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Fibulin 5: a key player in elastogenesis and emphysema development in COPD?

Setup of processing lung tissue samples and optimizing LC-MS method for targeted proteomics analysis

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Abstract

Chronic obstructive pulmonary disease (COPD), a chronic and progressive lung disease, has become a worldwide public health problem, with rising morbidity and mortality. The main characteristic of COPD is air flow limitation caused by a combination of small airway wall thickening and emphysema. Current treatments are not able to induce repair or to prevent further lung tissue damage since the exact mechanisms and pathological events are unknown. Therefore a better understanding is needed about how these repair processes are regulated in lung tissue and airways. A previous study performed gene expression analyses of COPD and control lung tissue samples. They found an increased expression of 112 genes and 61 genes of which expression was decreased in COPD patients compared to controls. Results showed that the most significantly increased gene was fibulin-5 (FBLN5). FBLN5 is known as an extracellular-matrix protein and plays an essential role in elastogenesis. Since FBLN5 gene expression is upregulated in COPD and an increased level of cleaved fibulin-5 has been shown in COPD samples, we hypothesize that increased protein cleavage of FBLN5 and consequently high levels of non-functional FBLN5 protein are present in COPD, which hampers elastogenesis and thus contributes to emphysema development. The overall aim of this study is to measure the levels of (cleaved) FBLN5 protein in a large set of COPD and control lung tissue samples using a targeted quantitative mass spectrometry analysis. After liquid chromatography–mass spectrometry (LC-MS) analysis and determination of the protein levels, the levels of cleaved FBLN5 can be correlated with the presence of COPD and degree of emphysema. To establish this a procedure for sample selection had to be created and a specific LC-MS method had to be set up. In this study a workflow was established to select and prepare lung tissue samples. Furthermore, a LC-MS method to measure the proteins of interest was created. After the optimization procedure, 123 tissue samples were prepared for LC-MS measurements.

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Introduction

Chronic obstructive pulmonary disease (COPD), a chronic and progressive lung disease, has become a worldwide public health problem[1]. Nowadays 64 million people are suffering from COPD and its rank as a cause of death has been increased since 1990 [1] [2,3]. The increased mortality of COPD is mainly directed by the enlarging epidemic of smoking and the impact of the rapid ageing of the world population[1]. The current available treatments are symptom relieving and aim to prevent disease progression to improve the patients' health status[4,5]. Unfortunately, still no cure has been found [6] [7]. Smoking cigarettes is the main cause of COPD, but since there exists large variability in the susceptibility to develop COPD in smokers, presence of genetic influences is suggested[8]. Currently alpha-1 antitrypsin deficiency (ATT) is the only proven genetic risk factor of COPD[9]. Additionally, it has been shown that particulate matter air pollution correlates with respiratory illness, especially in urban- and industrialized areas. Several studies demonstrate that traffic- and industry-related air pollutants may have a significant influence on the decline of pulmonary function and the growing rate of respiratory diseases as COPD or lung cancer [10–14].

The main characteristic of COPD is air flow limitation caused by a combination of small airways disease and emphysema, which ratio is different per person[1,15–17]. Airflow limitation is used as an indicator of the severity of COPD, done by spirometry. During the spirometry both forced vital capacity (FVC) and forced expiratory volume in one second (FEV_1) has to be measured. FVC stands for the volume of air forcibly exhaled after maximal inspiration. FEV_1 is the volume of air exhaled during the first second after maximal inspiration. When these to values are determined, the ratio of these two (FEV_1/FVC) can be calculated. The presence of a FEV_1/FVC < 0.70 indicates the presence of airflow obstruction. When this airflow obstruction is irreversible, based on the change in FEV_1 after administration of a bronchodilator, patients are diagnosed with COPD[18]. The parameter FEV_1 is determined to indicate the severity of airflow limitation. Based on the outcome the severity of COPD can by classified in GOLD stages 1 to 4, shown in Table 1[7]. Another parameter which is often taken in account is patients smoking history, determined by pack-years. Pack-years are calculated by dividing the number of cigarettes smoked per day by twenty and multiply this by the amount of smoking years.

