

**SHORT THESIS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY (PH.D.)**

**BIOMARKER CANDIDATES CORRELATE WITH
LUNG FUNCTION IN COPD**

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INTRODUCTION AND REVIEW OF LITERATURE

COPD (chronic obstructive pulmonary disease) a common preventable and treatable disease is characterized by progressive development of airflow limitation that is not fully reversible. The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs. The disease includes chronic bronchitis with sub-epithelial fibrosis and obstruction of small airways and emphysema leading to the enlargement of airspaces, destruction of lung parenchyma, and loss of lung elasticity.

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality worldwide. The World Health Organization (WHO) predicts that by 2020 COPD will rise from being the 12th to the 5th most prevalent disease world wide, and to the 3rd most common cause of death. Both genetic and environmental factors could be responsible for the observed geographical differences which show the highest incidence of death directly caused by COPD in the male population in Hungary.

Diagnosis of COPD is built on clinical symptoms and decreased level of lung function as tested by spirometry. COPD patients are usually diagnosed late when the disease is already in an advanced stage.

The main risk factor for COPD is cigarette smoking, and at present, cessation of smoking is the only way to attenuate lung function impairment. Only about 15-20% (or more) of smokers are susceptible to develop COPD, and 85-90% of COPD patients are smokers, indicating the possibility of additional, perhaps genetic factors play important role in the development of COPD. Identification of biomarkers will result in the eventual development an early diagnostics for the asymptomatic COPD patients. An appropriate early diagnostic applied for screening smokers with high susceptibility to develop COPD, may improve COPD morbidity and related death rate. Preventing environmental cause e.g. quitting smoking is the obvious preferred approach; this has proved to be very difficult for the majority of patients. Screening of the susceptible smoker population for early signs of COPD may lead to better success of COPD treatment via intensive efforts to manage smoking habits in the population at risk.

Current treatment of COPD is not curative; it addresses the symptoms of the disease only. There is a pressing need to develop new treatments because none of the currently available therapeutic drugs have been shown to slow the relentless disease progression. However, there has been disappointingly little therapeutic progress of COPD, in contrast to the enormous advances made in asthma management. The slowly progressive nature of COPD has meant that clinical trials aimed at assessing the efficacy of new treatments have been long and expensive.

Biomarkers and biomarker discovery in COPD

Biomarker refers to a detectable and quantifiable biological parameter: any molecule or measurable function. In the field of medicine, biomarkers that reflect the disease process are pursued. In this respect biomarkers are tools for the detection of specific molecular changes in pathophysiological processes; i.e., reflect the increase or decrease of blood protein levels in disease states or during clinical drug testing. The ultimate utility of COPD biomarkers is that after a tedious qualification and validation process they provide a new source of valuable diagnostics for early detection or companion to drug and general treatment regime.

In COPD, several types of biomarkers have been detected and measured that are related to disease pathophysiology and the destructive inflammatory process in the lung tissue. Relevant pulmonary biomarkers were tested in bronchial biopsies, bronchoalveolar lavage (BAL), and sputum and exhaled breath. Almost all of these biomarkers have been selected by hypothesis driven approaches. A review of 600 published studies finds that only a few of the biomarkers were validated rigorously but none has entered to clinical practice, thus there still remains void continued research for new ones.

Novel validated biomarkers will be specific and sensitive enough to discriminate clinical endpoints, e.g., healthy state from asymptomatic stages of disease and can be utilized as candidates to monitor the therapeutic effect during drug trials, disease management, early diagnosis, staging, and stratification. In evaluating COPD biomarkers it is important to compare findings in patients with cigarette smokers matched for exposures who do not have significant airflow limitation (COPD negative smokers without airways limitation) and with age-matched non-smoking apparently normal subjects. This is rarely performed accurately, making

interpretation of findings difficult. Importantly; after the necessary and time consuming validation process COPD biomarker discovery efforts should result in the discovery of new diagnostics that may be more simple to use than current expensive and complicated imaging and spirometry tests.

