary Antibodies only) in the test-system will be important additional parameters to define the specificity of the detection system and will allow a stepwise fine-tuning of this ELISA test (positive control, control sera, negative controls, isotype controls…).

3. Comparison of patients´ versus control group sera

If there were measurable significant level differences in the sera, this could be a potential candidate-marker, which can be prospectively used in further clinical serum-tests. At the end of this work phase it was determined which proteins, which antibodies, and which serum detection method could have the potential for a serum tumor marker. Provided that the methodology (antibody quality) can be established during the thesis, it will be possible to measure the protein concentration within two days after blood taking from the patient.

6.3 Cytoglobin Detection

6.3.1 Establishment of Direct ELISA using recombinant CYGB

In the first experiment with Cytoglobin, we gained to establish the optimum concentrations of the respective antigen and antibodies. A checkerboard titration is the ideal system to investigate which dilution is proper for a significant signal to perform a quantitative evaluation of an ELISA test. As seen in **Fig. 10**, we worked with a CYGB-antigen dilution starting at **250 ng/ml** and ending at **15.6 ng/ml**. The antibody (*Santa Cruz* rabbit-anti-CYGB antibody) dilution range started at **1:500**, and subsequent **1:1** dilutions were performed. The POX-labeled Secondary Antibody was diluted **1:4000**.

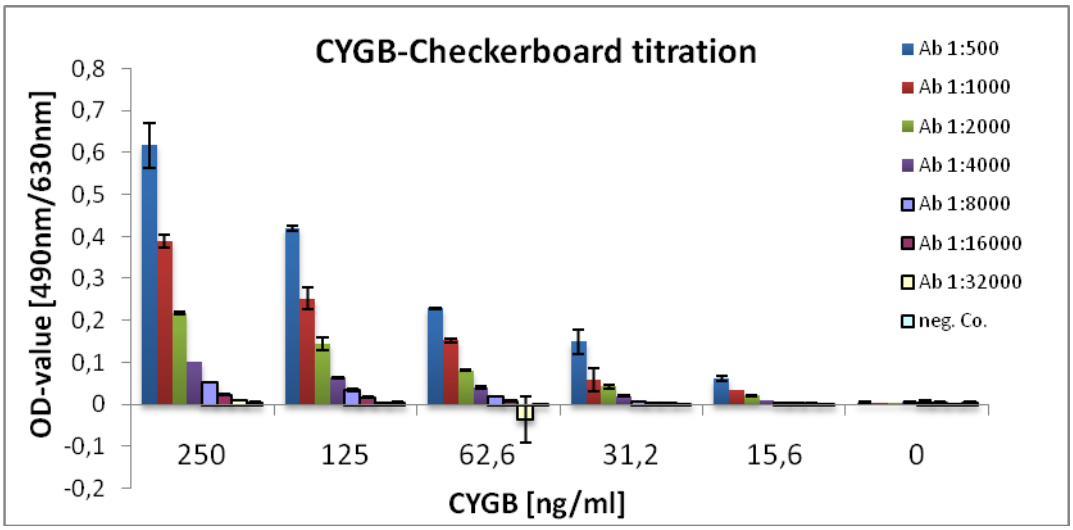

Fig. 10: Checkerboard titration of Cytoglobin concentrations

Different antigen and antibody dilutions were used to establish optimal antigen and antibody concentrations in ELISA.

As the optimum antigen concentration range we detected the range between **250 ng/ml** and **125 ng/ml** with an antibody dilution of **1:500 – 1:1000**. OD-values with lower concentrations of antigen and antibody were too low to represent a dilution that can be utilized in further assay experiments. The graphic shows a perfect linear decrease of the OD-values, which was determined with the respective concentrations and dilutions. The negative control showed that no unspecific process of antigen or antibody binding disturbed the binding reaction in the ELISA.

6.3.2 First Direct CYGB-ELISA with 10 serum samples

After the successful pretest with the checkerboard titration of Cytoglobin, we immediately tried to test 10 serum samples. As a positive control we also included a CYGB standard series starting at **250 ng/ml** and ending at **15.6 ng/ml** which is seen in **Fig. 11**. The graphic shows a perfect continuous decrease of the values, which means that the positive control worked fine on the ELISA plate.

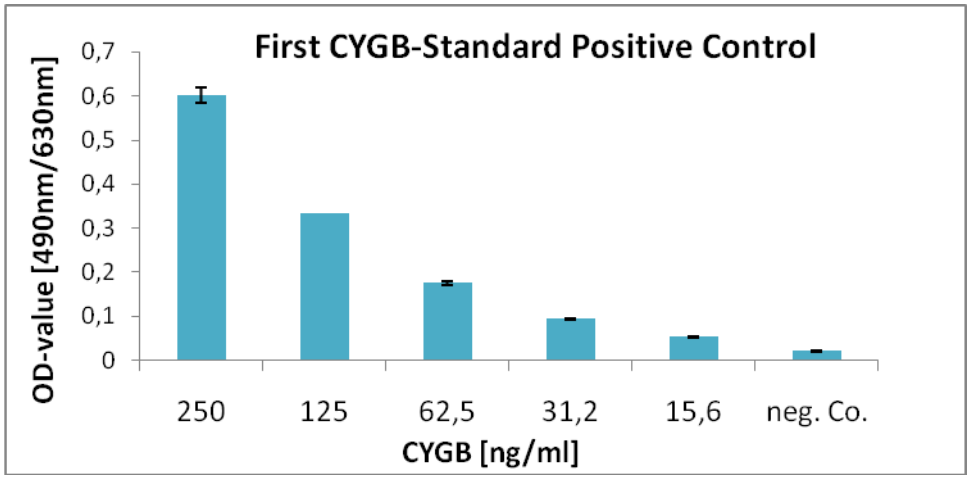

Fig. 11: First Cytoglobin Positive Control-ELISA

This dilution series was tested on the same ELISA plate as the serum samples.

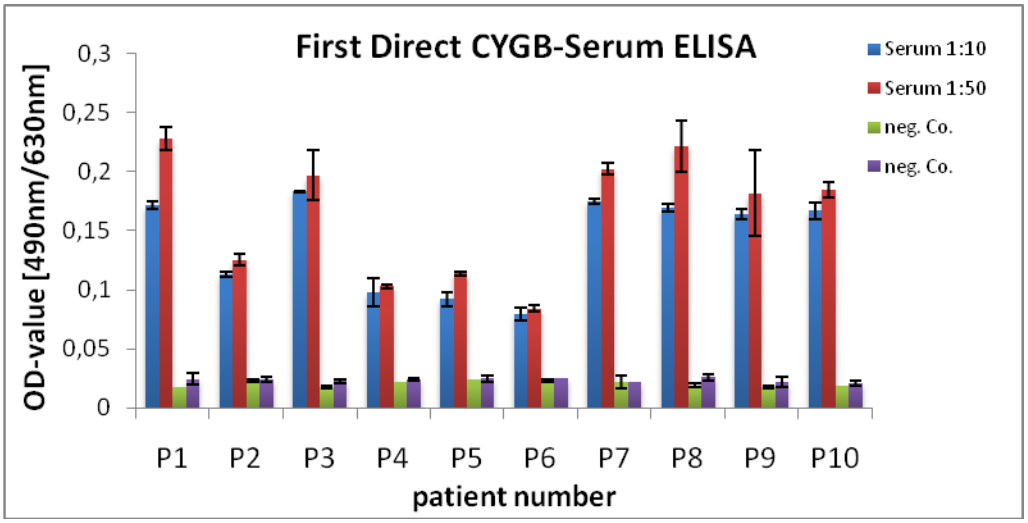

Fig. 12: First Direct Cytoglobin-Serum ELISA

10 patient's sera were tested in 2 dilutions. Negative controls contained no Primary Antibody.

In **Fig. 12** we see the ELISA results of the tested serum samples (**P1-P10**). Two dilution steps of the sera, **1:10** and **1:50**, were applied, to receive information about the ELISA sensitivity. Indeed, we detected specific positive signals versus the negative controls. We worked with a dilution of **1:500** of the Primary Antibody (*Santa Cruz* rabbit-anti-CYGB antibody) and with a dilution of **1:4000** of the POX-labeled Secondary Antibody (rabbit).
Anyway, an interesting phenomenon occurred between the two serum dilutions. It was expected that the signals of the dilution of **1:50** would be lower than those of **1:10**. Interestingly, with the of **1:50** dilution showing a significant higher value compared to the **1:10** dilution. The negative control was below detection threshold, excluding unspecific effects of the antibodies. In further experiments we planned to use higher serum dilutions in order to see whether this we were able to leave the supposed plateau pahse signal intensity.

6.3.3 Second Direct CYGB-ELISA with the same 10 serum samples

The test arrangement of this experiment were the same as in the pretest concerning the dilutions of Primary Antibody and POX-labeled Secondary Antibody. The interesting phenomenon, that higher dilutions showed at least higher OD-values, was further analyzed. As a positive control we also included CYGB standard starting at **250 ng/ml** and ending at **15.6 ng/ml** that can be seen in **Fig. 13**. The graphic shows a continuous decrease of the values, again indicating that the positive control worked fine on the ELISA plate.

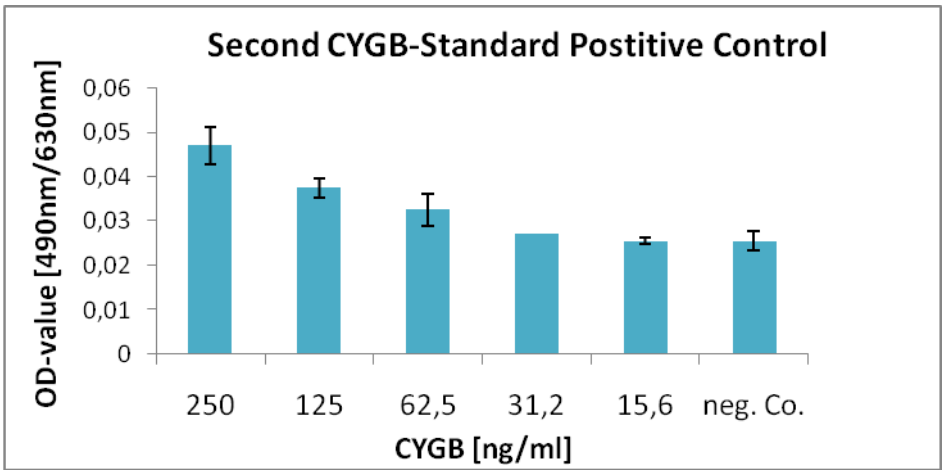

Fig. 13: Second Cytoglobin Positive Control-ELISA

This dilution series was tested on the same ELISA plate beside the serum samples.

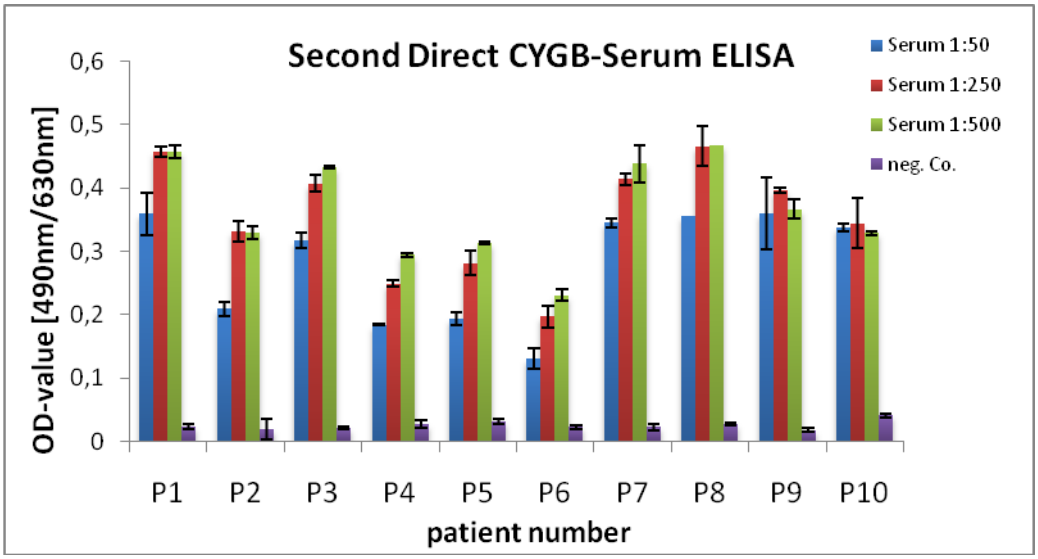

Fig. 14: Second Direct Cytoglobin-Serum ELISA

10 patient's sera were tested in 3 dilutions. Negative controls contained no Primary Antibody.

In **Fig. 14** the results of the same tested serum samples (**P1**-**P10**) as in **Fig. 10** are presented. This time, three higher dilution steps of the sera (**1:50**, **1:250** and **1:500**) were applied with the aim to receive more information about this observation.

Interestingly, the phenomenon of increasing OD-values with even higher serum dilutions again occurred: The bar diagram of **1:50** shows a significant lower value compared to the dilution of **1:250** and **1:500**. The negative control worked well.

As mentioned before, the reason for the observed signal intensity phenomenon remained unclear. We suggested that the antibody might recognize additional antigens or organic material in the human sera. Another reason was that we were still working in the saturation region. In the following experiment we planned to use even higher serum dilutions to investigate this question.

6.3.4 Third Direct CYGB-ELISA with the same 10 serum samples

The test arrangement of this experiment concerning the dilutions of Primary Antibody and POX-labeled Secondary Antibody were the same as in the previous test. The phenomenon, that higher dilutions lead to increased OD-values, was further analyzed.

As a positive control we also included a CYGB standard series starting at **250 ng/ml** and ending at **15.6 ng/ml** that can be seen in **Fig. 15**. The graphic shows a well done continuously decrease of the OD-values, which means that the positive control worked fine.

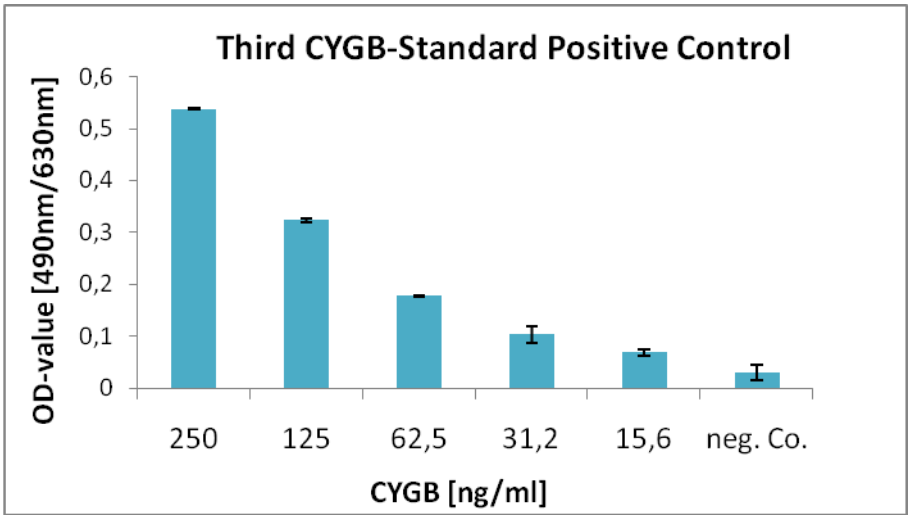

Fig. 15: Third Cytoglobin Positive Control-ELISA

This dilution series was tested on the same ELISA plate beside the serum samples.

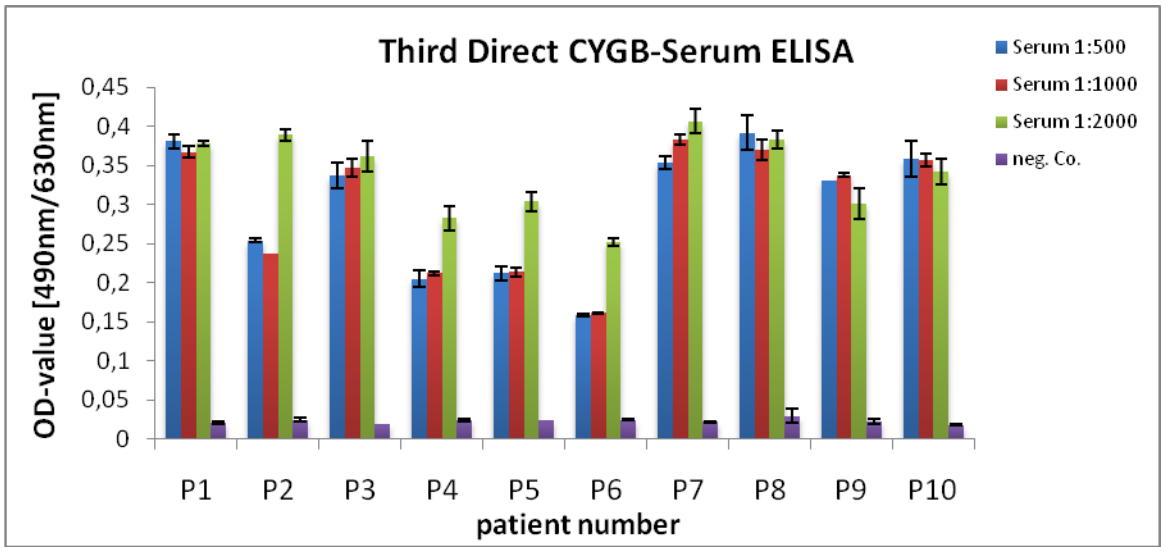

Fig. 16: Third Direct Cytoglobin-Serum ELISA

10 patient's sera were tested in 3 dilutions. Negative controls contained no Primary Antibody.