Gold stage	Severity of airflow limitation	FEV_1
Stage I	Mild	≥80%)
Stage II	Moderate	50% ≤ FEV_1<80%
Stage III	Severe	30 % ≤FEV_1 < 50
Stage IV	Very severe	FEV_1< 30%

Table 1 Classification of severity of airflow limitation in COPD (based on post-bronchodilator FEV1) in patients with FEV1/FVC < 0.70. [7]

Lung tissue remodeling in COPD, causing the limitation of breathing, is due to pathological events. Chronic inflammation is an important feature in COPD, affecting both large and small airways and lung parenchyma[4]. Emphysema and (small) airway wall thickening are the specific remodeling processes in COPD, which are seemingly paradoxical processes. Airway wall thickening results from a fibrotic response with increased extracellular matrix (ECM) production, while emphysema is characterized by damaged alveoli, destruction of tissue and after this features insufficient ECM production and inadequate repair. Current treatments are not able to induce repair or to prevent further lung tissue damage since the exact mechanisms and pathological events are unknown. Therefore a better understanding is needed about how these repair processes are regulated in lung tissue and airways.

Background

A previous study performed gene expression analyses of COPD and control lung tissue samples and found increased expression of 112 genes and 61 genes of which expression was decreased in COPD patients compared to controls. Results showed that the most significantly increased gene was fibulin-5 (FBLN5). [19] FBLN5 is known as an ECM protein and plays an essential role in elastogenesis. Nakamura *et al*. confirmed this essential role of FLBN5 by investigating fibulin-5-deficient mice, which resulted in a human aging phenotype such as loose skin and emphysematous lungs.[20] Furthermore, Katsuta *et al*. found that overexpression of elastin and fibulin-5 induced similar levels of elastic fibers in fibroblasts as cells overexpressing only fibulin-5, suggesting that fibulin-5 is a critical component in the control of elastic fibers.[21] Interestingly, FBLN5 protein can be cleaved by serine proteases resulting in a nonfunctional protein (Figure 1A), which was demonstrated to be increased in aging mice (1B)[22].

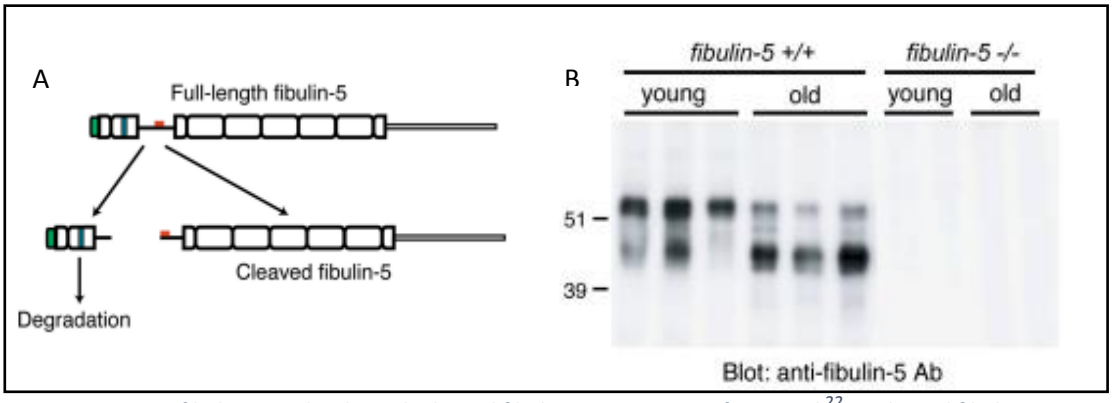

Figure 1 A: Intact fibulin-5 can be cleaved. Cleaved fibulin-5 protein is not functional.[22] B: Cleaved fibulin-5 is increased in old mice.[22]

Hirai *et al*. reported that FBLN5, when deposited on microfibrils, promotes assembly of elastic fiber components such as tropoelastin molecules and also interacts with LOXS enzymes that cross-link elastin. Microfibrils are mainly composed of fibrilin-1 and -2 and are considered to serve as scaffold for tropoelastin deposition. They have found that cleavage of FBLN5 destabilizes the FBLN5-microfibril interaction, leading to failure of the FBLN5 organizing ability in elastic fiber assembly, showed in Figure 3.[22]