Protein biomarker discovery technologies

The most important and relevant source of biomarkers are proteins because these are the molecules that carry function. The blood plasma is routinely used and it is the most accessible specimen. We expected that the complex COPD disease process will alter the human plasma proteome; therefore we may find useful COPD biomarker candidates.

Appropriate hypothesis free biomarker discovery technologies should be global, in this case, covering as much as possible of the human plasma proteome. At the same time, the candidates should be easily translatable to simple clinical assays. To this end, we applied a new antibody based biomarker discovery technology for measuring the plasma proteome in COPD. Until recently, the standard methodology of protein biomarker discovery involved 2-D gel electrophoresis with staining, followed by in-gel digestion of individual spots and peptide mass fingerprinting. While being a powerful separation approach, the method has known limitations of throughput and ability to detect low level markers. A second technology that was previously strongly advanced, particularly by the clinical community, was the Ciphergen protein chip approach. While there have been some promising results, for the most part, the results have been disappointing in terms of finding relevant markers and the robustness of the assay. There are a number of reasons, not the least being the inadequacy of the time of flight mass spectrometer.

There is general agreement within the community that LC/MS (liquid chromatography/mass spectrometry) is a powerful discovery tool for finding potential markers. However, as emphasized more and more often,, a critical bottleneck is the validation of these markers in large populations. Some advocate using MRM (multiple reaction monitoring) with added isotope labeled internal standards for quantitative purposes. Indeed, the use of antibody enrichment prior to MRM analysis has also been suggested by Anderson in the SISCAPA (Stable Isotope Standards with Capture by Anti-Peptide Antibodies) method. But others note that

detection of low level markers can be an issue, even if the method is focused on known species. Furthermore, the issue of throughput remains, as LC/MS runs can often take 1-2 hours each.

A second strategy is to attempt to generate antibodies (Abs) to the potential markers and conduct ELISA assays. Of course, this strategy is time consuming, and there is not a 100% likelihood of the generation of appropriate Abs. Nevertheless, the familiarity and confidence of the clinical and biological community with the ELISA format is a strong argument in favor of immunoassay approaches in the validation studies. Furthermore, based on appropriate monoclonal antibodies protein arrays can be constructed or fluorescent beads (LapMap, Luminex) can be used for assaying multiple proteins in subsequent marker validation assays and thus meet the expected need for multiplex marker validation.

Readily available libraries of epitope level characterized antibodies would then offer a powerful approach to biomarker validation, and indeed the potential discovery of new markers. Since there are a variety of antibody types, one has to question as to what is the most suitable type of antibody to be used in this endeavor. Monoclonal antibodies (mAbs) are often viewed as the optimum immunoassay reagents because they can be generated against the actual protein molecule present in the “in-vivo” sample and state; i.e. 3-dimensional structure, including any PTM’s (posttranslational modification) present. This is an important point, given that 50% of the proteins are glycosylated in blood and that the glycan is known to be able to affect the conformation of the protein. Thus, we choose to formulate our strategy with the goal of generating mAb libraries against natural proteins of the normal and COPD plasma proteome.

Lipid mediators

COPD pathology involves an inflammatory cascade, events, which start with exposure to smoke and end in the destruction of the lung tissue. Mediators of the early phase of this process include lipid metabolites. Therefore we focused a part of our studies on lipid mediators of inflammation. Amongst the many different classes of inflammatory mediators which have been suggested to play a role in COPD, lipid mediators derived from phospholipase metabolites appear to play an important role. Many of the lipid mediators as well as many chemokines, have been implicated in inflammatory processes in COPD, thus it is