In **Fig. 16** the ELISA results with the same tested serum samples (**P1-P10**) are presented. This time, further even higher dilution steps of the sera (**1:500**, **1:1000** and **1:2000**) were performed to see the effect in this dilution range. The phenomenon of non-decreasing OD-values again occurred with the three serum dilutions. The bar diagram of **1:500** showed a significant higher value compared to the **1:1000** and **1:2000** dilutions. The negative control worked well. As mentioned before, the reason why that phenomenon can be observed was unclear. Therefore, in the following experiment we intended to explore via Western Blot the abundance of Cytoglobin in the sera.

6.3.5 Cytoglobin Western Blot with 13 serum samples

Since we were not able to answere the question of the phenomenon of increasing bar diagrams, we intended to perform a Western Blot. We used patient **P1**-**P13** to observe the protein bands contained in the sera after resolving them on an acrylamid SDS-gel running through the gel at 23V overnight. The proteins were then transferred to nitrocellulose and this blot was incubated, as in the ELISA, with anti-CYGB and POX-labeled anti-rabbit antibody. The *Santa Cruz* rabbit-anti-CYGB antibody was diluted **1:200** and the POX-labeled rabbit secondary antibody was diluted **1:3000**.

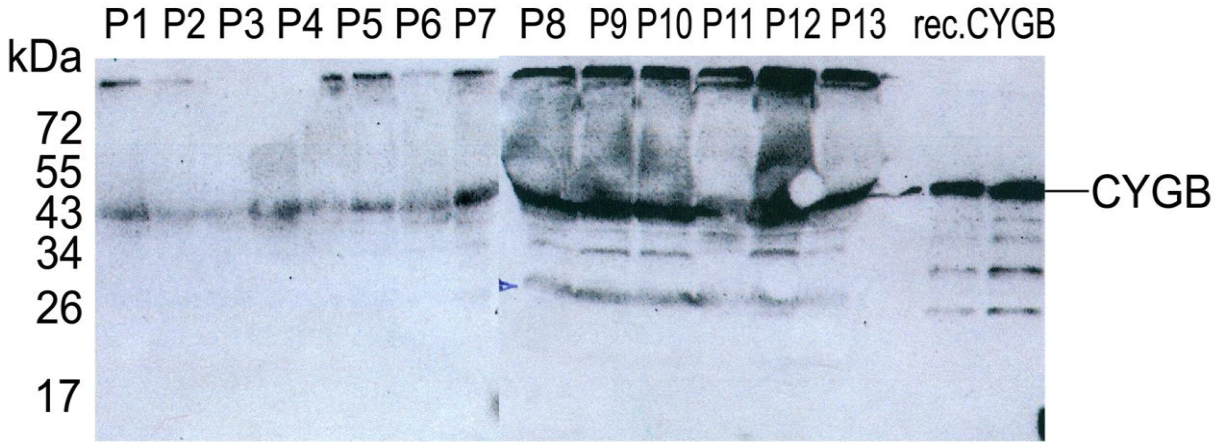

Fig. 17: Western Blot of patients´ sera probed with anti-CYGB antibody

Fig. 17 shows the results of this analysis. The proteins have been separated by size, which is determined in *kiloDalton* (kDa), as shown on the left margin of the blot. As a control, we also loaded recombinant CYGB on the blot to see, to which band the antibody would bind. Interestingly, the *Santa Cruz* antibody reacted predominantly with a protein band migrating at approximately 43 kDa, whereas its proposed molecular weight should reside at 48 kDa (http://www.abnova.com/products/products_detail.asp?Catalog_id=H00114757-P01).
This discrepancy can be explained by the presence of the GST-tag fused to the full-length recombinant CYGB protein. This blot proved that Cytoglobin was present in human sera and that the *Santa Cruz* antibody recognized it specifically.

6.3.6 Establishment of Sandwich ELISA using recombinant CYGB

As the antibody unsed in the preliminary direct serum ELISA seemed to be quite unspecific, we aimed to establish a Sandwich ELISA. This was the next option to maybe receive more sensitive results. The CYGB in serum might possibly be caught better with a Capture Antibody than by direct coating onto the wells. In the first experiment we tried to establish the optimum antibody and antigen coating conditions, that could be used in further test. For preparing a geometric dilution series, we started at **25 ng/ml** and ended at **0.3 ng/ml**.

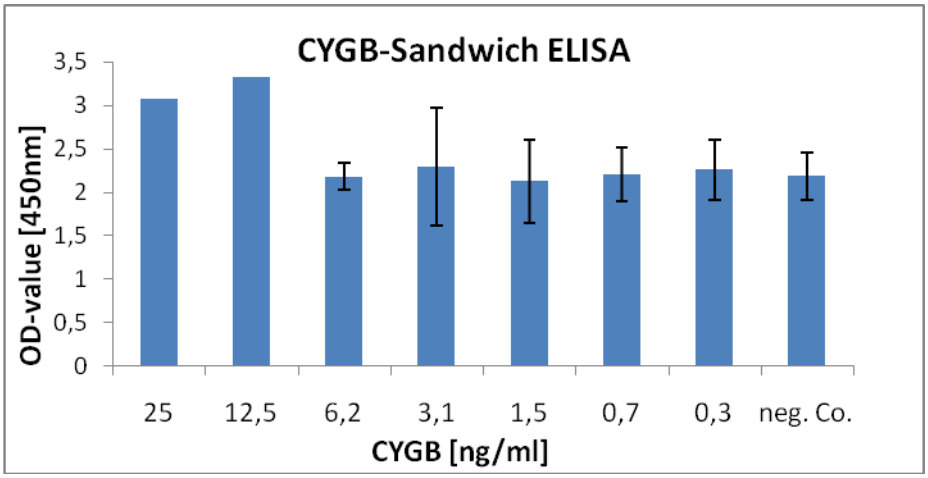

Fig. 18: Cytoglobin Sandwich ELISA

Establishment of a Sandwich ELISA for Cytoglobin. The negative control was performed without antigen.

Fig. 18 shows the result of the S-ELISA, where we used the *Abnova* mouse-anti-CYGB antibody as Capture Antibody, which was diluted **1:250**. As a Detection Antibody we used a *Santa Cruz* rabbit-anti-CYGB antibody at a **1:500** dilution. The POX-labeled rabbit Secondary Antibody was diluted **1:4000**. Strikingly, we observed high OD-values, but there was no significiant OD-value difference between the negative control wells and the recombinant CYGB containing wells. Therefore, we concluded that the two applied antibodies (*Abnova* and *Santa Cruz*) recognized each other, leading to unspecific background signals. Therefore, in the next experiment we intended to compare the S-ELISA and D-ELISA side by side on one plate with alternating Capture and Detection Antibodies, once with *Abnova* mouse and then with *Santa Cruz* rabbit.

6.3.7 Comparison of Cytglobin S-ELISA and D-ELISA

We divided the ELISA plate into 4 rows, so that we were able to change the order of the Capture- and Detection Antibody, as well as to observe which one of the two different antibodies was better to use as a Capture- and which one was better as a Detection Antibody. The important expected accessory effect was to allegorize whether the S-ELISA system is more sensitive than the D-ELISA.

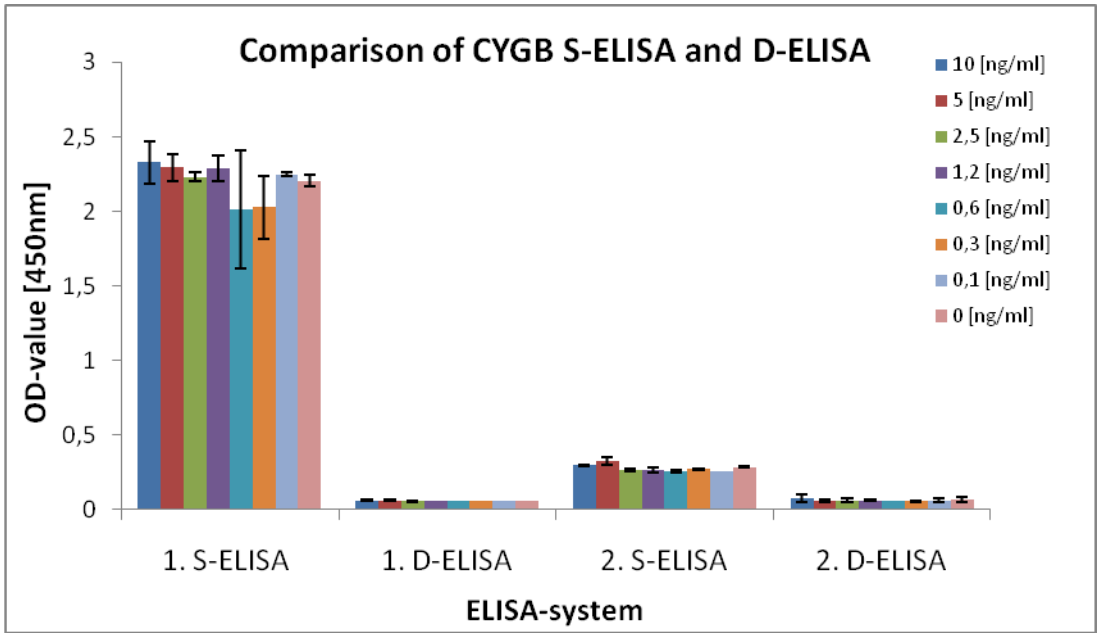

Fig. 19: CYGB S-ELISA and D-ELISA in comparison

This experiment was tested on one ELISA plate. The negative control was performed without antigen.

In this experiment (**Fig. 19**), alternatively *Abnova* mouse anti-CYGB or *Santa Cruz* rabbit anti-CYGB antibody were used as Capture Antibodies. Both antibodies were diluted **1:250**. For preparing a geometric dilution series, we started at **10 ng/ml** and ended at **0.15 ng/ml**. The rabbit anti-CYGB and mouse anti-CYGB antibodies were diluted **1:500**. Both POX-labeled Secondary Antibodies were diluted **1:4000** (mouse respectively rabbit).

In the **1. S-ELISA**, plates were coated with *Abnova* mouse-anti-CYGB antibody as the Capture Antibody and as a Detection Antibody we used *Santa Cruz* rabbit-anti-CYGB antibody. In this test system, we can observe that there are no significant differences between the dilutions and that even the negative control wells present a too high background signal. It is possible that the antibodies recognize each other, and therefore we got such a high signaling.

As expected, the **1. D-ELISA** (we used *Abnova* mouse-anti-CYGB antibody) and **2. D-ELISA** (we used *Santa Cruz* rabbit-anti-CYGB antibody) show extremely low signals, because of the absence of Capture Antibodies. That confirmed our opinion that direct coating of the recombinant protein does not work efficiently enough.

The wells of **2. S-ELISA** were coated with *Santa Cruz* rabbit-anti-CYGB antibody as the Capture Antibody and as a Detection Antibody we used *Abnova* mouse-anti-CYGB antibody. As seen before with S-ELISA, in this interaction we can observe that there are no significant differences between the dilutions and that even the blank wells present a too high background signal. We assumed that *Santa Cruz* antibody is unusable as a Capture Antibody.

In that case with our two antibody products (*Abnova* and *Santa Cruz*), we cannot define the optimum ELISA system, since both of them do not work very effective in combination.

We thought the S-ELISA system would remain to be the better choice in seeking the optimum concentrations for the establishment. Unfortunately we cannot go on with the S-ELISA in combination of these two antibodies, because of the fact that the results view quite unfeasible bar diagrams. In summary, since the antibody from *Santa Cruz* turned out to be more ineffective as a Capture Antibody, we intended to give the *Abnova* product a try, because anyway there are the best results seen in the graphic. It is much better than the antibody from *Santa Cruz* as a Detection Antibody. Maybe the direct coating of the human sera would work better with the *Abnova* as a Detection Antibody. In the next experiment, the standard dilution series will be set higher, because in this experiment, we used very low antigen concentrations.

6.3.8 CYGB Dot Blot

In this experiment we intended to test whether the antigen from *Abnova* operates appropriately with the antibodies from *Abnova* and *Santa Cruz*. **Fig. 20** shows the result of this experiment. Using a geometric dilution series of CYGB, starting at **20 ng** and ending at **1.25 ng**, we observed an expected decrease of the color intensity of each spot. On blot number **1** we observed a stronger background. There seems to be a decrease of the color, but not as strong as in blot number **2**. Especially on blot number **2** we saw two dark spots with the lowest dilutions, going on with higher dilutions that lead to a decreasing color intensity.

The result of the above signal row of the Dot Blot (number **1)** can be interpreted that *Abnova* mouse anti-CYGB antibody might react more intensely than the *Santa Cruz* anti-CYGB antibody. Fact is that both antibodies are capable for further experiments.

1

2

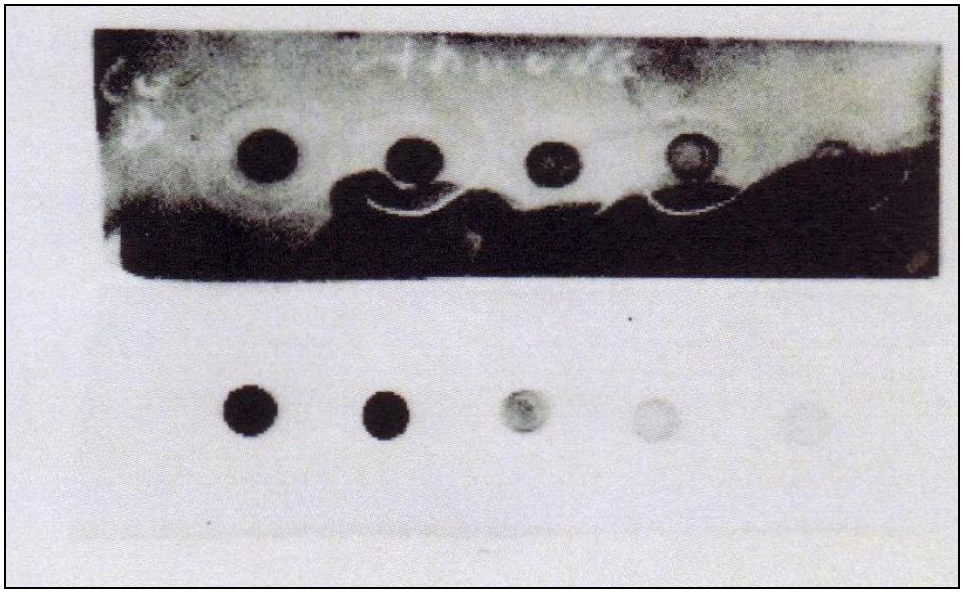

Fig. 20: CYGB-Dot Blot with recombinant protein and two antibodies

The upper blot (number **1**) shows the **1:500** dilution of the *Abnova* mouse-anti-CYGB antibody.
The blot below (number **2**) shows the **1:200** dilution of the *Santa Cruz* rabbit-anti-CYGB antibody.
POX-labeled Secondary Antibody (mouse respectively rabbit) was diluted **1:3500**.

6.3.9 New Establishment of Direct ELISA with recombinant CYGB and Abnova mouse antibody

After the decision to go on with the *Abnova* mouse-anti-CYGB antibody, we needed a standard curve that shows us the optimum dilution, so that we can go further with coating the human sera without a Capture Antibody. **Fig. 21** represents the standard curve with the antibody dilution of **1:500**. For preparing a geometric dilution series, we started at **200 ng/ml** and ended at **12.5 ng/ml**. POX-labeled Secondary Antibody (mouse) was diluted **1:4000**.
Because of the fact that this assay worked very well, we continued with the direct coating of human sera and chose *Abnova* mouse as the Detection Antibody.