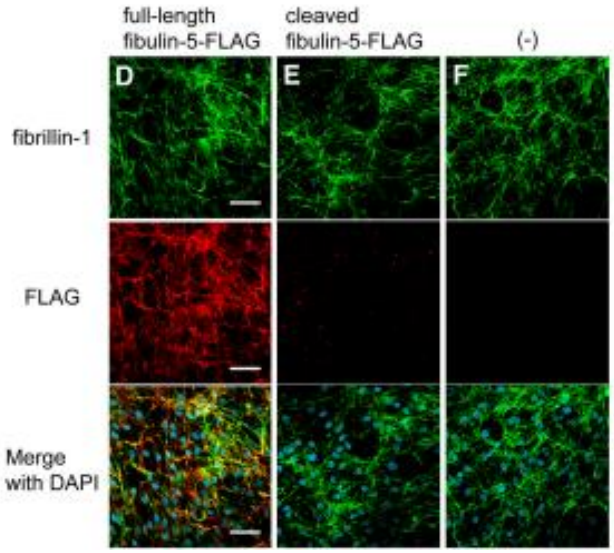

Figure 3 Recombinant fibulin-5 protein was added to 293T cell culture, fibulin-5 co-localized with fibrillin-1 micro fibrils. When recombinant truncated fibulin-5 protein was added to the culture, the added truncated fibulin-5 did not deposit on fibrillin-1 microfibrils. [22]

The functional role of FBLN5 in elastogenesis and tissue repair in COPD has to be demonstrated. However the increased levels of cleaved FBLN5 in COPD patients has to be demonstrated first. Further the link between cleaved FBLN5 levels and emphysema severity has to be determined. Brandsma *et al*. already have found higher levels of cleaved FBLN5 protein in a small subset of COPD lung tissue samples compared to controls (Figure 2)[19]. Since FBLN5 gene expression is upregulated in COPD and an increased level of cleaved fibulin-5 has been shown in COPD samples, we hypothesize that increased protein cleavage of FBLN5 and consequently high levels of non-functional FBLN5 protein are present in COPD, which hampers elastogenesis and thus contributes to emphysema development.

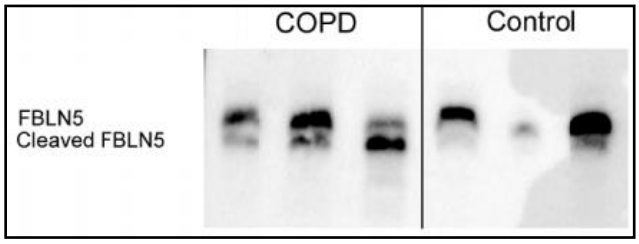

Figure 2 Brandsma et al. Western blot showing full length FBLN5 protein and cleaved from of FBLN5 in lung tissue from 3 COPD patients and 3 control subjects. [19]

To test this hypothesis, the levels of (cleaved) FBLN5 protein in a large set of COPD and control lung tissue samples will be measured. This will be done by a sensitive and targeted quantitative mass spectrometry approach. To complete our protein validation for our elastogenesis gene signature, we included additionally protein levels measurements of microfibrillar-associated protein 4 (MFAP4), latent transforming growth factor beta binding protein 2 (LTBP2) and elastin (ELN). Lung tissue samples from well characterized COPD patients with different disease severities and non-COPD control subjects were used. Furthermore, we have included a group of COPD patients with α-1-anti-trypsin deficiency. These patients develop COPD due to high protease activity in their lungs and therefore are expected to have the highest levels of cleaved FBLN5.

The overall aim of this study is to measure the levels of (cleaved) FBLN5 protein in a large set of COPD and control lung tissue samples using a targeted quantitative mass spectrometry analysis. After LC-MS analysis and determination of the protein levels, the levels of cleaved FBLN5 can be correlated with the presence of COPD and degree of emphysema. To establish this a procedure for sample selection had to be created and a specific LC-MS method had to be set up. Therefore, the main goal of this study was to setup a workflow to select lung tissue samples. Furthermore, a LC-MS method to measure the proteins of interest had to be created. Optimization is performed during the workflow- and method setup. After the optimization procedure, the selected tissue samples were prepared for LC-MS measurements.