important to study the potential profile changes of the specific lipid classes, and chemokines to search for novel inflammatory mechanisms that may work through GPCRS (G protein-coupled receptors). Almost 50% of known drug targets belong to the group of GPCRS, justifying special attention, as the likelihood of finding a drug gable target within this family is about fifty times higher than in the entire genome. Among others, lipidic ligands, arachidonic acid metabolites (prostaglandins, protacyclins, thromboxanes, leukotriens and eicosateranoic acids), lysophospholipids, fatty acids and endocannabionoids serve as ligands for known and perhaps for still orphan GPCR receptors. Yet another class of GPCRS serves as chemokine receptors. Among the various groups of lipid like GPCR ligands arachidonic acid metabolites (ecosinoids) play specific role in inflammatory processes and have been implicated in COPD. From this group PGE₂, PGD₂ and 5-oxo-ETE are of special interest. The eicosanoid 5-oxo-6E, 8Z, 11Z, 14Z-eicosatetraenoic acid (5-oxo-ETE) has recently been identified as the ligand for the oxoeicosanoid (OXE) receptor. In vitro and in vivo studies have suggested that 5-oxo-ETE has a role in the asthmatic inflammatory response and it has been shown to stimulate eosinophil migration to the airways. New data suggest that eosinophils have an important role in the pathogenesis of asthma, being required for mucus accumulation, airway hyper responsiveness and remodeling of the airways. However, there are several mediators that can stimulate the recruitment of eosinophils to the airways and the development of antagonists against the OXE receptor is required to evaluate the potential of the OXE receptor as a new therapeutic approach for asthma. COPD and asthma share similar symptoms and perhaps overlapping etiology, thus it is important to examine the role of the lipid metabolite receptors and its family members in COPD. Instead of a global and prohibitively costly research endeavor we choose to test a limited number of lipid mediators, eicosapentaenoic acid (EPA), lysophosphatidyl choline (LPC), 15 hydroxy-eicosatetraenoic acid (15-HETE) and prostaglandin D₂ (, PgD₂).

BEGINNING OF COPD RESEARCH AT UD MC AND GRANTS

The current research activity at the Medical and Health Science Center of the University of Debrecen (DEOEC) started in 2001. My clinical research group in the Department of Pulmonology of the University of Debrecen obtained a collaborative research grant* from the Genomics and Bioinformatics group under the leadership of Laszlo Takacs at the Pfizer Fresnes Laboratories in France. The goal of the collaboration was to find biomarkers and new drug targets in COPD with proteomics, metabolomics and genomic research.

***E. Csanky M.D**, L. Nagy MD.Ph.D., P. Gergely Ph.D.DSc.: Identification of disease relevant target and biomarker candidate genes by comprehensive interrogation of the genome and proteome in COPD.

The first results were very exciting, and accordingly with the same group at Pfizer, we started the second grant, in 2003**. Monoclonal antibody mediated biomarker discovery: The aim of the study is the simultaneous identification of plasma proteins as biomarkers of COPD and suitable antibody candidates for assay development.

****E. Csanky M.D**, L. Nagy MD.Ph.D., P. B. Scholtz Ph.D, Gergely Ph.D.DSc.: Discovery and validation of biomarkers and drug targets for COPD: a clinical genomics, proteomics and genetics collaboration with the University of Debrecen 2003. (A9001156 Pfizer grant).

Biosystems International in 2005 proposed a comprehensive pilot study*** that aims at the long term goal of a potential clinical surrogate marker and novel lipid or chemokine mediator driven disease mechanism that works through GPCRS. For securing the best discovery outcome we compared “healthy” COPD symptom free smokers with those COPD patients who smoke. We chose a set of precursor and end-stage, biologically active lipid metabolites and tested these in the BAL fluid of COPD patients and controls by mass spectrometry based quantitative methods.