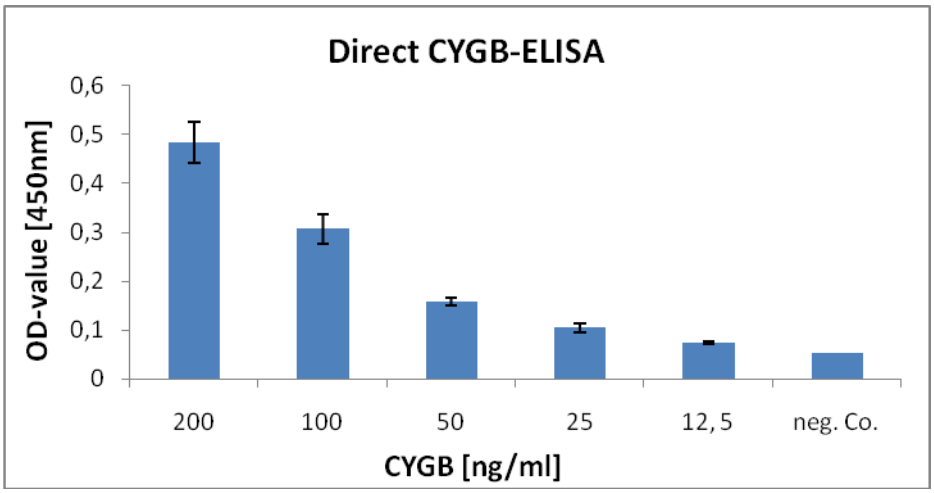

Fig. 21: Direct Cytoglobin ELISA

Establishment of Direct ELISA with *Abnova* mouse antibody. The negative control was performed without antigen.

6.3.10 Direct CYGB-ELISA with all serum samples

As a positive control we created a standard starting at **200 ng/ml** and ending at **25 ng/ml** that is seen in **Fig. 22**. The graphic shows a curve continuous decrease of the OD-values, which means that the positive control worked fine.

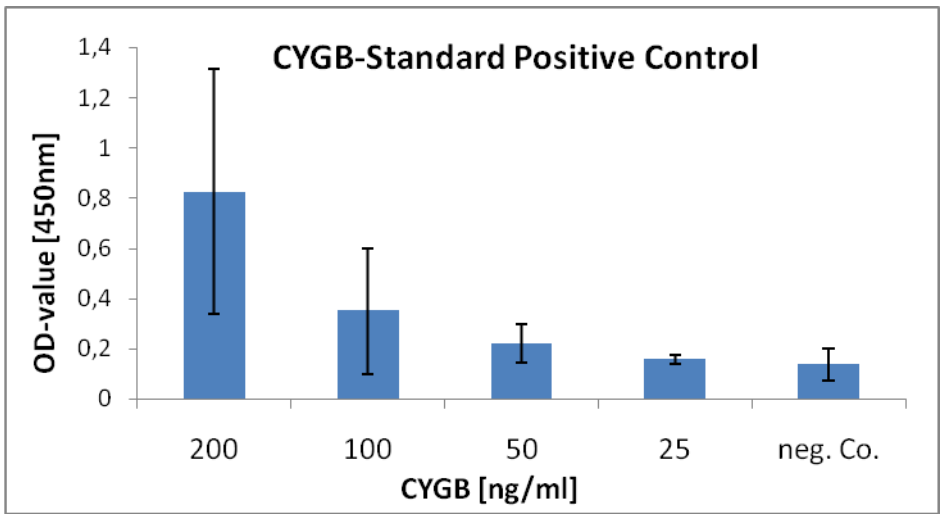

Fig. 22: Cytoglobin Positive Control-ELISA

This dilution series was tested on the same ELISA plate beside the serum samples.

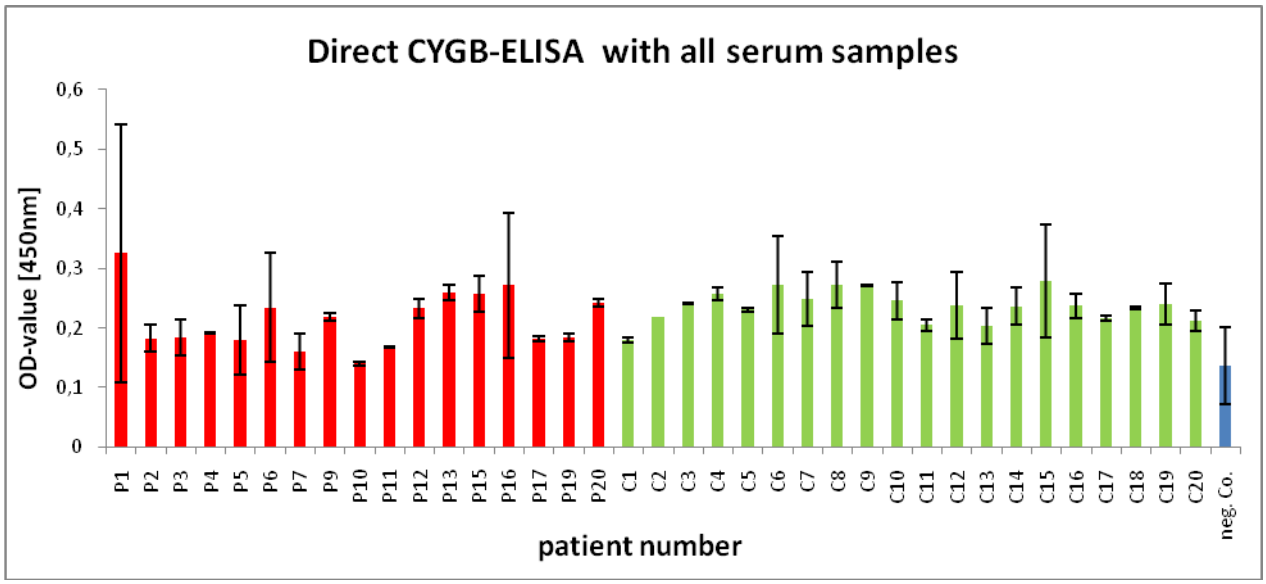

Fig. 23: Direct Cytoglobin-Serum ELISA with all serum samples

Patient sera (*red*) and control group (*green*) was tested on one ELISA plate. The negative control (*blue*) was performed without serum.

We worked with **1:2** sera dilution. The *Abnova* mouse-anti-CYGB antibody was diluted **1:500** and POX-labeled Secondary Antibody (mouse) was diluted **1:4000**. The results can be seen in **Fig. 23**. It can be mentioned, that the negative control wells were significantly lower than the rest of the values, which tells us that the negative control did work out. Interestingly the control group seemed to have higher values than the patients. After these actual results, it can be said that we were able to establish a Cytoglobin ELISA.

After calculating the average OD-values of the patients and the control sera seen, a t-test analysis was performed to evaluate the overall statistic relevant difference between Cytoglobin levels in the sera of patients versus the control group, which is also termed *p-value*. **Fig. 24** shows the mean OD-values of both sample groups, and the corresponding p-value was **0.076**. As in biological systems a p-value of **0.05** is generally considered as significant, which means that there is at least a 95% chance that the observed results are different. Our t-test result indicated that the CYGB-ELISA analysis did not reveal a significant higher level of Cytoglobin in the patients´ versus the control group sera. On the contrary, the values of the control group values seemed to be slightly but unsignificantly higher compared to those of the patients.

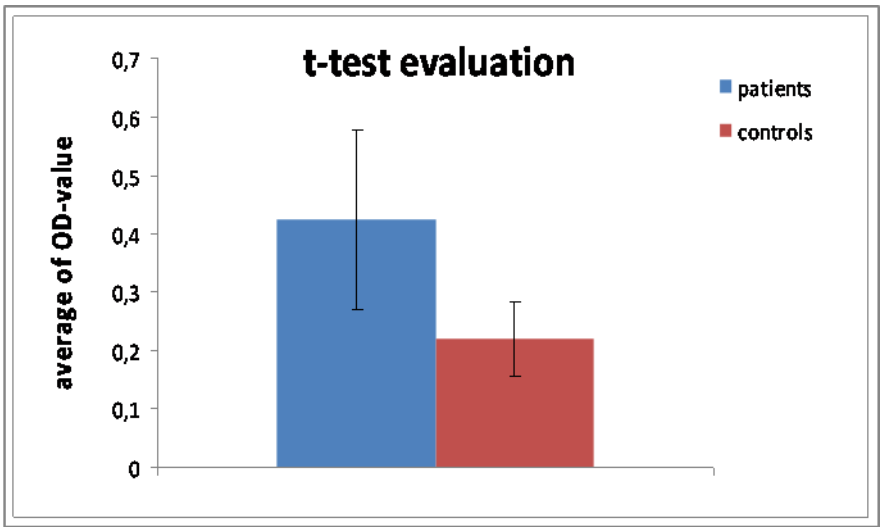

Fig. 24: Average OD-values of patients´ versus control sera in the CYGB-ELISA

6.4 Interleukin 18 Detection

6.4.1 Establishment of Direct ELISA using recombinant IL-18

In the first experiment with Interleukin 18, we gained to establish the optimum concentrations of the antigen and antibodies. A checkerboard titration is the ideal system to investigate which dilution fits for a significant signal in the quantitative evaluation of an ELISA test. We worked with an antigen dilution starting at **250 ng/ml** and ended at **15.6 ng/ml**. The antibody (*Everest Biotech* goat-anti-IL-18 antibody) dilution range started at **1:500** and subsequent **1:1** dilutions were performed. POX-labeled Secondary Antibody (goat) was diluted **1:4000**.

As seen in **Fig. 25** the optimum antigen concentration range was between **250 ng/ml** and **125 ng/ml** with an antibody dilution of **1:500 – 1:1000**. The other values were too low to represent a useful dilution that can be utilized in further assay experiments. The graphic shows a perfect linear decrease of the values, which was estimated with these concentrations and dilutions. The negative control showed that no other process of the antigen or antibody binding disturbed the binding reaction.

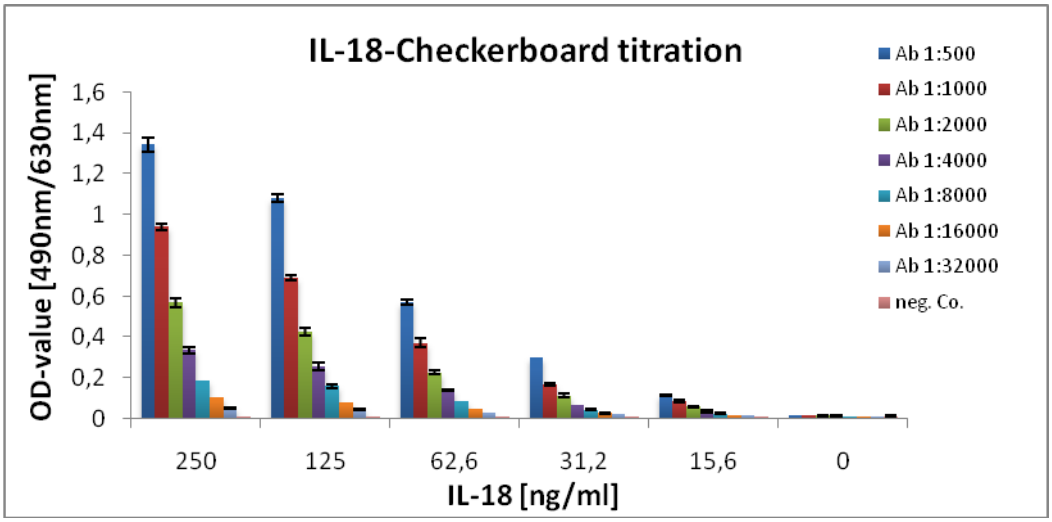

Fig. 25: Checkerboard titration of Interleukin 18 concentrations

Different antigen and antibody dilutions were used to establish optimal antigen and antibody concentrations in ELISA.

6.4.2 First Direct Interleukin-18 ELISA with 10 serum samples

After the successful pretest gaining optimal positive signals with the checkerboard titration of Interleukin 18, we tried to test 10 serum samples. As a positive control we also created an IL-18 standard starting at **250 ng/ml** and ending at **15.6 ng/ml** that is seen in **Fig. 26**. The graphic shows a continuous decrease of the values, which means that the positive control worked fine on the ELISA plate.

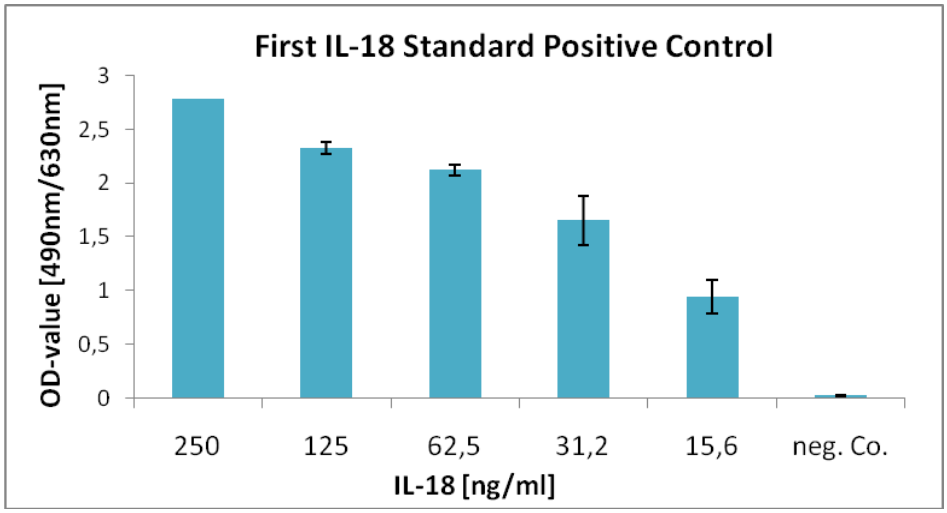

Fig. 26: First Interleukin 18 Positive Control-ELISA

This dilution series was tested on the same ELISA plate beside the serum samples.

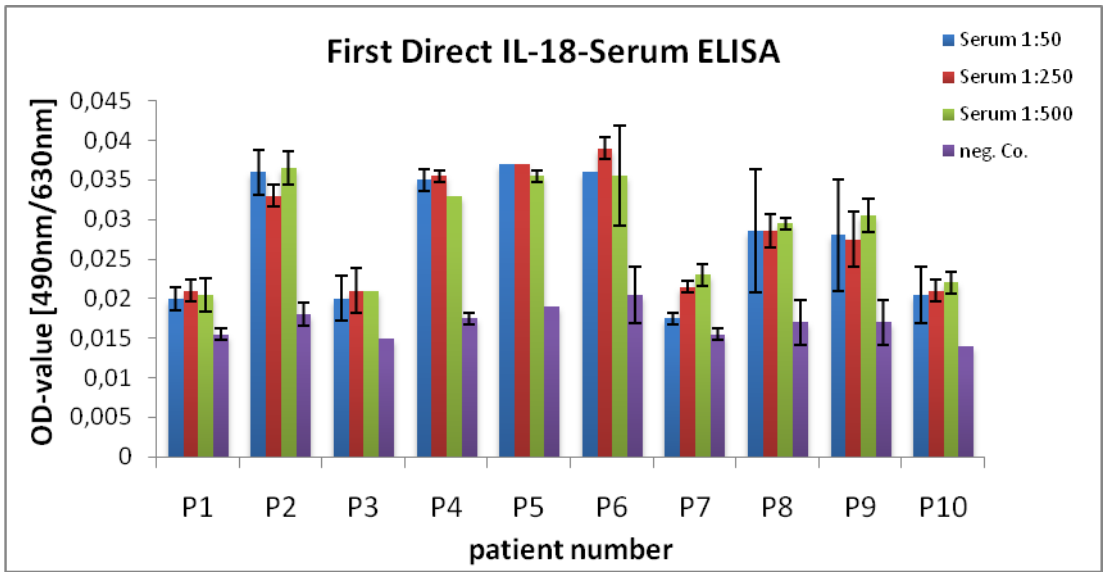

Fig. 27: First Direct Interleukin 18-Serum ELISA

10 patient's sera were tested in 3 dilutions. Negative controls contained no Primary Antibody.

In **Fig. 27** we see the ELISA results of the tested serum samples (**P1-P10**). We worked with three sera silutions (**1:50**, **1:250** and **1:500**). The dilution of the Primary Antibody (*Everest Biotech* goat-anti-IL-18 antibody) was **1:500** and the dilution of POX-labeled Secondary Antibody (goat) was **1:4000**. We detected positive signals versus the neagitve controls.
Following the three different sera dilutions, we could not observe an expected decrease of signals. Sometimes the values seen in the graphic increased, although the dilution was higher. Generally the measured OD-values were too low, indicating that no significant result was obtained. In the next experiment, we tried to work with higher serum concentrations in order to gain higher signals.

6.4.3 Second Direct Interleukin-18 ELISA with the same 10 serum samples

The test arrangement of this experiment concerning the dilutions of Primary Antibody (*Everest Biotech* goat-anti-IL-18 antibody) and POX-labeled Secondary Antibody (goat) was the same as in the pretest. Just the dilution range of the standard curve and the dilutions of the sera differed in this experiment. This time, we started the dilution of the standard at **30 ng/ml** and ended at **0.93 ng/ml** that is seen in **Fig. 28**. The graphic shows a continuous decrease of the standard values, which means that the positive control worked fine on the ELISA plate.