Proteomics approach

The quantification of the protein levels is carried out by a proteomics approach. Proteomics is defined as the large-scale characterization of the entire protein complement of a cell line, tissue or organism[23]. In proteomics several approaches can be applied. One approach is to convert an entire protein mixture to peptides and purify the peptides before running them for analysis by mass spectrometry, either in a discovery- or targeted proteomics setting. Where discovery proteomics experiments are intended to identify as many proteins as possible, targeted proteomics experiments are designed to quantify less proteins but with very high precision, sensitivity and specificity[24]. As said, based on the previous study of

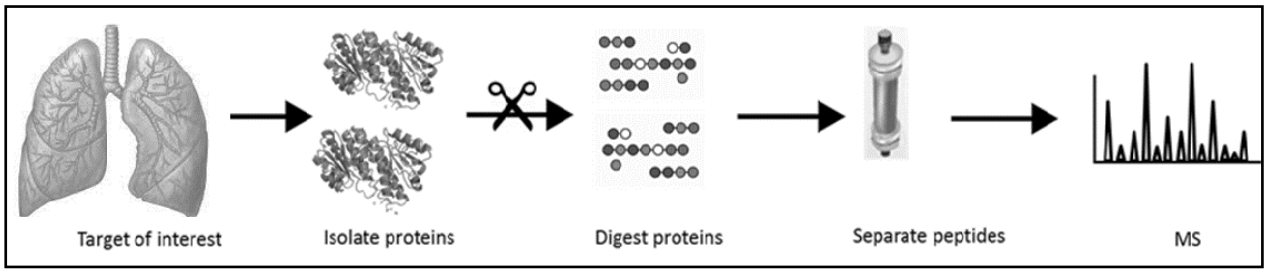

Figure 4 Proteomics approach workflow

Brandsma *et al*., the proteins of interest selected for targeted quantification in this study are FBLN5, MFAP4, LTBP2 and ELN[19]. In proteomics proteins are usually detected as peptides. Detection will be performed in a mass spectrometer coupled to a liquid chromatography (LC) system (Figure 4). The LC system is used for separating peptides based on their hydrophobicity. Peptides bind to a non-polar stationary phase and are eluted by increasing a non-polar character of the mobile phase[25]. This results in peptide separation because the more non-polar peptide elute later than more polar peptides. The timeframe of binding and elution of the peptide is called retention time. The peptides are obtained by digesting protein mixtures into peptides using a protease, in this case trypsin. An important characteristic of trypsin is high cleavage specificity and therefore is the main protease used in proteomics. Trypsin cleaves C-termini to arginine or lysine residues and places the highly basic residues at the C-termini of the peptides. This generally leads to high y-ion series (Figure 5)[26].

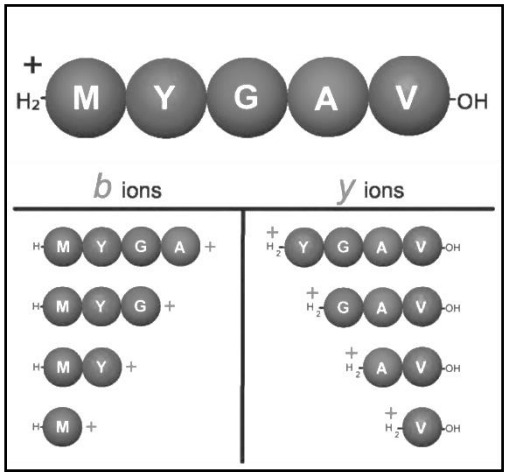

Figure 5 Example of b ions and y ions that may appear in MS spectrum. [27]

In targeted proteomics for absolute quantification, isotopically labeled peptides are added to determine the concentrations of the proteins in the sample. In isotopic peptides Lysine (K) and Arginine (R) are labeled with ^{13}C and can be distinguished via this mass shift. The LC retention time of the peptide and the quantitative mass spectrometer response will not be affected. [28] Due to this mass shift the labeled peptide is separately shown from the endogenous peptide in the mass spectrum. Since the concentration of the isotopically labeled peptide is known, the concentration of the endogenous peptide can be calculated. The software Skyline provides a function of determining the ratio between the endogenous and the labeled

peptide based on the intensity measure by the MS, an example is shown is in Figure 6. Using this method the concentration of the endogenous peptide can be calculated.