*** L. Takács L, **E Csányk**: Comprehensive Pilot and Biomarker Early-Validation (50-50) studies for COPD GPCR target and biomarker discovery. (2005. Biosystems International grant)

THE AIM OF THE DISSERTATION

The long term goal of my hypothesis free and hypothesis driven biomarker research is to enable the discovery, qualification and validation of an analyte panel based new laboratory diagnostics for COPD

My specific questions (protein biomarkers):

- Does the plasma proteome contain COPD specific biomarkers?
- Do we find protein biomarkers capable to discriminate smoking but COPD free subjects from smoking COPD patients?
- Can we apply the monoclonal antibody research for the discovery and qualification of new biomarkers in COPD?

Additional goal of my work was to explore and introduce the less popular metabolom studies in COPD research.

My specific questions are:

- Do we find COPD specific lipid mediators in the BAL fluid with targeted hypothesis driven biomarker research?
- Is the hypothesis driven metabolom marker research applicable for the discovery and qualification of new markers in COPD?
- Whether any correlation can be observed between lung function parameters and biologically relevant lipids obtained directly from the diseased tissue compartment (BAL fluid)

PATIENTS, MATERIALS AND METHODS

Patient's selection for sample collection

Patients were obtained from the Department of Pulmonology, Medical and Health Science Center, University of Debrecen, Hungary. The clinical protocol was prepared in compliance with EU regulations and the necessary approval was obtained from the regional and institutional ethics committees. Patient selection was driven by a lung function test, particularly FEV₁, (<80% predicted). The lung function parameters were observed by routine spirometry tests where the patient's ability to forcefully exhale and inhale is measured. Maximal volume and maximal velocity were tested.

Pulmonary function tests and reversibility testing

Using a pneumotachograph based system, dynamic and static lung volumes were determined. Measurements were performed repeatedly until values within 5% variation were obtained at least 2 times. The tests were performed according to the European Respiratory Society reproducibility criteria. The tests were performed when the patients were clinically stable and free from infection.

First, pre-bronchodilator spirometry was performed as described previously. Subjects were then treated with 400 µg of salbutamol by inhalation, and after 15 minutes, post-bronchodilator spirometry was performed. In order for the subject to be designated as having irreversible airway obstruction, the post bronchodilator FEV₁/FVC should remain < 0.7, and the FEV₁ may not improve more than 12% or 200 mL in FEV₁, over the pre-bronchodilator value (as a diagnostic criteria of COPD).

Blood plasma samples

Samples of venous blood was drawn after an overnight fast from the forearm from each subject in the free-living state and collected in polypropylene tube, placed immediately on ice, no longer than 30 min and centrifuged. After centrifugation, the plasma was aliquoted immediately in pre-bar-coded tubes, then frozen and stored at -80°C until analysis.

BAL fluid collection

We performed the BAL only the case of independent clinical diagnostic indications. After the diagnostic tests we utilized the left over BAL fluid

for our experiments. Briefly, after local anesthesia of the upper respiratory tract with topical lidocaine spray (2%), the tip of the bronchoscope was wedged into a sub segment of the right middle lobe, and 6 serial aliquots of sterile saline, 50 ml each, were introduced and immediately aspirated. Afterwards, the volume of the recovered lavage fluid was measured.

Sample preparation monoclonal antibody generation

Tracer and immunogen preparations were produced via identical methods. First, an Agilent MARS-6 column was used as recommended by the producer to remove the six most abundant plasma proteins. Next, a polyclonal antibody affinity column was applied for the normalization of COPD specific proteins. For immunization and hybridoma generations, standard procedures were used with minor modifications. Spleen cells were prepared by gentle teasing the tissue between sterile frosted microscopic slides and hypotonic shock to remove red blood cells. Splenocytes were fused to Sp2/Ag0 myeloma cells with PEG. Hybridoma supernatants were generated via standard procedures.

Screening of hybridoma supernatants

Capture assays detect biomarker hits: in this assays no inhibitors were used and the tracer was prepared from pooled plasma samples. Inhibition assays also detect biomarker hits, however, the nature of these biomarkers are different from those based on capture detection: in these assays dilution of individual plasma samples were added to the assay. Biomarker levels were expressed as % inhibition.