In **Fig. 29** the results of the tested serum samples (**P1-P10**) are presented. This time three more concentrated dilution steps of the sera (**1:10**, **1:25** and **1:50**) were applied to receive more information about the reaction. Following the three different sera dilutions, we could not observe an expected decrease of the OD-values although we worked with higher concentrated dilutions. Generally the measured OD-values were too low again, so that no significant result could be obtained. In the next experiment, we tried to work with even higher concentrated serum dilutions, to hopefully gain higher signals.

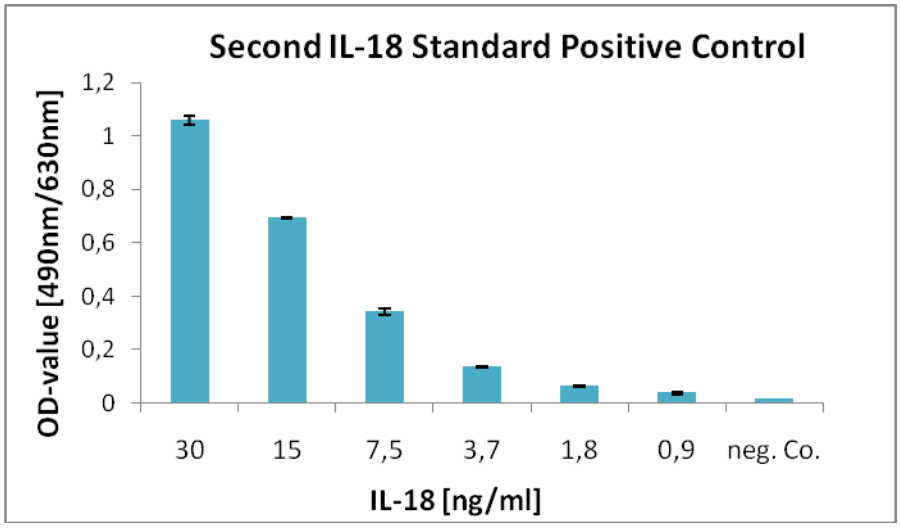

Fig. 28: Second Interleukin 18 Positive Control-ELISA

This dilution series was tested on the same ELISA plate beside the serum samples.

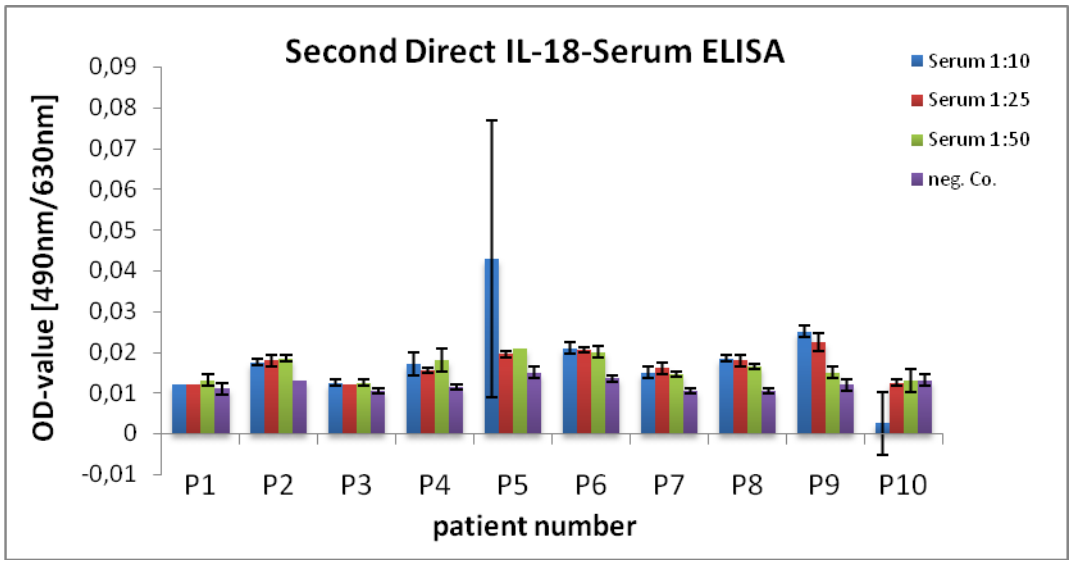

Fig. 29: Second Direct Interleukin 18-Serum ELISA

10 patient's sera were tested in 3 dilutions. Negative controls contained no Primary Antibody.

6.4.4 Third Direct Interleukin-18 ELISA with the same 10 serum samples

The test arrangement of this experiment concerning the dilutions of Primary Antibody (*Everest Biotech* goat-anti-IL-18 antibody) and POX-labeled Secondary Antibody (goat) were the same as in the previous test. Just the dilution range of the IL-18 standard curve and the dilutions of the sera differed to the other experiment. This time, we started the dilution of the standard at **10 ng/ml** and ended at **0.31 ng/ml** which is seen in **Fig. 30**. The graphic shows a well done continuously decrease of the values, which means that the positive control worked fine on the ELISA plate. We detected significant positive signals versus the negative controls.

In **Fig. 31** the results of the tested serum samples (**P1-P10**) are presented. This time, three even more concentrated serum dilution steps (**1:2**, **1:5** and **1:10**) were applied, to receive more information about the reaction. Following the three different sera dilutions, we could not really observe a strong increaese of the OD-values, although we worked with higher concentrated dilutions. Generally, the measured OD-values were very low again, but there seemed to be a little signal increase. From these data we concluded that the IL-18 amounts were very low in the sera. As a conclusion to our results with IL-18, we decided to order matched antibody pairs, that could be more sensitive with the human sera. Following the instance, described in the product information, we should be able to gain positive and significial results. The following experiment described the working results with the IL-18 Module Set.

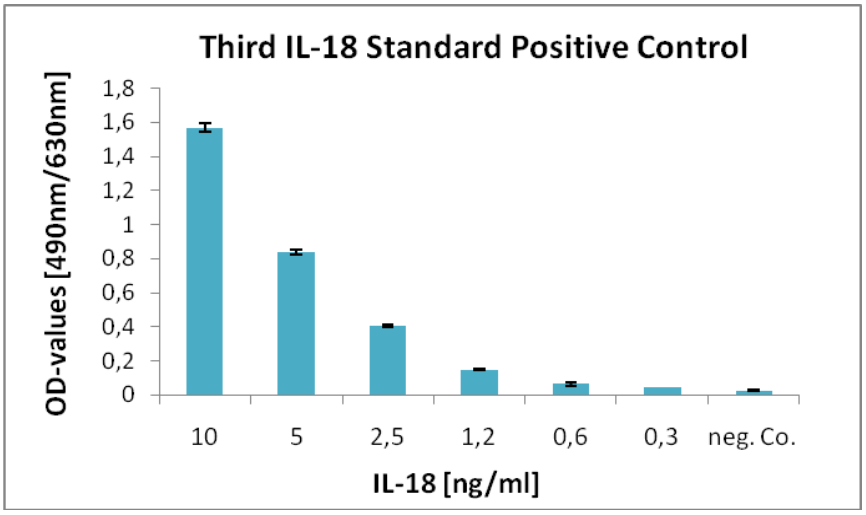

Fig. 30: Third Interleukin 18 Positive Control-ELISA

This dilution series was tested on the same ELISA plate beside the serum samples.

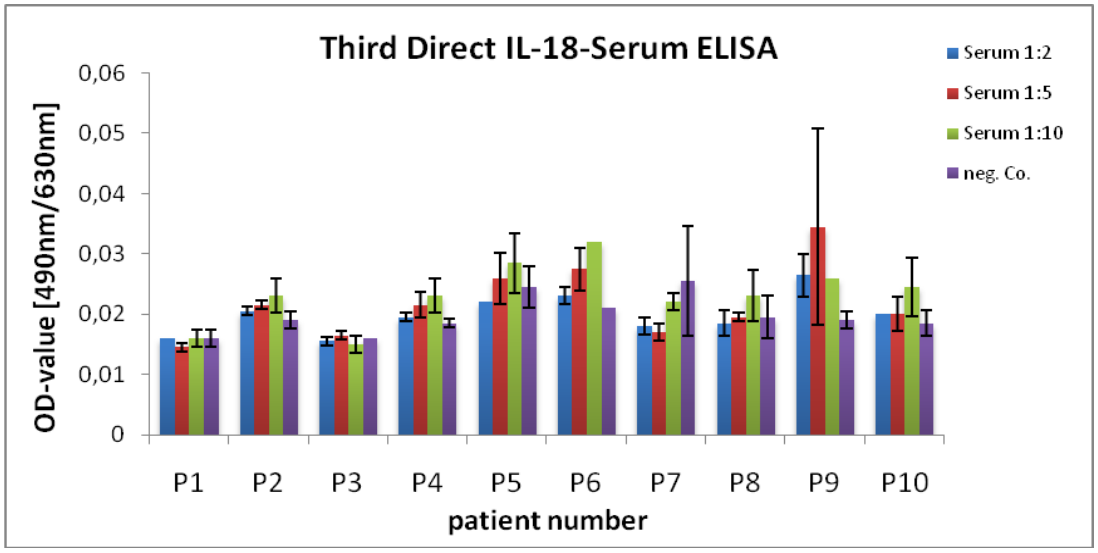

Fig. 31: Third Direct Interleukin 18-Serum ELISA

10 patient's sera were tested in 3 dilutions. Negative controls contained no Primary Antibody.

6.4.5 Sandwich Interleukin-18 ELISA with the same 10 serum samples

The test arrangement of this experiment was now achieved with the reagents of the ordered *Bender MedSystems*® IL-18 Module Set. The dilution range of the standard curve started at **10 ng/ml** and ended at **0.62 ng/ml**. The positive control can be seen in **Fig. 32**. The graphic shows a continuous decrease of the values, which means that the positive control worked fine on the ELISA plate. We detected positive signals versus the neagitve controls.

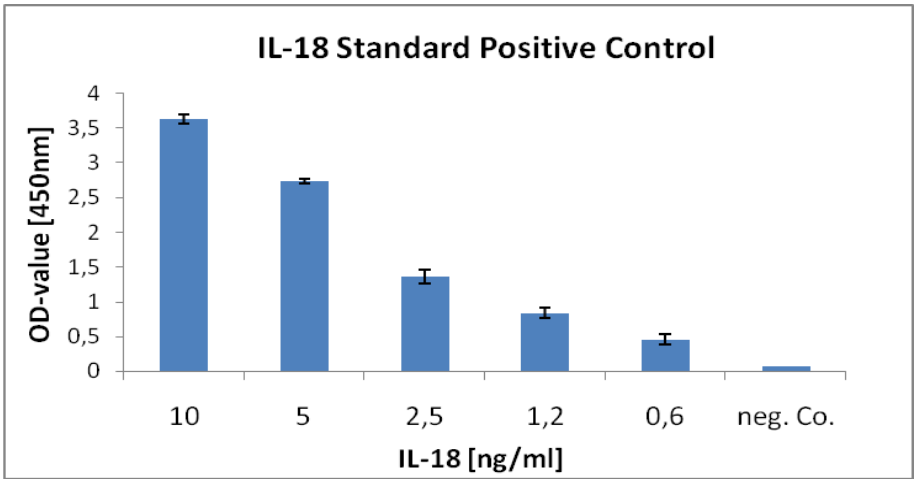

Fig. 32: Interleukin 18 Positive Control-ELISA

This dilution series was tested on the same ELISA plate beside the serum samples.

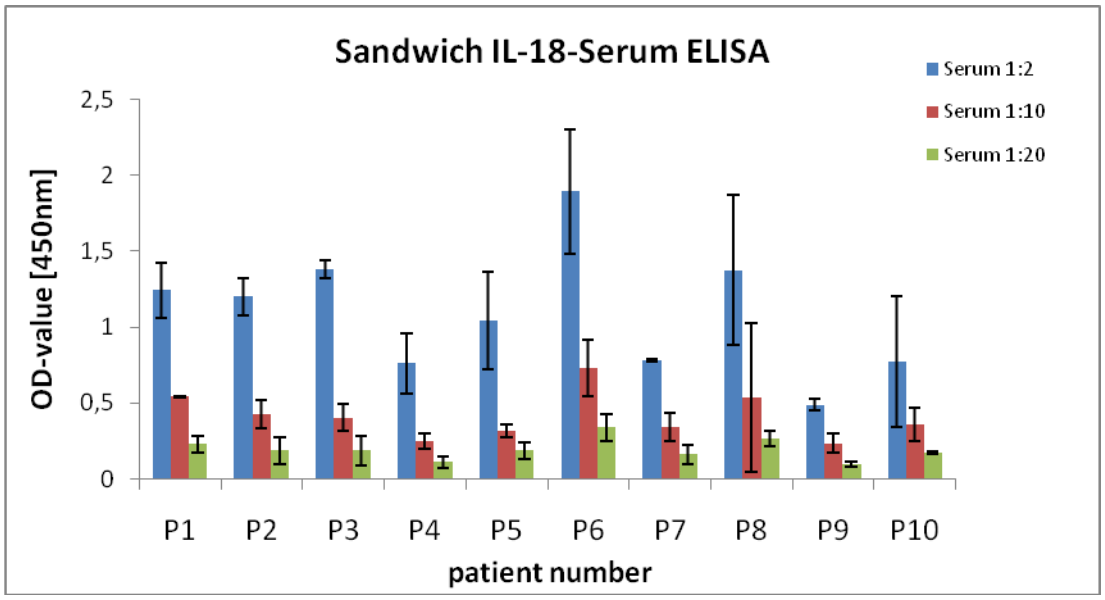

Fig. 33: Sandwich Interleukin 18-Serum ELISA

10 patient's sera were tested in 3 dilutions.

6.4.6 Sandwich Interleukin-18 ELISA with 40 serum samples

Again the test arrangement of this experiment was achieved with the reagents of the ordered *Bender MedSystems*® IL-18 Module Set. The dilutions of Concentrated Biotin-Conjugate and Concentrated Streptavidin-HRP was the same as in the pretest. The dilution range of the standard curve started at **5 ng/ml** and ended at **0.07 ng/ml**. We decided to go on with sera dilutions of **1:2**, since working with these showing the best OD-values. The positive control can be seen in **Fig. 34**, showing continuous decrease of the values, which means that the positive control worked fine on the ELISA plate. We detected positive signals versus the negative controls.

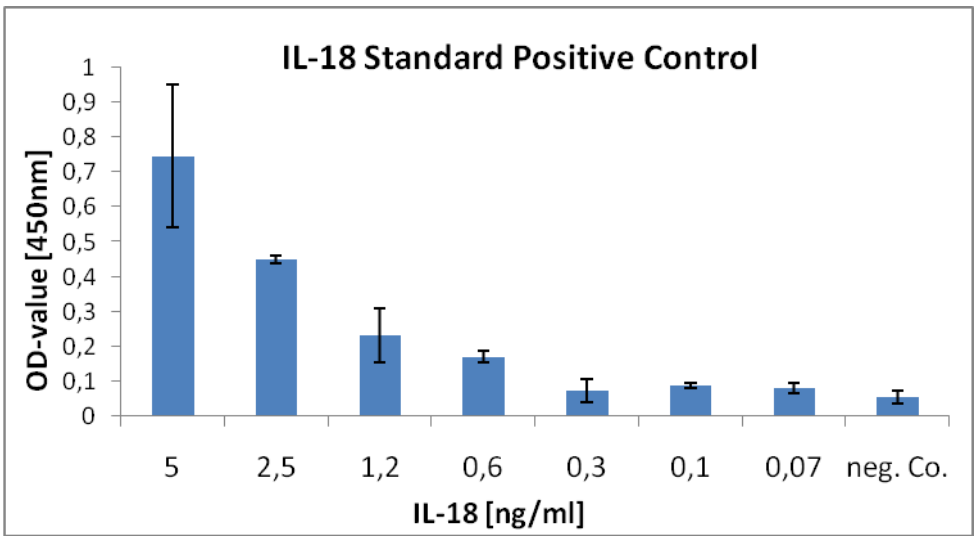

Fig. 34: Interleukin 18 Positive Control-ELISA

This dilution series was tested on the same ELISA plate beside the serum samples.