Setup and optimization methods for LC-MS

To be able to measure the concentration of the proteins of interest, a specific method for the LC-MS had to be set up. In this project we aim to develop a method to measure each selected FBLN5 peptide and the other peptides of interest. This setup of the method requires optimization steps on several variables.

Per protein, several peptides were selected on forehand, considering uniqueness and optimal properties for LC-MS detection. Each peptide has its own characteristics, which are used to develop the LC-MS method to measure the peptide. As shown in figure 6, a peptide can be cut into numerous different peptides , called product ions. These product ions are given a letter and a subscript is added to indicate the number of amino acid residues present in the product ion. For example, the first ion showed in figure 6 retains four amino acids from the intact peptide. Counted from the C-terminus the ion is labeled as y4 and from the N-terminus is called the b4 ion. To break the bonds of the peptide for generating product ions energy is required. This energy is different for each peptide and is the second characteristic. The required energy, named collision energy, has to be optimized for each peptide to get the highest intensity of transitions. The third characteristic of peptides is the retention of the peptide to the LC column, so-called retention time, has to be determined per peptide to complete the LC-MS method.

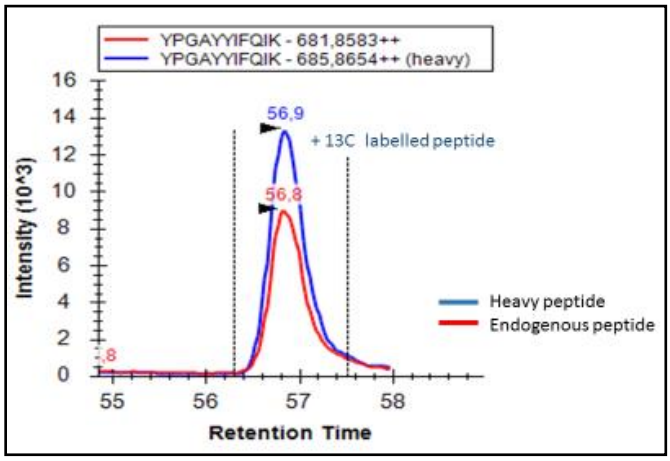

Figure 6 Example Skyline graph of one peptide with in red the measured intensity of endogenous peptide, in blue the isotopically labeled peptide

Methods & Materials

Experimental groups

Tissue samples (n=123) from well characterized COPD patients with different disease severities and non-COPD control subjects were included (Table 2). Lung tissue samples are obtained from the lung tissue biobank from the UMCG, Pathology department, which were previous received from lobectomy or transplantation.

Experimental group (n)	Characteristics
Control subjects (n=17)	never smokers + lung disease history, normal FEV1 and FEV1/FVC >0.7
Control subjects (n=41)	>10 pack years, normal FEV1 no lung disease history, current+ ex-smokers
Stage II COPD patients (n=30)	>10 pack years, $50\% \leq$ FEV1$< 80\%$ current + ex-smokers
Stage III COPD patients (n=13)	>10 pack years $30\ \% \leq$FEV1$< 50\%$
Stage IV COPD patients (n=34)	>10 pack years, FEV1$< 30\%$
Stage IV COPD patients (n=19)	alpha-1-antitrypsin deficiency, $FEV_1 < 30\%$, ex-smokers

Table 2 Experimental groups and characteristics.

Lung tissue samples

Frozen peripheral lung tissues are used and sliced into $\pm$10x5µ[1] slices using a cryostate, considering tissue size variation. Hematoxylin and eosin (HE[2]) stained tissue sections taken before and after the section of slides used for proteomics analysis were checked for abnormalities (i.e. excessive inflammation, fibrosis, tumor cells, large airways) by a pathologist before further processing.