Protein identification using LC-MS/MS

Protein mixtures from normal and COPD patients were solubilized. Proteins were digested overnight. Nanocapillary LC of the tryptic peptides was performed. MS analysis was executed using a LCQ Deca XP PLUS™ IT mass spectrometer. The mass spectrometer was operated in dynamic exclusion mode with three MS/MS scans after each MS scan. The BioWorks software was used to analyze all data with the SEQUEST algorithm against Swiss-Prot human database <http://www.expasy.org/sprot>

Lipid metabolite assays

Entire BAL samples were extracted with ethyl-acetate. The dried down material was reconstituted in methanol, centrifuged and the supernatant

was again dried down in the Speedvac™. The resulting material was reconstituted in ethanol and isotope mix in methanol and 10µl of the extract was analyzed immediately by HPLC-MS.

HPLC-MS analysis

The HPLC-MS analysis was performed using an HPLC-MS system consisting of a Waters 2695X HPLC separation module including a gradient pump, a degasser and a heat-able column compartment. The column was maintained at 40°C and connected directly to the MS-MS detector including an electrospray ionization option. The system was controlled via the MassLynx software. MRM (multiple reaction monitoring) with ESI (ESI (-) settings) was performed with the HPLC eluate flowing into the ESI source at a temperature of 85°C.

Standardization

Stock solutions of lipid standards were prepared from commercially available standards of 15-HETE, dX-15-HETE, dX-PgD₂ and, PgD₂ and EPA, to give a final concentration of 1000 ng/ml. All stock solutions were stored in the dark at -80°C. Multilinear calibration was carried out using the reference retinoids by measuring different injections of ethanol standard solutions at four different concentrations (1, 10, 100, 1000 ng/ml) using a 1 µl injection volume. The detection limit was 5 ng/ml for LPC, 15-HETE and EPA, and 10 ng/ml for, PgD₂ with a linear coefficient of regression of greater than 0.99 over the entire concentration range.

RESULTS

PLASMA PROTEIN BIOMARKER DISCOVERY

Patient cohort

The two clearly distinguishable clinical points selected for the biomarker discovery study were 30 stage-II COPD smoking male patients and 30 age and sex matched smoking control individuals with apparently healthy lung functions, respectively. The goal was to identify patients with active disease evolution (not the end stage), with the expectation to find disease mechanism relevant biological markers. The most important clinical parameters that discriminate COPD from the control population are spirometric results. Maximal volume and maximal velocity were tested.

Global protein biomarker discovery

A total of 3500 hybridoma supernatants were generated and screened by ELISA assays (“capture” first followed by “inhibition” assay). The first step ELISA screening was based on hybridoma IgG capture and its tracer binding. The first level screening identified 250 biomarker hits. Each of the 250 hits was tested by the inhibition assay using individual patient or subject plasma samples. In these assays tracer binding was inhibited by plasma samples of individual patients or subjects. The second screening step reduced the number of hit candidates to ten high quality biomarker leads. All ten individual biomarker leads showed statistically significant discrimination power with the non-parametric Mann Whitney test.

An integrated, high-throughput, disease-specific monoclonal antibody (mAb)-based biomarker discovery platform has been developed to provide new biomarker leads with the focus on large scale discovery and production of mAb-based, disease-specific clinical assay candidate biomarkers. The monoclonal antibody mediated proteomics approach within a short time (eight month) delivered ten of mAb-s that, in simple ELISA assays discriminate the two populations with high degree of statistical significance. The outcome of the biomarker discovery process is applicable for testing of clinical validation paradigms, like response to treatment or correlation with other clinical parameters. In contrast to mass spectrometry (MS) based or systems biology based strategies, our process produced pre-validated clinical assays as the outcome of the discovery process. The encouraging results clearly demonstrate the efficiency of the approach described here, and set the grounds for the next

steps of studies, namely, the hunt for candidate biomarkers that respond to drug treatment.