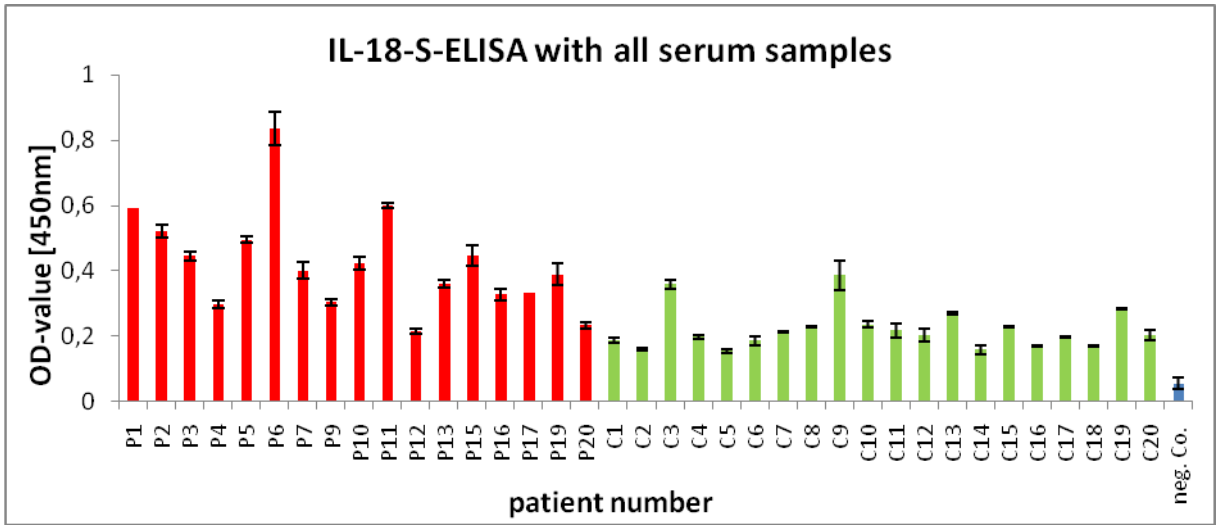

Fig. 35: Interleukin 18-Serum ELISA with all serum samples

Patient sera (*red*) and control group (*green*) was tested on one ELISA plate. The negative control (*blue*) was performed without serum.

Fig. 35 shows the results for all serum samples. The negative control wells were compared to the other values very low, so the negative control was supported. Generally, it can be said that this established IL-18 ELISA can be used for further analyses.

Further, a t-test analysis was performed to represent the overall statistic relevant difference between the patient and the control group. **Fig. 36** shows the mean OD-values of both, the patient's and the healthy control group, and the corresponding p-value was **0.000047**. As shown in the graphic, IL-18 levels were significantly higher in the patients than in the control group. Therefore, we concluded that IL-18 might be a promising candidate biomarker.

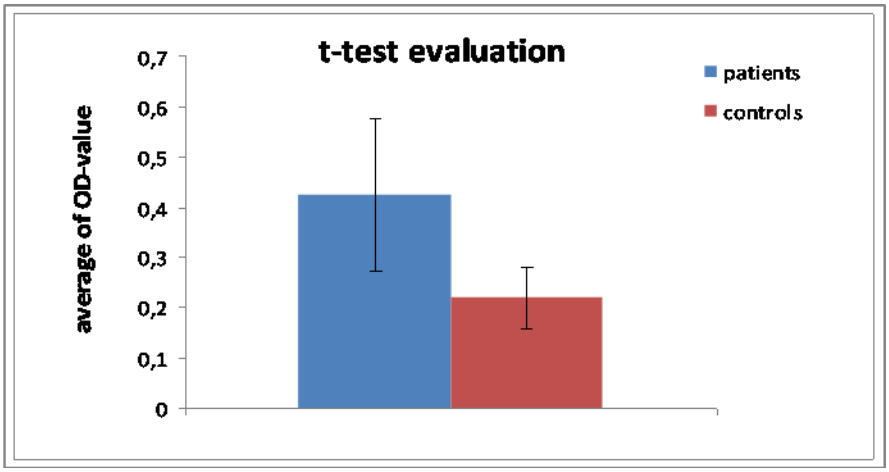

Fig. 36: Average OD-values of patients´ versus control sera in the IL-18-ELISA

6.5 Mesothelin Detection

6.5.1 Establishment of the ELISA with two recombinant MSLN

Because of the fact, that we had purchased two different *Abnova* MSLN products (*full-length* and *partial recombinant protein*), we aimed to compare the performance of these recombinant proteins by applying both solutions on one microtiter plate.

To establish the optimum standard curve, we calculated the stock-solution of the MSLN proteins. The series started at **250 ng/ml** and ended at **15.6 ng/ml**. To establish the optimum dilution of the Primary Antibody (*Abnova* mouse-anti-MSLN antibody), we started the dilution range at **1:500**, followed by **1:1000** and **1:2000**. POX-labeled Secondary Antibody (mouse) was diluted **1:4000**.

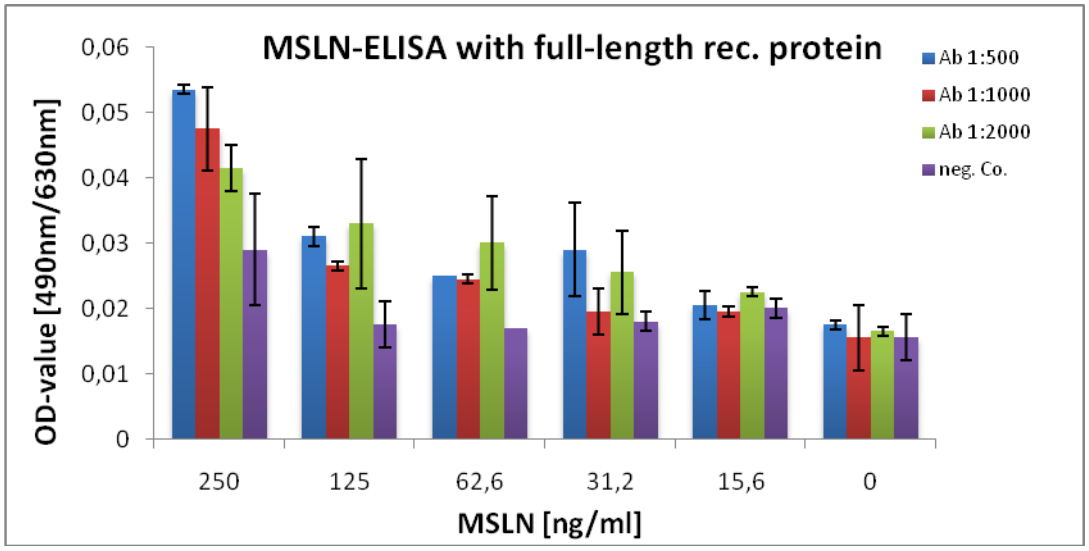

Fig. 37: Direct Mesothelin ELISA

Establishment of a Direct ELISA of Mesothelin with the full-length recombinant protein. The negative control was performed without antigen.

Unexpectedly, we observed in that experiment (**Fig. 37**), that the MSLN full-length recombinat protein was not recognized by the antibody. The OD-values were very low. Although the antibody should be able to recognize all of the protein, we did not gain significant positive results with this combination. Therefore we tested the partial recobinant protein, in ELISA, whose results can be seen in **Fig. 38**

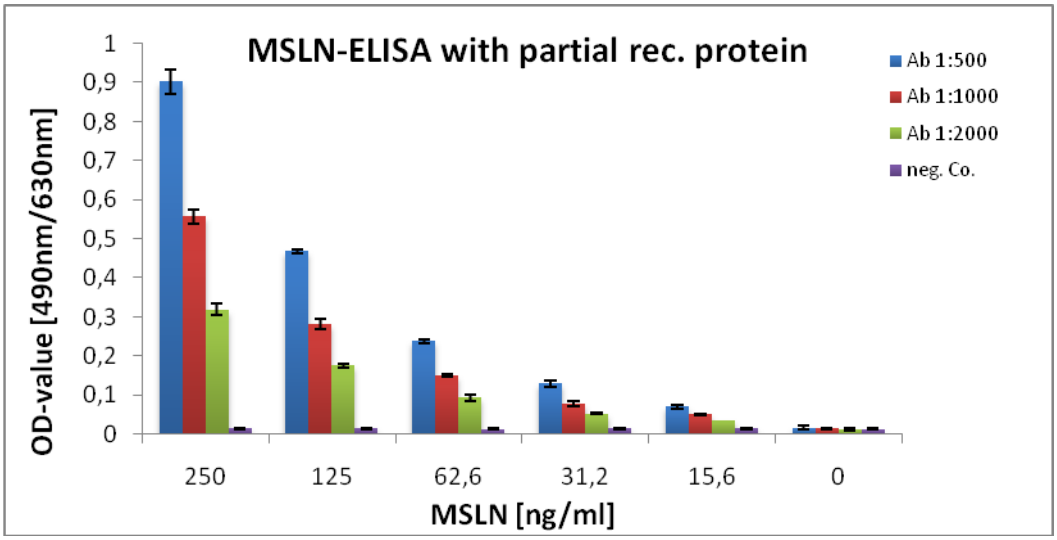

Fig. 38: Direct Mesothelin ELISA

Establishment of a Direct ELISA of Mesothelin with the partial recombinant protein. The negative control was performed without antigen.

Here, the interaction of the partial recombinant protein with the antibody worked fine. The dilution series showed a perfect decrease of the OD-values. The negative control showed that no other process of antigen or antibody binding disturbed the binding reaction. Due to these results, we aimed to continue to use the partial recombinant protein in further experiments.

6.5.2 Direct ELISA with MSLN and 10 serum samples

After a successful established standard series with the MSLN partial recombinant protein, we intended to go further with the human sera in Direct ELISA. First, only 10 sera were used in three different dilutions. The standard series for positive control started at **125 ng/ml** and ended at **7.8 ng/ml**. **Fig. 39** shows a continuous decrease of the values, which means that the positive control worked fine.

The sera were diluted **1:50**, **1:250** and **1:500**. We prepared the dilution of the Primary Antibody (*Abnova* mouse-anti-MSLN antibody) of **1:1000**. POX-labeled Secondary Antibody (mouse) was diluted **1:4000**. **Fig. 40** shows the results of the direct ELISA, showing very low and unspecific signals.
We concluded that it was not possible to gain signals with the sera working with direct ELISA. As an alternative, we tried to establish an S-ELISA in the next experiment. We suggested to receive better results, if we would use a Capture Antibody that could catch more of the protein out of the serum sample.

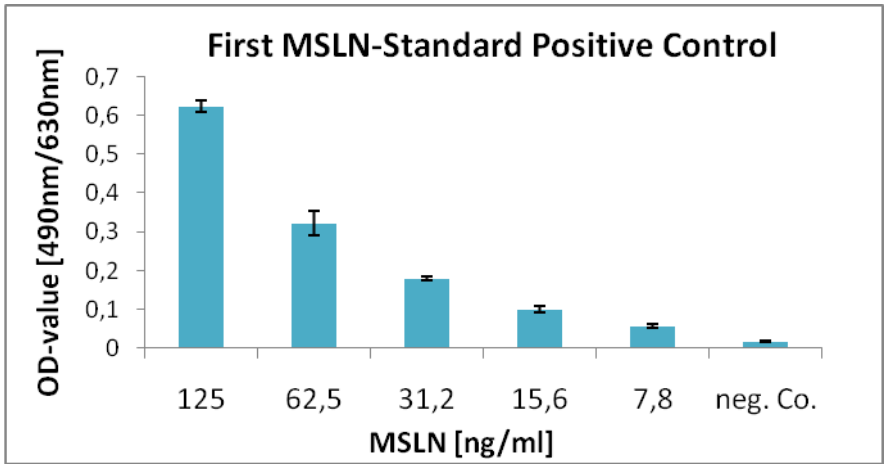

Fig. 39: First Mesothelin Positive Control-ELISA

This dilution series was tested on the same ELISA plate beside the serum samples.

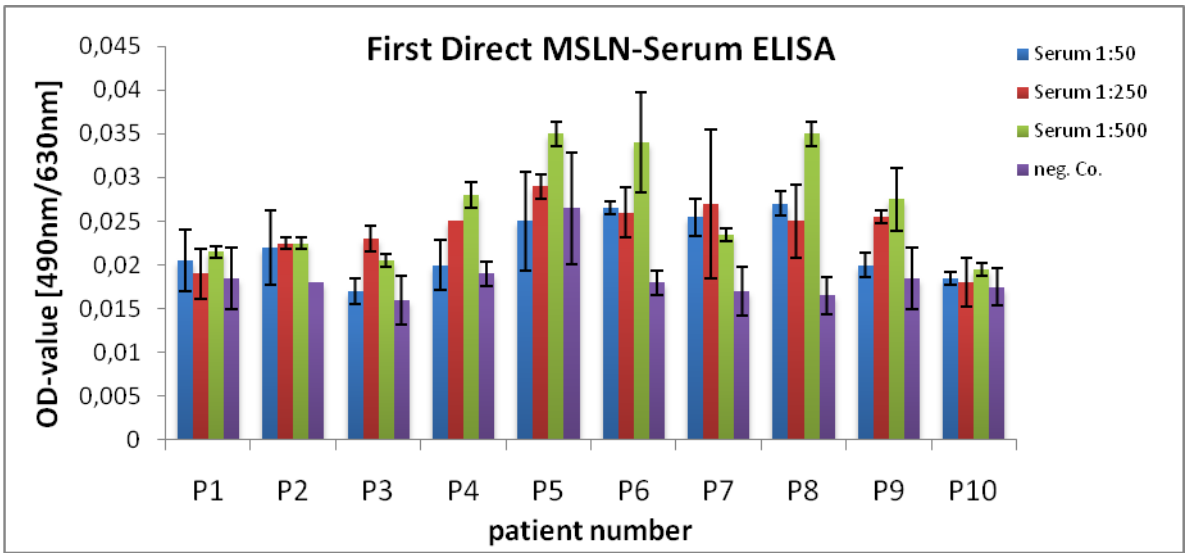

Fig. 40: First Direct Mesothelin-Serum ELISA

10 patient's sera were tested in 3 dilutions. Negative controls contained no Primary Antibody.

6.5.3 Establishment of Sandwich ELISA with recombinant MSLN

We intended to gain better results with an S-ELISA system, because chances are high to get higher OD-values, when there is a Capture Antibody coated that might catch the wanted protein. The Capture Antibody (*Abnova* rabbit-anti-MSLN antibody), was diluted it **1:250**. The standard dilution series started at **25 ng/ml** and ended at **0.39 ng/ml**. The Detection Antibody (*Abnova* mouse-anti-MSLN antibody) was diluted **1:500**. The POX-labeled Secondary Antibody (mouse) was diluted **1:4000**.

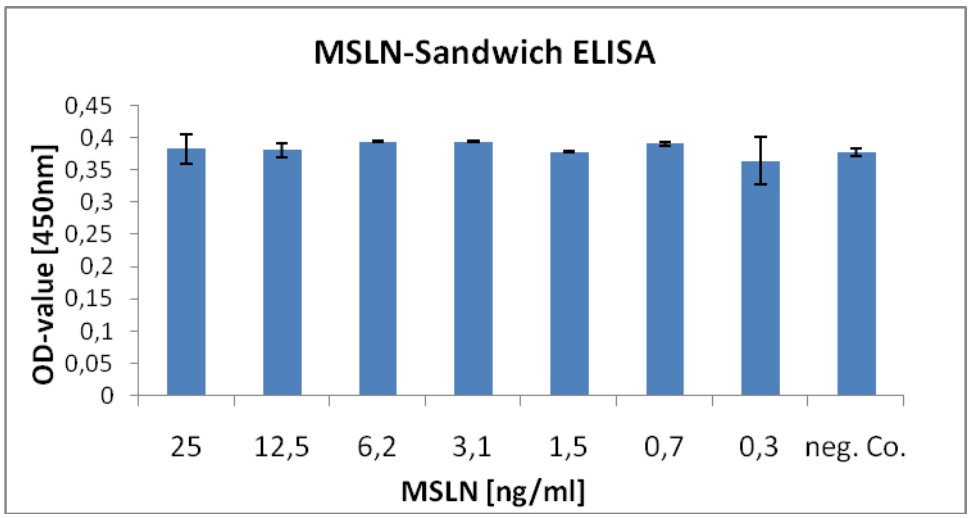

Fig. 41: Mesothelin Sandwich ELISA

Establishment of a Sandwich ELISA of Mesothelin. The negative control was performed without antigen.

Unfortunately we could not successfully establish a Sandwich ELISA with the available anti-Mesothelin antibody combination. All values shown in **Fig. 41** have the same levels and we could not observe a decrease. Even the blank wells as a negative control showed a signal, indicating a strong unspecific background. We concluded from these data that establishing an S-ELISA with MSLN was not possible with the available antibody combination.

6.5.4 MSLN Dot Blot

In this experiment we intended to test whether the antigen from *Abnova* operates appropriately with the antibodies from *Abnova*. Using a geometric dilution series of recombinant MSLN, starting at **20 ng** and ending at **1.25 ng**, we observed the expected decrease of the color intensity of each spot. On blot number **1** we observed a decrease of the color intensity of each spot. The first one at **20 ng** was the lowest dilution and showed the best result. The other dilutions decreased faster, which can be interpreted that the dilution of spot number **1** is the best one that worked. On blot number **2** we didn't see a signal, indicating that the *Abnova* rabbit anti-MSLN did not recognize the recombinant protein.