Tissue sample preparation

Lung tissue samples were distributed over 3 batches by random sampling, with approximately uniform distribution of each experimental group in each batch. The tissue slices were collected in a screw cap tube (2.0 Screw cap tube, Conical, #B91211), including 3 or 4 beats (2,3 mm diameter, ZIRCONIA/SILICA, #11079125z, BioSpec Products) and kept frozen until bead beating. Tissue was lysed in 100 µl of lysis buffer containing Ripa buffer + protease inhibitor cocktail (100x dilution). The tissues were then homogenized with the Precellys 24 bead beater for two times at 6000 rpm (2x 15 seconds) and then centrifuged at 12.000 rcf for 10 minutes at 4°C. The supernatant containing the homogenized tissues were collected and stored at -80°C. Protein concentration in the lysate was determined using the microplate bicinchoninic acid (µBCA) assay kit (Thermo Fisher Scientific), following the manufacturer's instructions. Bovine Serum Albumin (BSA) was used as standard.

[1] Fine: settingmode of cryostate; thickness per cut
[2] HE-staining: haematoxylin and eosin staining, respectively colors nuclei and cytoplasm

Protein preparation

Samples were prepare for separation by SDS-PAGE at a concentration of 50 µg total protein concentration. Protein mixtures containing 50 µg of protein and LDS loading buffer were loaded, after heating at 90 °C for 5 minutes, onto a 12 wells RunBlue 4-12% Bis-Tris Gel (Expedeon, #NBT41212), run for 5 min at 100V and stained with InstantBlue staining (Expedeon, #ISB1L) for at least 30 minutes. The band containing all proteins was excised from the gel and sliced into smaller gel pieces. Gel pieces were washed with a decreasing concentration ammonium bicarbonate (ABC) and increasing concentration acetonitrile (ACN). During these 30 minutes washing steps, samples were incubated in a thermomixer (500 rpm) at 30 °C. Washing started with 70% 100mM ABC + 30 % ACN. This step was repeated if the gel pieces still were blueish. Then 50% 100 mM ABC + 50% ACN and last 5 minutes with 100% ACN, before drying in an oven at 37 °C. Proteins were reduced with 20 µl 10 mM diothithreitol (DTT) for 30 minutes at 55-60 °C. Alkylation of the reduced disulfide bridges is done with 20 µl 55 mM iodoacetamide (IAA) at room temperature in the dark for 30 minutes. The gel pieces were washed with 300 µl 100% ACN while mixing at 900 rpm for 30 minutes and dried in oven at 37°C. Protein digestion was performed by overnight digestion with 30 µl trypsin (Sequencing grade modified trypsin, Promega) in a 1:100 w/w trypsin:protein ratio at 37 °C. The residual liquid was collected. Peptide extraction was performed with 30 µl 75% ACN + 5% formic acid (FA). Liquid was collected and combined with previous residual liquid and diluted in 800 µl 0.1% FA for SPE cleanup with C18-Aq columns (Gracepure TM SPE, 50mg/1ml, #5141486). The columns were conditioned with 2x 1 ml 100% ACN + 0.1% FA and re-equilibrated with 2x 1 ml 0.1% FA. The diluted tryptic digest was applied to the packing bed and washed with 2x 1 ml 0.1% FA. Peptides were eluted with 2x 0.4 ml 50% ACN + 0.1% FA. The eluates were dried in SpeedVac under vacuum at 60 °C. For measurement, the dried samples were resuspended in 25 µl 0.1% FA, resulting in a protein concentration of 2 µl/µl per sample. For measurement 5 µl of the sample was put in a MS-vial plus 5 µl of the mix of synthetic peptides.

Mass spectrometry analysis

Mass spectrometric analyses was done on a triple quadrupole mass spectrometer (TSQ Vantage, Thermo Scientific), coupled to a nano-liquid chromatography system (Ultimate 3000, Dionex) with a nano-LC column (Acclaim PepMap RSLC 100 C18, column i.d. 75 µm x 15 cm, particle size 2 µn, pore size 100 Å, Dionex) for the separation of the peptides. Samples were injected from a cooled autosampler (5 °C) and loaded onto a trap column (Acclaim Pepmap100 C18, column i.d. 300µm x 5 mm, particle size 5 µm, pore size 100 Å). Peptides were separated on the nano-LC column using a lineair gradient from 3% building up to 60% acetonitrile with 0.1% formic acid in 100 minutes at a 300nL/min flow rate.