LIPID BIOMARKER STUDY

Patient's selection for the BAL study

The pulmonary function results had shown clearly segregated patient populations, one with, the other without COPD. The lung function parameters which were used in the analysis include FEV₁, %FEV₁, PEF, and TLC.

Lipids in BAL fluid

The lipids which were used in this determination were those that gave measurable values in most or all of the BAL fluid samples tested and include eicosapentaenoic acid (EPA), lysophosphatidyl choline (LPC), 15 hydroxy-eicosatetraenoic acid (15-HETE) and prostaglandin D₂ (PgD₂).

We noted that although all patients received a similar amount of BAL fluid via a flexible bronchoscope, the recovered fraction of the fluid was variable and we found no apparent correlation between the estimated metabolite concentration and the results of the lung function test with the exception of total lung capacity. We observed some correlation between the total amount of some of the recovered analytes and the amount of BAL fluid recovered, in particular, EPA ($R^2 = -0.77$) and 15-HETE ($R^2 = -0.75$), and to a lesser extent, PgD₂ ($R^2 = -0.57$).

BAL compartmentalization

These observations have prompted us to develop a hypothetical model of BAL fluid compartmentalization. We assume that the delivered lavage fluid partitions to two compartments; in compartment-1, which is termed "recoverable" because it represents the recovered fraction, the fluid reaches the target tissue environment where sufficient degree of equilibration of soluble metabolites takes place. The recoverable compartment is likely to represent the large and mid-size bronchi. The fraction of metabolites present in the recoverable compartment and which equilibrates with the lavage fluid is assumed to be fairly constant. Consequently, it is the total recovered lipid metabolite amount and not the concentration of the metabolite, which may show correlation with

functional parameters. The second compartment, named “non-recoverable” may not interfere with the metabolite recovery from the recoverable compartment. The non-recoverable compartment is likely to represent the bronchoalveolar space. It is also likely that the majority of the liquid from this compartment is quickly reabsorbed. Variability of the compartment size could be due to variation in BAL technique, individual differences and variable re-absorption speed. It is likely that this latter factor has the highest impact.

Correlation of Lung function parameters with recovered amount of lipid biomarkers

To consider our model, the lung function values were plotted against the total recovered BAL lipid levels for each of the four lipids. For the total recovered, PgD₂ amount there is a striking inverse correlation with FEV₁, %FEV₁ and PEF which in all cases gave an R² value above 0.6. On the other hand, there was no correlation between, PgD₂ levels and total lung capacity. Given the observed correlation between total lung capacity and both the concentration of lipids recovered in BAL fluid as well as total lipid recovery the total recovered lipid values were normalized as a function of lung capacity and reanalyzed. The values obtained following this analysis demonstrate that the correlation between PgD₂ levels and the FEV₁ and %FEV₁ values are independent of total lung capacity whereas the correlations between PgD₂ and PEF as well as EPA and all of the lung function parameters were slightly dependent upon this parameter. The correlations for LPC and 15-HETE were not affected.

DISCUSSION

Our work is a first step to find novel biomarkers with the hope that these will serve as clinical surrogates during drug development and may also identify the treatment responsive population at an early stage. To focus our work we aimed at two important factors to strengthen our approach in comparison to those published in the relevant literature so far.

We choose hypothesis driven and hypothesis free discovery methods. As a hypothesis driven approach we choose to measure a selected group of lipid mediators. And as a hypothesis free approach we screened the entire human plasma proteome.