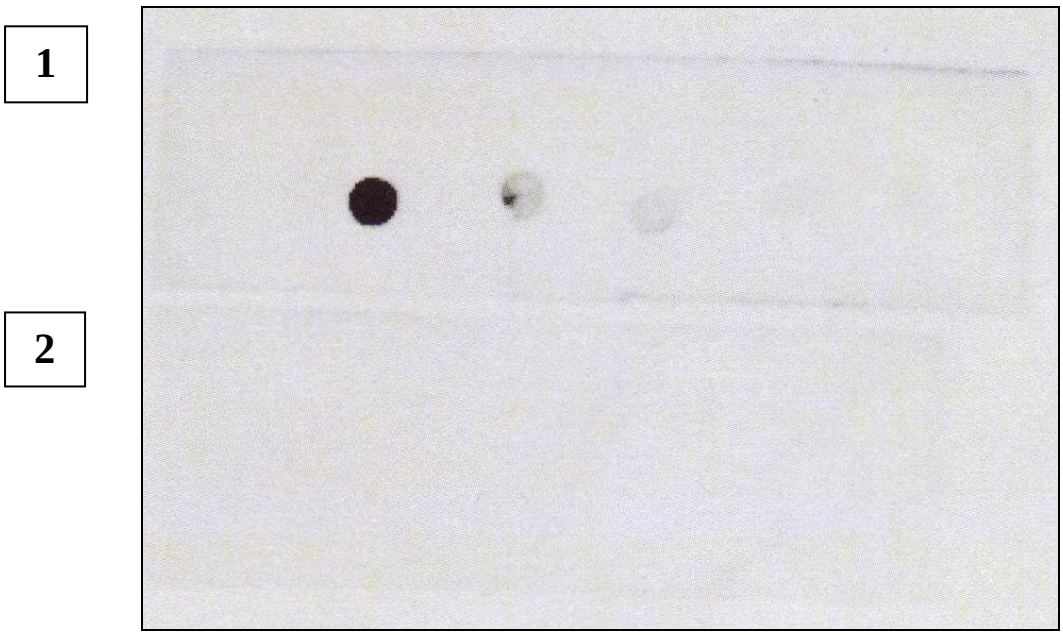

Fig. 42: MSLN-Dot Blot with recombinant protein and two antibodies

The upper blot (number **1**) shows the **1:500** dilution of the *Abnova* mouse-anti-MSLN antibody.
The blot below (number **2**) shows the **1:1000** dilution of the *Abnova* rabbit-anti-MSLN antibody.

The result of blot number **1** can be interpreted that *Abnova* mouse-anti-MSLN antibody reacted well with the antigen. The dilution of **1:500** seems to be the best choice. The result of blot number **2** showed that the *Abnova* rabbit-anti-MSLN antibody did not function. We couldn't observe any reaction with the antigen.
In the next experiment we turned back to the D-ELISA by coating higher amounts of sera, and this time we used three different antibodies, to look which one worked best with the coated sera.

6.5.5 Direct MSLN-ELISA with sera and 3 different antibody dilutions

Here, we aimed to test whether we could detect MSLN directly in the sera. In this experiment we analyzed the different performances of our three different antibodies with the sera. The sera were diluted **1:2** and the three antibodies were all diluted **1:500**. The POX-labeled Secondary Antibodies (mouse respectively rabbit) were diluted **1:4000**. As the antibodies did not recognize the recombinant MSLN, we omitted to include a standard dilution series.

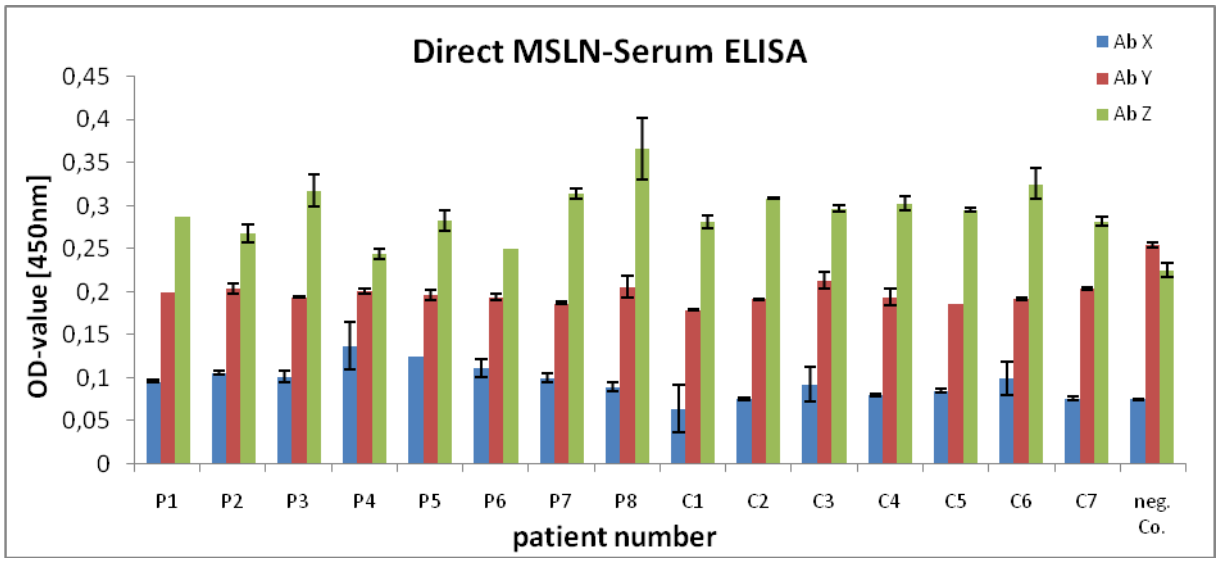

Fig. 43: Direct Mesothelin-Serum ELISA

Ab X: *Abbiotech* rabbit-anti-MSLN antibody

Ab Y: *Abnova* mouse-anti-MSLN antibody

Ab Z: *Abnova* rabbit-anti-MSLN antibody

15 patient's sera were tested in with 3 different antibodies. Negative controls were performed without serum.

As **Fig. 43** shows, the *Abnova* rabbit antibody (**Ab Z**) delivered highest signals, compared to the other two. Unfortunately we were not able to gain a difference between patient sera and control group. Concerning the negative controls, the background values were definitely too high.

A t-test analysis (**Fig. 44**) was performed to represent the overall statistic relevant difference between the patients´ and the control group, yielding a p-value of **0.64**.

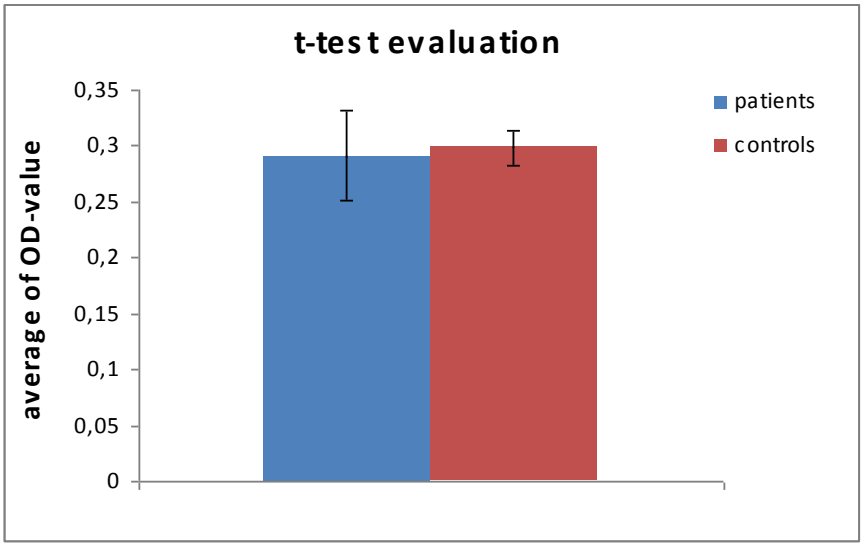

Fig. 44: Average OD-values of patients´ versus control sera in the MSLN-ELISA

Conclusively, although we could establish a valuable MSLN-Positive Control ELISA, we encountered difficulties to implement the system for the serum samples, and to establish a Sandwich ELISA for Mesothelin with the commercially available antibody systems.

6.6 Mucin 5B Detection

6.6.1 Establishment of Direct ELISA with recombinant Mucin 5B

To establish the optimum standard curve, we started the MUC5B standard series at **250 ng/ml** and ended at **15.6 ng/ml**. To establish the optimum dilution of the Primary Antibody (*Abnova* mouse-anti-MUC5B antibody), we started the dilution range at **1:500**, followed by **1:1000**, **1:2000**, **1:4000**, **1:8000** and **1:16000**. The POX-labeled Secondary Antibody (mouse) was diluted **1:4000**.

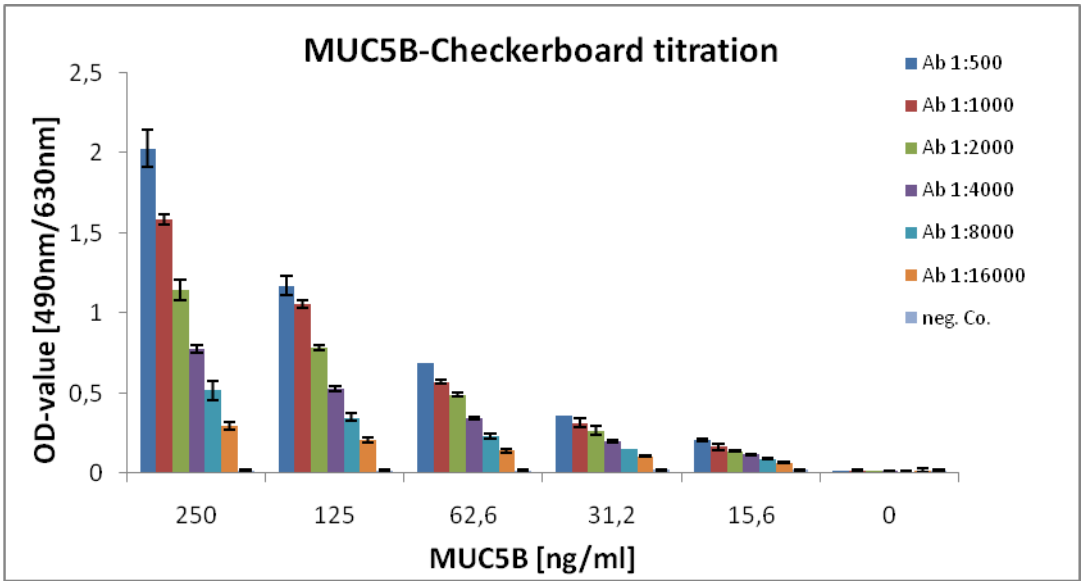

Fig. 45: Checkerboard titration of Mucin 5B concentrations

Different antigen and antibody dilutions were used to establish optimal antigen and antibody concentrations in ELISA.

As seen in **Fig. 45**, the optimum antigen concentration range was between **250 ng/ml** and **125 ng/ml** with an antibody dilution of **1:500 – 1:2000**. The other values were too low to represent a useful dilution that can be utilized in further assay experiments. The checkerboard titration shows a perfect continuous decrease of the values, which was estimated with these concentrations and dilutions. The negative control showed that no other process of the antigen or antibody binding disturbed the binding reaction.

6.6.2 First Direct MUC5B-ELISA with 10 serum samples

We started the dilution of the standard at **125 ng/ml** and ended at **7.81 ng/ml**. As a positive control we also included MUC5B standard that is seen in **Fig. 46**. The graphic shows a continuous decrease of the values, which means that the positive control worked fine on the ELISA plate. We detected positive signals versus the negative controls.

In **Fig. 47**, the results of the tested serum samples (**P1-P10**) are presented. Furthermore, three dilution steps of the sera (**1:50**, **1:250** and **1:500**) were applied to receive more information about the reaction. The Primary Antibody (*Abnova* mouse-anti-MUC5B antibody) was diluted **1:2000**. The POX-labeled Secondary Antibody (mouse) was diluted **1:4000**.
Following the three different sera dilutions, we could not really observe the expected decrease of OD-values. Generally, the measured OD-values were too low, indicating that no significant result was was obtained. Even the negative control wells made a high signal, which told us, that maybe an unspecific binding took place. We suggested that very low levels of MUC5B were present in the sera. Therefore, in the next experiments, we tried to obtain a well working Sandwich ELISA. Maybe that could lead to better signals, because of using a Capture Antibody that catches more of the wanted protein.

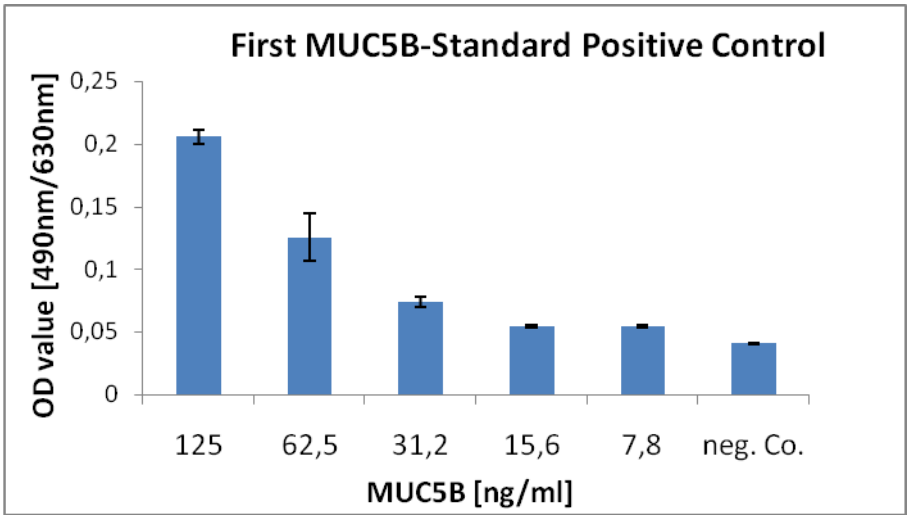

Fig. 46: First Mucin 5B Positive Control-ELISA

This dilution series was tested on the same ELISA plate beside the serum samples.

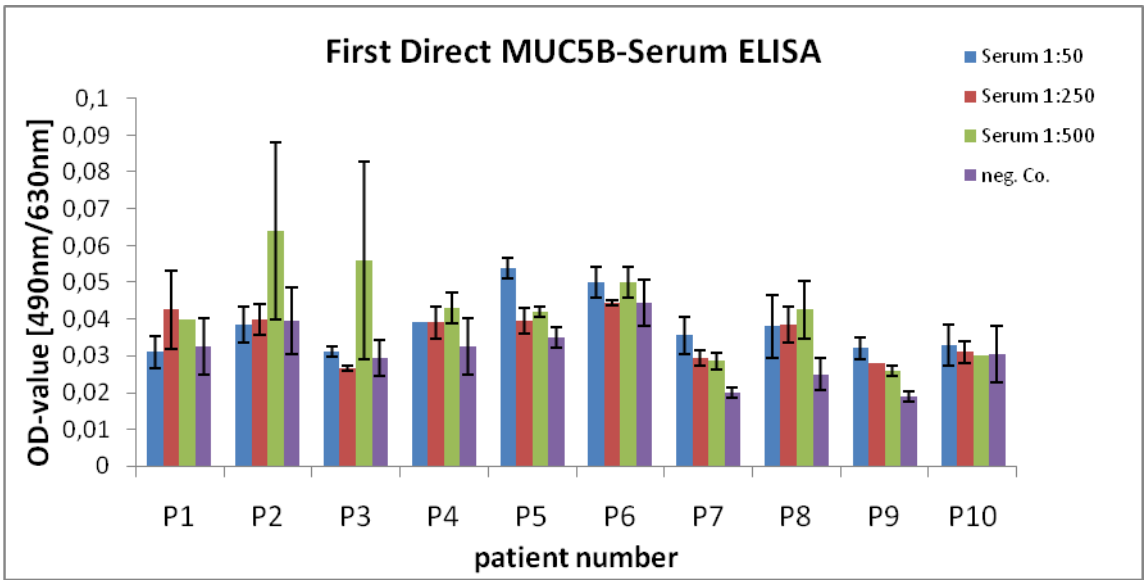

Fig. 47: First Direct Mucin 5B-Serum ELISA

10 patient's sera were tested in 3 dilutions. Negative controls contained no Primary Antibody.

6.6.3 Establishment of Sandwich ELISA with recombinant MUC5B

Because of the fact, that we did not gain positive results with direct serum coating, we intended to establish a Sandwich ELISA. Maybe the chance would be higher to catch more of the antigen compared to working with the D-ELISA.