Targeted peptide approach

^{13}C-lysine or arginine labeled standards were ordered from Thermo Scientific. Per protein at least two peptides were selected. Except for LBTP2, one peptide was selected due to no availability of the synthetic peptide for the second peptide (Table 3). In a later stadium of this project the proteins GAPDH, HPT, p63 and new peptide for LTBP2 will be added as controls for respectively housekeeping protein marker, blood marker and marker for bronchial cells.

Protein	Selected peptide(s)
MFAP4 Microfibrillar-associated protein 4	▪ GWNDY(K) ▪ VDLEDFENNTAYA(K)
LTBP2 Latent transforming growth factor beta binding protein	▪ QSTFTLPLSNQLASVNPSLV(K) ▪ *SLGPGTCTLPLAQ(R)*
ELN Elastin	▪ LPYGYGPGGVAGAAG(K) ▪ LPGGYGLPYTTG(K)
FBLN5 Fibulin- 5	▪ QSGQCLDIDEC(R) ▪ TIPEAC(R) ▪ CIDPI(R) ▪ YPGAYYIFQI(K)
GAPDH *Glyceraldehyde 3-phosphate dehydrogenase*	▪ *QASEGPL(K)*
HPT *Haptoglobin*	▪ *VTSIQDWVQ(K)*
p63 *Transformation-related protein 63*	▪ *LPSVSQLINPQQ(R)* ▪ *VEGNSHAQYVEDPITG(R)*

Table 3 Proteins of interest and selected peptides per protein. Proteins and peptides added in a later stadium are written in italic.

Data processing

Optimizing will be done using the specified software program named Skyline, designed for building quantitative method and analyzing targeted proteomics experiments[29]. Skyline works with predictive algorithms and libraries (NIST). After loading proteins and peptides in FASTA-format from UniProt Web site (http://www.uniprot.org/) into Skyline, by predictive algorithms the charge state and fragment ions for each peptide are chosen for the attained method. This methods, based on predictive libraries and algorithms, is used as starting point for further optimization. Skyline automatically selects precursors, transitions, collisions energy and retention time for each peptide. In this project transition selection per peptide is done, starting with the predicted top 7 transition by Skyline. Also determination of the optimal collision energy per peptide is performed with Skyline-predicted collision energy as starting point. Retention times of each peptide is determined for two LC-MS devices.

Results

I Optimizing sample conditions: Determination minimal acquired tissue input

To determine the number and thickness of the tissue slices and the amount of lysis buffer needed to get sufficient protein yield, we started with various numbers of slices and different volumes of lysis buffer (**Fout! Verwijzingsbron niet gevonden.**). Tissue from two different t-numbers were used: T15-16416 (α--anti-trypsin deficiency) and T15-9153 (COPD), respectively named sample 1 and 2.

In sample 2 the amount of 100µ and 50µ in 50µl lysis buffer result in the highest protein concentration and also the overall result is a higher protein concentration comparing to sample 1 (Table 4). We suggest that this is due to blood particles: after adding the lysis buffer, the supernatant of the samples of sample 2 were very reddish. Therefore, we only considered the results from sample 1, which show that 5x10µ in 100 µl is sufficient.

Sample 1/2	Slides (number x µ)	Lysis buffer (µl)	Protein concentration (mg/ml) sample 1	Protein concentration (mg/ml)sample 2
A	10x10	100	3,44	9,02
B	10x10	50 (100 in 1B)	4,08	17,26
C	5x10	100	5,80	8,21
D	5x10	50	6,50	12,91
E	2x10	100	4,19	4,21
F	2x10	50	4,42	6,50

Table 4 Results from test 1.