PLASMA PROTEIN BIOMARKER DISCOVERY

While we reported as a single study in this work, the results are typical for current experience base (e.g. mAbs specific for lung cancer. in preparation). Within eight months, our mAb-mediated proteomics approach delivered ten mAbs, which discriminated the normal and affected populations with high degree of statistical significance using a simple ELISA assays. The precision of the measurements was better than that of popular MS-based methods. Some of the proteins identified seem to be relevant to specific disease processes and have been verified on independent clinical cohorts in trial setting (Pfizer confidential source)

Based on our studies, we find the monoclonal antibody library based proteomics technology suitable for the discovery of disease specific biomarkers. We show four antibody biomarkers from the pool of ten we discovered with this technology. The monoclonal antibody biomarkers were found COPD specific on the cohorts we used. The new technology ensures the development of clinical laboratory tests without apparent bottleneck in contrast to MS based or targeted hypothesis mediated technologies. From this point of view our technology achieves discovery and early validation in one step. The inhibition test we present here for the first time, in a more generally applicable form has been marketed as QuantiPlasma biochip, which was developed by Biosystems International and Randox and has been tested successfully at the DEOEC.

We have developed a method that can generate disease-specific monoclonal antibodies (mAbs) to the native state of human plasma glycoproteins. Using a novel strategy, a large library of mAbs that can

discriminate COPD from controls was obtained. Furthermore, another important outcome of the study was the high quality of mAbs reagents that were produced. Immunological based assays are important tools for high throughput validation studies of biomarkers in a large sample cohort, and the availability of high-affinity and well characterized reagents are indispensable. Even though, the identification of the antigen for the COPD specific mAbs characterized in this study corresponded to relatively high level proteins, the success of the initial study suggests that this method could become a powerful approach to the rapid generation of disease-specific mAbs. The method had a high success rate and produced a large number of positive clones that discriminated COPD from normal. It is tempting to continue identifying the antigens for the remaining IgG secreting hybridomas. However, other methods for antigen identification, such as those based on epitope mapping via peptide libraries displayed on phage display technology may be more suitable for high throughput screening of antigens and are being explored. The experimental data collected in this preliminary research suggests that this methodology is very promising and can lead to the high throughput generation of a large bank of COPD specific mAbs with good specificity and high affinity. Therefore the mAbs can be excellent capture reagents for immunological based method in proteomic research and or biomarker validation and eventually for the development of novel diagnostics.

LIPID STUDY

We demonstrate for the first time that in the BAL a few lipid biomarkers (EPA, 15-HETE, LPC, PgD₂) quantified by LC-MS technology show an apparently good and positive linear correlation with TLC (Total Lung Capacity). The total PgD₂ quantity recovered from the BAL fluid showed good linear but inverse correlation with COPD specific lung function tests (FEV1 and FEV1%) after applying our hypothesis on the compartmentalization of BAL fluid. Important my work as evidenced from recent publications became one of the starting points for the development of PgD₂ inhibitors at the pharma industry. According to our new hypothesis on the BAL compartmentalization, a variable portion of the BAL fluid is reabsorbed quickly; however the “washed-out” lipid biomarkers are not reabsorbed. The hypothesis published here first is likely to be valid as supported strongly by the quality of the results. We plan to continue the qualification of the lipid biomarkers with special focus on PgD₂ and its involvement in COPD.

We found correlation between lung function parameters and biologically relevant lipids obtained directly from the diseased tissue compartment from BAL fluid. Although the number of samples was relatively small, this analysis demonstrates a good inverse correlation between the levels of PgD₂ and EPA measured in BAL fluid by mass spectrometry and several lung function parameters including FEV₁, %FEV₁ and PEF for PgD₂ and FEV₁ and %FEV₁ in the case of EPA. Furthermore, the linear correlation between the FEV₁ and %FEV₁ values and the level of PgD₂ was independent of total lung capacity, suggesting that this correlation is strongly associated with the lung function parameter being measured.