The MUC5B standard dilution series started at **20 ng/ml** and ended at **2.5 ng/ml**. In this experiment we used three different dilutions of the Capture Antibody (*Santa Cruz* rabbit-anti-MUC5B antibody). The Detection Antibody (*Abnova* mouse-anti-MUC5B antibody) was diluted **1:300**, **1:1500** and **1:3000**. Furthermore, we used four different dilutions of the Detection Antibody. This antibody was diluted **1:500**, **1:1000**, **1:2000** and **1:4000**.

The POX-labeled Secondary Antibody (mouse) was diluted **1:4000**.

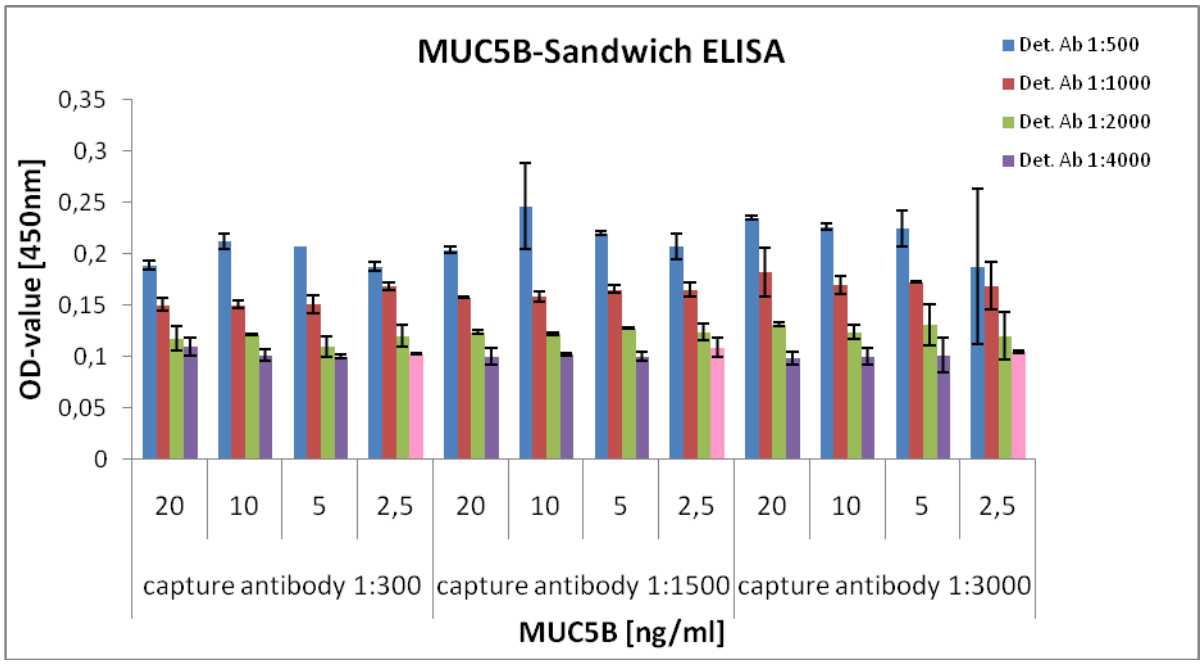

Fig. 48: Mucin 5B Sandwich ELISA

This experiment was performed on one ELISA plate. The *pink* bars represent the negative controls, which contained no antigen.

As **Fig. 48** shows, we used 3 different dilutions of Capture Antibody and 4 different dilutions of Detection Antibody. The **1:500** dilution of the Detection Antibody lead to the highest OD-values. We aimed to keep this dilution in further experiments. So we have successfully established this Sandwich ELISA.

6.6.4 Sandwich MUC5B- ELISA using 2 different capture antibody dilutions

In order to achieve even more specific signals with the serum samples, we worked with two different dilutions of the Capture Antibody (*Santa Cruz* rabbit-anti-MUC5B antibody). The Capture Antibody was diluted it **1:100** and **1:200**. This time, the MUC5B standard dilution series started at **5 ng/ml** and ended at **1.25 ng/ml**. The dilution of the Detection Antibody (*Abnova* mouse-anti-MUC5B antibody) was **1:500**. The POX-labeled Secondary Antibody (mouse) was diluted **1:4000**.

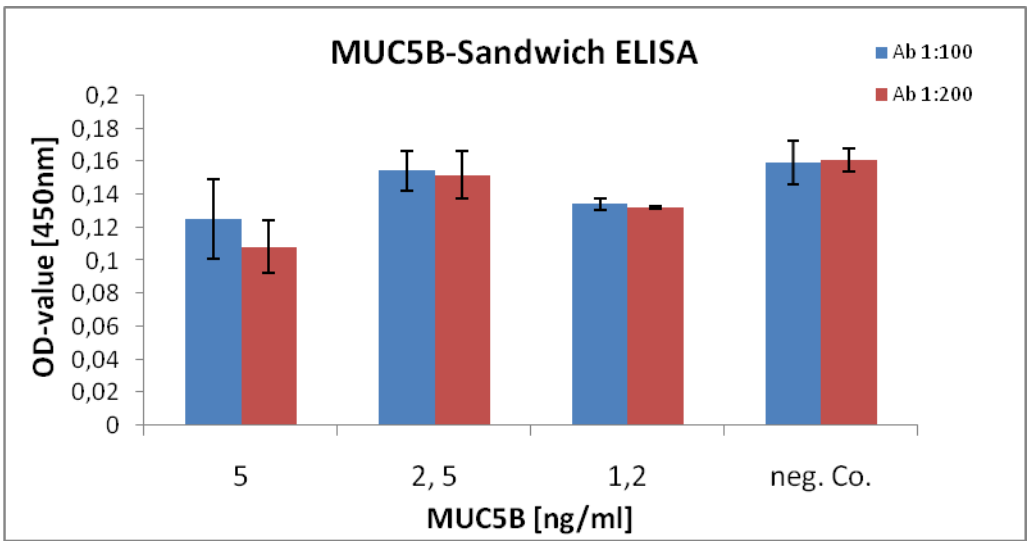

Fig. 49: Mucin 5B Sandwich ELISA

Establishment of a Sandwich ELISA of Muc5B. The negative control was performed without antigen.

As **Fig. 49** shows, we could not really improve the S-ELISA specificity in this setting. It might be possible that *Santa Cruz* rabbit-anti-MUC5B antibody did not recognize the recombinant protein. The values of the negative control were too high. Maybe this strong background signal was occurring because of other binding processes of the *Santa Cruz* antibody.

6.6.5 Comparison of MUC5B S-ELISA and D-ELISA with serum samples

Although we could not successfully establish a Sandwich ELISA system, we tried to test the sera. We suggested that the antigen in the serum interacted better with the two antibodies, than working with the recombinant protein. We also wanted to demonstrate that the Sandwich ELISA system worked better than the Direct ELISA.

In this experiment we diluted the Capture Antibody (*Abnova* mouse-anti-MUC5B antibody) **1:500**. The sera were diluted **1:2**. The dilution of the Detection Antibody (*Santa Cruz* rabbit-anti-MUC5B antibody) was **1:500**. The POX-labeled Secondary Antibody (rabbit) was diluted **1:4000**.

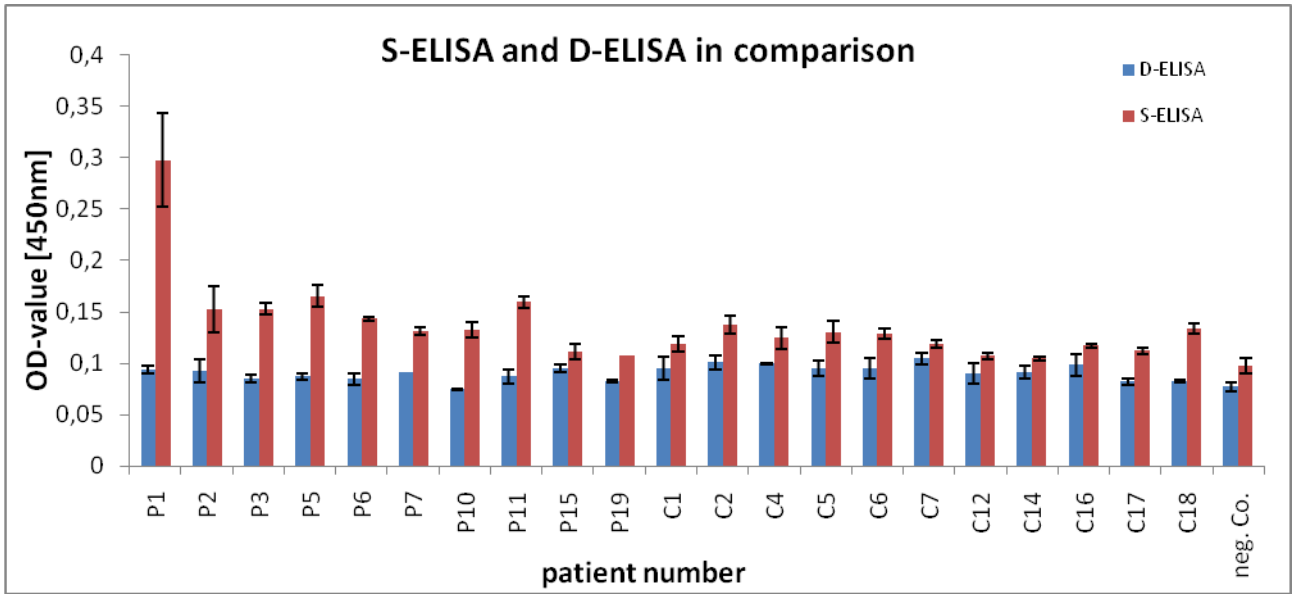

Fig. 50: S-ELISA and D-ELISA in comparison

This experiment was tested on one ELISA plate. The negative control was performed without antigen.

As the graphic (**Fig. 50**) shows, we were able to demonstrate that the S-ELISA system was more sensitive than the D-ELISA. Because of using a Capture Antibody for coating, we observed that more antigen was caught in the sera. The OD-values were not high, but still ranged above the background control. We also observed that the OD-values of the patient sera were obviously a little bit higher than the sera of the control group.

6.6.6 Sandwich MUC5B ELISA with all sera

We aimed to test all available sera.

In this experiment we worked with the same dilution factors as in the pretest. The Capture Antibody (*Abnova* mouse-anti-MUC5B antibody) was diluted **1:500**. The sera were diluted **1:2**. The dilution of the Detection Antibody (*Santa Cruz* rabbit-anti-MUC5B antibody) was **1:500**. The POX-labeled Secondary Antibody (rabbit) was diluted **1:4000**. This time, we intended to coat all sera to receive more information about the other serum samples in compare.

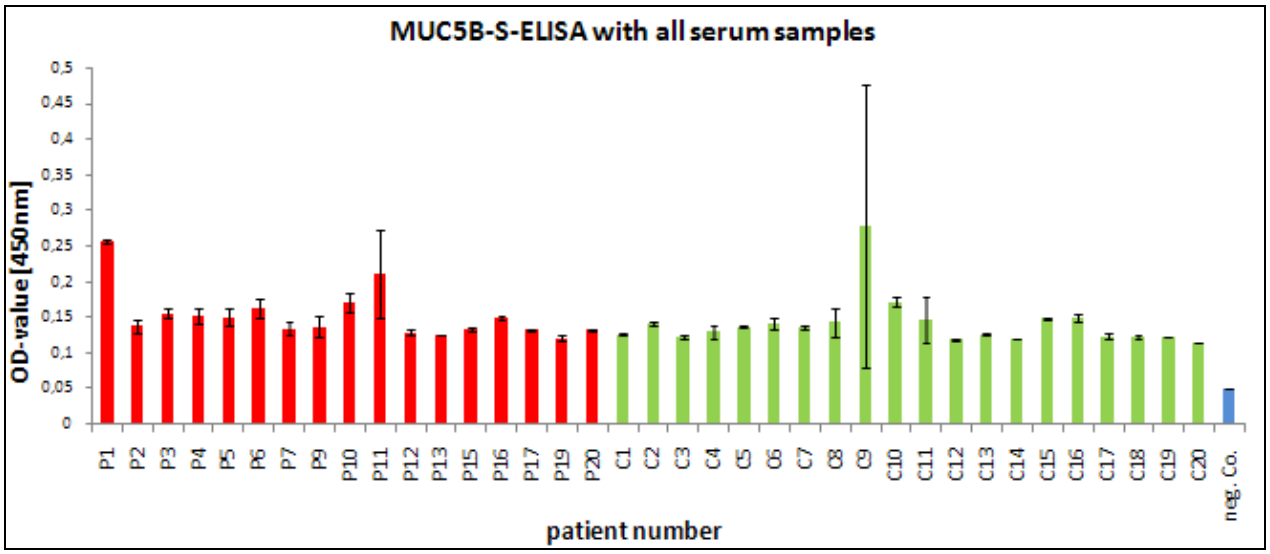

Fig. 51: Mucin 5B-Serum ELISA with all serum samples

Patient sera (*red*) and control group (*green*) was tested on one ELISA plate. The negative control (*blue*) was performed without serum.

In **Fig. 51** shows the results of this ELISA. **C9** seems to be an outlier of the control group. The negative control showed significant lower values, so that we could be sure, that the binding process worked fine.

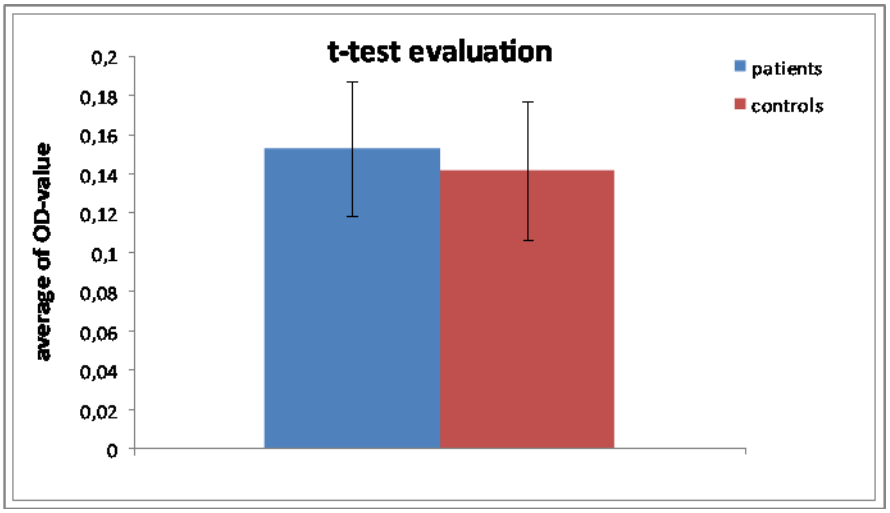

Fig. 52: Average OD-values of patients´ versus control sera in the Muc5B-ELISA

A t-test analysis was performed to represent the overall statistic relevant difference between the patient and the control group. **Fig. 52** shows the mean OD-values of both, the patient's and the healthy control group, and the corresponding p-value was **0.339**. This means that no significant differences between the patients´ compared to the control group were detected.

6.6.7 Sandwich MUC5B ELISA with sera and 3 Detection Antibody dilutions

Finally, we aimed to test whether a higher concentration of the detection antibody would lead to higher OD-values versus the negative control wells.

In this experiment we worked with the same dilution factors as in the pretest, just with three new dilutions of the Detection Antibody. The Capture Antibody (*Abnova* mouse-anti-MUC5B antibody) was diluted **1:500**. The sera were diluted **1:2**. The dilutions of the Detection Antibody (*Santa Cruz* rabbit-anti-MUC5B antibody) were **1:500**, **1:1000** and **1:3000**. The POX-labeled Secondary Antibody (rabbit) was diluted **1:4000**.