Many different classes of mediators of inflammation have been shown to play a role in COPD, including a number of lipids, chemokines and cytokines. In some cases, similar mechanisms of inflammation are shared between COPD and asthma whereas in other cases, some mechanisms are unique to one or the other disease. These mediators play a role in both the recruitment and activation of inflammatory cells. COPD is characterized by the presence in the lungs of macrophages, neutrophils and CD⁸⁺ T-cells, whereas in asthma the dominant inflammatory cell types present in the lungs include eosinophils, mast cells and CD⁴⁺ lymphocytes indicative of a largely allergic phenotype.

SUMMARY

Background: Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality worldwide. COPD is characterized by progressive development of airflow limitation that is not fully reversible. The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs. Diagnosis of COPD is built on symptoms and decreased level of lung function as tested by spirometry. COPD patients are usually diagnosed only when the disease is already in an advanced stage. Only about 15-20% (or more) of smokers are susceptible to developing COPD, and 85-90% of COPD patients are smokers, indicating the possibility of additional, genetic factors playing role in the development of COPD. Identification of biomarkers, in the longer term will result in the eventual development an early diagnostics for the asymptomatic COPD patients, for screening of smokers with high susceptibility to develop COPD and to follow therapeutic effects as new therapies will become available. Here, I present the first phase of the biomarker discovery work.

The aim of the study: The long term goal of my hypothesis free and hypothesis driven biomarker research is to enable the discovery, qualification and validation of an analyte panel based new laboratory diagnostics. My specific questions (protein biomarkers): Does the plasma proteome contain COPD specific biomarkers? Do we find protein biomarkers capable to discriminate smoking but COPD free subjects from smoking COPD patients? Additional goal of my work was to explore and introduce the less popular metabolom studies in COPD research. First we examined whether bronchoalveolar lavage fluid obtained from the site of the pathology, as the „direct imprint” of the disease process is applicable in the quest for lipid biomarkers? Specific questions on this subject: Do we find COPD specific lipid mediators in the BAL fluid with targeted hypothesis driven biomarker research? Is the hypothesis driven metabolom marker research applicable for the discovery and qualification of new markers in COPD?

Result and discussion:

Plasma – protein – biomarkers: Based on our studies, we find the monoclonal antibody library based proteomics technology suitable for the discovery of disease specific biomarkers. We show four antibody

biomarkers from the pool of ten we discovered with this technology. The monoclonal antibody biomarkers were found COPD specific on the cohorts we used. The new technology ensures the development of clinical laboratory tests without apparent bottleneck in contrast to MS based or targeted, hypothesis mediated technologies. From this point of view our technology achieves discovery and early validation in one step. The inhibition test we present here for the first time, in a more generally applicable form has been marketed as QuantiPlasma biochip, which was developed by Biosystems International and Randox and has been tested successfully at the Clinical and Molecular Laboratory of DEOEC.

BAL – lipid – biomarkers: We demonstrate for the first time that in the BAL a few lipid biomarkers (EPA, 15-HETE, LPC, PgD₂) quantified by LC-MS technology show an apparently good and positive linear correlation with TLC (Total Lung Capacity). The total, PgD₂ quantity recovered from the BAL fluid showed good linear but inverse correlation with COPD specific lung function tests (FEV1 and FEV1%) after applying our hypothesis on the compartmentalization of BAL fluid. Important my work as evidenced from recent publications became one of the starting points for the development of, PgD₂ inhibitors at the pharma industry. According to our new hypothesis on the BAL compartmentalization, the variable part of the BAL fluid is reabsorbed quickly; however the washed-out lipid biomarkers are not reabsorbed. The hypothesis published here first is likely to be valid as supported strongly by the quality of the results. We plan to continue the qualification of the lipid biomarkers with special focus on PgD₂ and its involvement in COPD.

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Candidate: Eszter Csánky

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Doctoral School: Doctoral School of Molecular Cell and Immune Biology

List of publications related to the dissertation

1. Csánky E.: A krónikus obstruktív tüdőbetegség patogenezise: Legfontosabb ismereteink 2011-ben. Orvostudományok. Szle. 78 (10), 11-18, 2011.
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