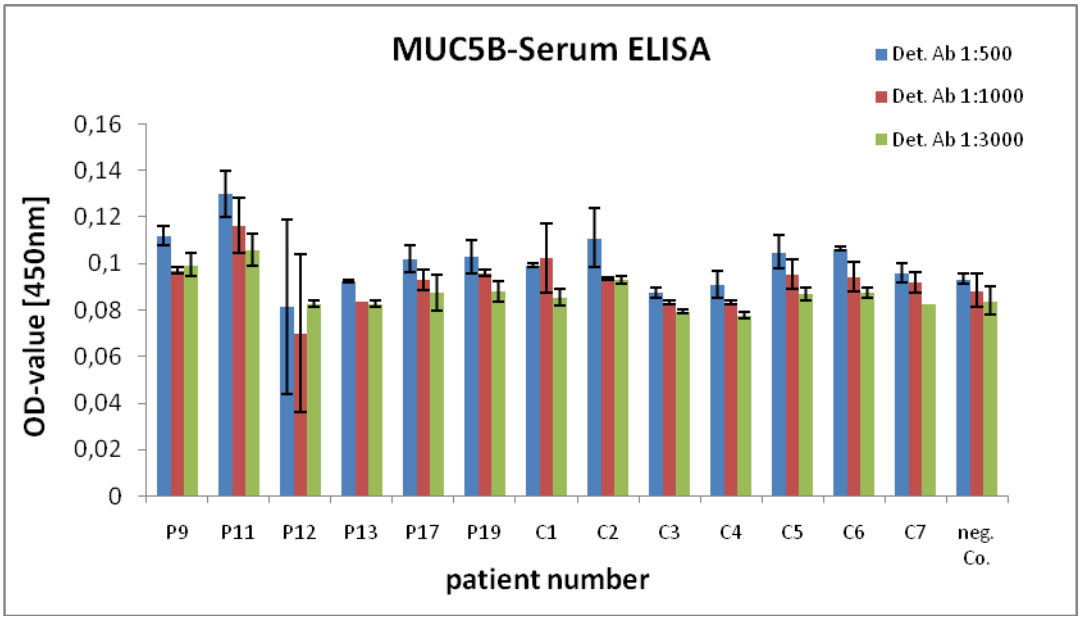

Fig. 53: Sandwich Mucin 5B-Serum ELISA

13 patient's sera were tested with 3 different dilutions of Detection Antibody (=**Det. Ab**).

The negative controls contained no serum sample.

As **Fig. 53** shows, we were not able to achieve a higher difference of the three antibody dilutions compared to the negative control wells. From these data we concluded that Mucin 5B levels were not significantly different in the sera of healthy versus cancer patients.

7. Discussion

In this thesis work, we aimed to draw novel conclusions from the effects seen in our established ELISAs. The biomarkers that we discovered in the earlier achieved surgical resection were chosen, because of their probable high occurrence in the human serum of patients suffering from lung cancer. Further, the four described antigens **Cytoglobin**, **Interleukin 18**, **Mesothelin** and **Mucin 5B** have been associated generally to be a candidate lung cancer biomarker.

In this work we firstly tried to establish the particular ELISA for each antigen. We generally tested the sera in **Direct ELISAs** and furthermore experimented with **Sandwich ELISAs** as the second choice to reach the goal to develop a clinical blood test in order to enable an earlier diagnosis of lung cancer.

Tumor markers in sera:

Detection and treatment of lung adenocarcinomas at an earlier stage would highly reduce the death rate of this type of lung cancer. However, lung x-ray screenings and bronchial needle biopsies have limitations. The development of fast and reliable non-invasive tumor marker detection methods could improve the outcome of this disease. During this diploma thesis work, we tried to detect new proposed lung tumor markers in human serum by using recombinant proteins, serum samples and commercially available antibodies. The proposed biomarkers had been identified in an earlier achieved proteome analysis of surgical lung resections of tumor versus healthy tissue samples. The candidate proteins were chosen based on their highly probable occurrence in human serum of patients suffering from lung cancer. Here, we showed the efficiency to detect these candidates in serum samples derived from clinical practice. Their potential to be an important cancer biomarker was evaluated in this study. The final goal of this project would be the development of a clinical blood test to enable an earlier diagnosis of lung cancer.

Specific Antigen-ELISA establishment:

The ELISA is one of the most prominent methods to trace proteins in biological fluids in clinical laboratory analysis. It relies on the simple principle of a specific binding between an antigen and its corresponding antibody, one of which has been immobilized on a solid (plastic) phase. Colorimetric detection of this binding reaction by enzymatic antibody labeling enables a direct correlation of optical density measurements in a spectrophotometer with the

concentration of bound antigen. The sensitivity of this method can be very high, reaching ng/ml detection thresholds. Besides being a rather cheap method, the ELISA is also a fast, reliable and easy analytical method, as no pre-separation steps of the biological fluids is necessary. Further, several different antigens can be detected at once in one sample, making this method ideal for large scale patient screenings. Its execution can be standardized and performed by a working roboting workstation. Being aware that certain plasma or serum proteins can be unstable over longer storage time, we added the protease inhibitor trasylol to the samples. Throughout the whole time course of this diploma thesis, we did not observe any gross signal instabilities, indicating that – at least regarding our proposed tumor markers – no degradation phenomena occurred. This indicated that our ELISA method would be a robust test system, being applicable to larger cohorts of clinical patients´ blood samples deriving from daily blood taking routine.

In the beginning this work we tried to establish particular detection ELISAs for each of the four antigens produced by recombinant protein expression technology. Various conditions were tested to optimize the detection protocol. As a starting point, we tested the coating frequency of **Direct ELISA** and furthermore experimented with **Sandwich ELISA** as the second choice offering a higher signal sensitivity.

Cytoglobin-ELISA:

Direct detection of recombinant Cytoglobin was immediately successful with the respective chosen antigen and antibody concentrations (**Fig. 10**). We therefore immediately tried to detect Cytoglobin in serum samples. Surprisingly, when trying to detect Cytoglobin in the sera with Direct ELISA, we obtained unusual high OD-values, although we used higher serum dilution steps in each experiment. The reason for that phenomenon was unclear. We suggested that the reaction was in the saturation region, which would mean that the Cytoglobin levels in the sera would be so high that even with a **1:2000** dilution we would be in the so-called **plateau-phase** (**Fig. 54**) of the ELISA detection range, but not in the linear range, where the OD-value directly correlates with increasing dilutions, which would lead to a linear decrease of the OD-value readings.

A Western Blot of serum samples probed with the *Santa Cruz* rabbit anti-CYGB antibody revealed that this antibody worked quite unspecific (**Fig. 17**). Interestingly, a strong band became visible at 53 kDa, while the CYGB size was determined to be 26 kDa. Either the antibody shows strong cross reactivities with unrelated other serum proteins, or this 53 kDa band represenst a CYGB isoform or a protein containing a homologous domain.

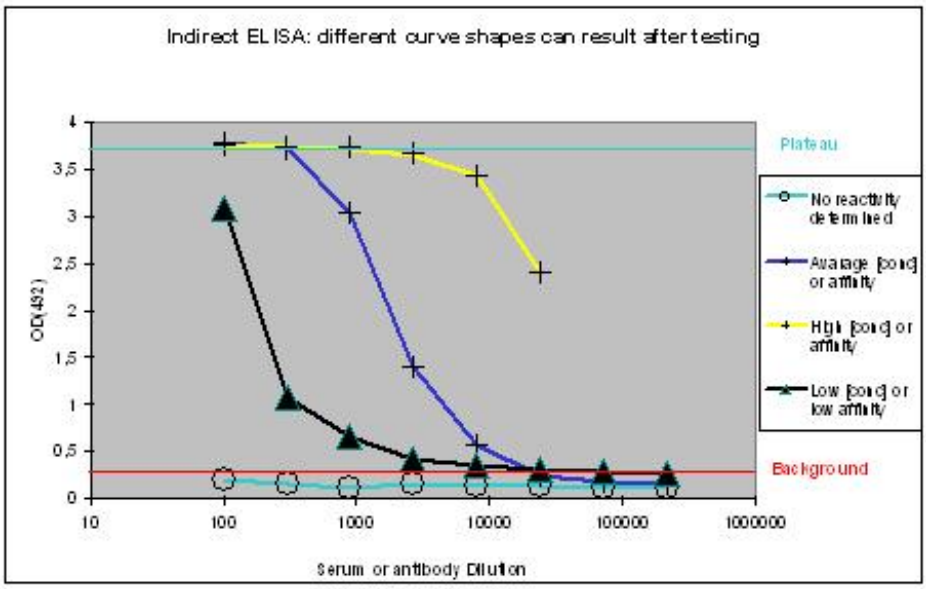

Fig.54: Different outcomes of ELISA-tests

(adapted from www.eurogentec.com/EGT/Images/elisa1.jpg)

However, based on these observations, we hoped that a Sandwich ELISA system would lead to more sensitive results by capturing CYGB with antibodies on the plastic surface. We thought that the S-ELISA system would be a better choice to seek the optimum concentrations for the establishment, and we compared both methods on one ELISA plate. **Fig. 19** shows a comparison between Direct and Sandwich ELISA using all possible antibody combinations. We were able to show that direct coating of the antigen delivered very low signals compared to the signals when using the Sandwich ELISA. We could also differentiate the working condition between the antibodies of *Santa Cruz* and *Abnova*. Working with both, *Abnova* mouse-anti-CYGB antibody as a Capture and with *Santa Cruz* rabbit-anti-CYGB antibody as a Detection Antibody, we observed that there are no significant signal differences between the antigen dilutions and that even the negative control wells present a too high background signal. It could be possible that the antibodies bind to each other, thereby generating such high OD-values. Also when turning round the experimental setting the result was not significant. From these data we assumed that the *Santa Cruz* antibody is unqualified as a Capture as well as a Detection antibody, since the signal-background noise is not separated. Conclusively, we have to say that we could not define an optimal Sandwich ELISA system by applying these two antibodies (*Abnova* and *Santa Cruz*).

Nonetheless, after having tested in a Dot Blot that the *Abnova* antibody worked at least ten times more sensitive than the *Santa Cruz* antibody (**Fig. 20**), we changed strategy and tried to perform the CYGB-test again with a Direct ELISA, applying the *Abnova* antibody.

In principle, this ELISA-test showed significant results above background threshold level, but these preliminary results indicated that Cytoglobin-levels were not critically different in patient versus control serum samples (**Fig. 23** and **Fig. 24**).

Interleukin 18-ELISA:

When establishing the ELISA test system for IL-18 using the recombinant antigen, we also immediately gained optimal OD-values. When extending the test to the serum samples, we had to realize that the serum-concentrations of this cytokine were very low, so that a Direct ELISA was not sensitive enough (**Figs. 27, 29** and **31**). As we could immediately dispose of a commercially available Sandwich test system, we were not concerned with establishment problems as we had encountered them with CYGB. Using this system from *Bender MedSystems*®, we were instantly able to gain reliable and positive test results for IL-18 from the sera even with dilutions of 1:10 (**Fig. 33**).

Concerning the determination of cytokine concentration in bodily fluids, it has been noticed that their half-life (i.e. the time, after which half of the amount has degraded) is often very short. Therefore, cytokine measurements in plasma or serum can be very sensitive, especially when the samples have been stored over longer time periods. In the case of IL-18, we did not observe any instabilities of sample signals in the ELISA measurements. As all samples were treated in the same manner and stored over a comparable time period, we can say that this interleukin is a suitable serum target. Of course, the measurements performed here represent an actual "snapshot" of the IL-18 level in human serum samples and do not betray the cellular source of this inflammatory response. Therefore, the origin of IL-18 is obscure and might indicate (1) a strong inflammatory response of the immune system against the tumor, or (2) an inflammatory process of the tumor itself regulating cancer cell survival. Further, it is unknown whether serum levels of IL-18 are changed in other inflammatory or malignant diseases.

Interestingly, our study indicated a significant elevated level of IL-18 amounts in sera of lung cancer patients, indicating that it might be used a valuable screening marker. We additionally tried to find out whether there is a correlation between the clinical parameters and the outcome of the ELISA. Surprisingly, we detected exceedingly high OD-values for patients´ sera **P1**, **P6** and **P11**, whose primary tumors showed different sizes, but who all had a **N2** status. A very cautious interpretation of these data would indicate a higher incidence of serum IL-18 levels in patients with advanced lymph node metastatic progress of lung adenocarcionoma.

However, further extensive test series have to clarify the observed significance, also in the context of tumor stage and prognosis in the presence/absence of metastases.

Mesothelin-ELISA:

Establishment of a positive control test-ELISA using recombinant MSLN generated interesting protein-chemical questions and problems. First, we had to realize that only the partial recombinant protein was recognized by the antibody, though it had been generated against the full-length variant. Further, we revealed that the rabbit anti-MSLN antibody did not detect recombinant MSLN at all (**Fig. 42**), explaining the outcome of the test-Sandwich ELISA shown in **Fig. 41**, which did not yield specific signals. Surprisingly, this same rabbit anti-MSLN antibody then lead to highest OD-values in a subsequent Direct ELISA, where we tried to detect MSLN in the serum samples without parallel running of a positive control series, as it would not work with this antibody (**Fig. 43**). This test-system finally showed specific positive results with MSLN from human sera. We found a commercially available MSLN ELISA-test system: *Mesomark* ™ [35]. As this system is quite expensive, we decided not to order this precoated ELISA plates.

However, we were not able to derive a significant different level of MSLN amounts in tumor patients versus controls. This result is interesting, as previous studies have identified MSLN as a tumor marker for patients with mesothelioma and ovarian cancer [39]. Our data indicate that either our test-system is not sensitive enough yet, or that MSLN is rather a marker for tumors of mesothelial rather than of ektodermal origin.

Mucin 5B-ELISA:

Regarding the establishment of a sensitive positive test-ELISA, we were immediately successful to see strong positive signals with the recombinant antigen (**Fig. 45**). Unfortunately, similar to MSLN, we were not able to obtain specific positive signals when applying the serum samples (**Fig. 47**) and immediately decided to switch to a Sandwich ELISA system. As with CYGB, we encountered detection problems with a *Santa Cruz* antibody as capturing agent (**Fig. 48**), though it seemed to be usable as a Detection Antibody (**Figs. 50** and **51**). Á very cautious preliminary interpretation of the serum test-ELISAs might indicate that there are indeed elevated MUC5B levels in the sera of lung tumor patients (**Fig.52**), though this result is not statistically significant. Therefore, a larger sample cohort has to be tested to extend and confirm our results.

Suitability of ELISAs as a serum test:

In summary, in this thesis work we have established, which of the four potential candidate tumor biomarkers, which commercially available antibodies and which detection method could have the potential for a future serum test. Then, we aimed to draw conclusions from the effects seen with the patients´ sera in the previously established ELISAs.

For all of the four potential tumor antigens we focused on, we were able to establish a sensitive positive control test with the recombinant proteins. When switching to test the serum samples, we had to envision discrepancies between the performances of the antibodies with recognizing the recombinant proteins versus detecting the native proteins in human sera. Generally, we can say that the application of a Sandwich ELISA should be the method of choice, as it is more sensitive than a Direct ELISA. The major drawback of this method is the availability of a cheap and practicable matching antibody pair system.

From the data we retrieved with the patients´ sera, we sometimes recognized higher test-signals, when the tumor was marked with **T1**/**T2**/**T3**/**T4** or **N1**/**N2**. Further, sometimes, but not in all cases, the **TX** or **N0** stage tumors showed lower levels of the signals. We did not have information from all patients concerning the **G**-staging, but from the data which we compared, there was no significant connection between the test-signals and **G1**/**G2** and **G3**.

Conclusively, at the moment, we can say that especially Interleukin 18 and maybe Mucin 5B, but neither Cytoglobin nor Mesothelin seem to have a potential as a critical serum marker for lung adenocarcinoma patients.

Outlook-Summary:

In future and ongoing collaboration with the clinical partner, our optimized serum detection protocol shall be verified in a prospective clinical trial using a larger patient cohort. If it is possible to correlate tumor staging with the amount of tumor marker in the patients´ sera, these results might be the basis to develop diagnostic strategies for lung tumors. Further, the results will be compared with the clinical parameters (tumor grade and stage, metastasis, etc.) to better estimate the clinical potential of the developed test system. There is a series of additional potential marker proteins in the lung tumor proteome database that have not been explored yet. These could represent additional lung cancer indicators. Only a whole panel of markers might probably form a useful set for detailed and meaningful tumor characterizations. Especially, we have identified a differential expression of IL-18 in lung adenocarcinoma tissue and serum. This preliminary result suggests that IL-18 could be a useful marker for the early diagnosis and treatment of lung cancer.

8. List of abbreviations

Ab	Antibody
BSA	Bovine serum albumin
C	Control group
CF	Cystic fibrosis
COPD	Chronic obstructive pulmonary diseases
CT	Computer-Tomography
CYGB	Cytoglobin
D-ELISA	Direct ELISA
Det. Ab.	Detection Antibody
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
HE-staining	Haematoxylin and Eosin staining
HRP	Horseradish peroxidase
ICE	IL-1beta-converting enzyme
IFN	Interferons
Ig	Immunoglobulin
IL-18	Interleukin 18
MSLN	Mesothelin
MUC5B	Mucin 5B
neg. Co.	Negative control
NK-cells	Natural killer cells
OD	Optical density
P	Patient
PAS-staining	Periodic Acid Schiff
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with Tween 20
PET	Positron emission tomography
SD	Sample Diluent
S-ELISA	Sandwich ELISA
SMRP	Soluble mesothelin-related peptides
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline with Tween 20

Th1	T-lymphocyte helper type 1
TMB	3,3’,5,5’-Tetramethylbenzidine
TNF	Tumor necrosis factors
TR	Tandem repeats
Tween 20	Polysorbate surfactant

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10. Curriculum Vitae

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Work experience

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