Nevertheless a second test to determine the minimal acquired tissue is performed. In the second test both 2x10µ and 5x10µ of tissue was included. In this test the thickness of the slices were reduced from 10 to 5 fine to make the lysis buffer more efficient. To maintain the total amount of tissue (20µ and 50µ), the number of slices are increased. A previous test for tissue preparation, done by others, showed that bead-beating twice instead of once resulted in a higher intensity of the elastin peptides in MS measurements. They also tested lysis only and sonication to increase the intensity of elastin peptides. Figure 7 shows that bead-beating results in higher intensity of elastin compare to lysis and sonication.

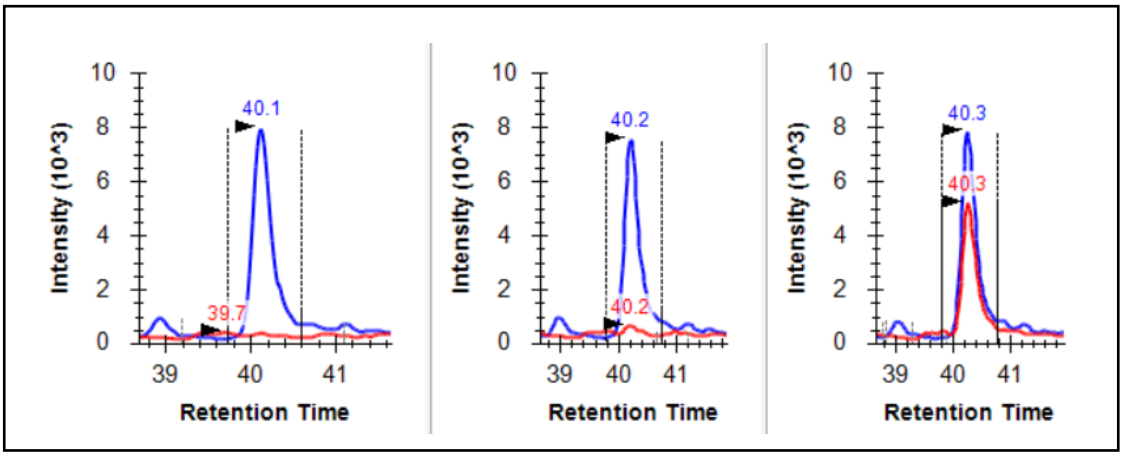

Figure 7 Results from respectively shown: Lysis, sonication, bead-beating.

In the second test to determine the minimal acquired tissue input, we included the bead-beating method as variable (Table 5). In sample 1A the high protein concentration is outstanding, which was bead-beaded two times. Sample 1B and 1C were bead-beaded once and show a lower protein concentration. The amount of lysis buffer causes no difference in protein concentration, comparing B and C. It seems that the higher protein concentration of sample A is due to the bead-bead-beating. This difference is not seen in sample 2 in test 2. Though it shows that the amount of tissue influences the protein concentration; a higher protein concentration in the samples containing 50µ tissue. This could be caused by blood particles as suggested previously. Noticeable, the protein concentration for 1F and 2F is aberrant compare to the other two samples containing 10x5µ of tissue. The bead-beating didn't result in a higher protein concentration in the samples containing 10x5µ of tissue. Considering each outcome of the test we concluded that 50µ of tissue in a ratio of 10x5 and a volume of 100 µl lysis buffer is sufficient to give a decent protein concentration. Despite it didn't result in a higher protein concentration in samples containing 10x5µ, we decided to bead-beat each sample two times, to improve the intensity of elastin in MS measurement.

Sample 1/2	Slides (number x (µ))	Lysis buffer (µl)	Bead-beader (times)	Protein concentration (mg/ml) sample 1	Protein concentration (mg/ml) sample 2
A	4x5	50	2	9,76	7,07
B	4x5	50	1	2,21	8,59
C	4x5	100	1	1,36	7,15
D	10x5	100	2	7,93	12,31
E	10x5	100	1	5,37	12,87
F	10x5	100	1	8,59	13,56

Table 5 Results from test 2.

Setup sample selection procedure

After deciding which number of slices, volume lysis buffer and times of bead-beading, we started with the t-number selection and cutting of all subjects (Table 6). A list of potential subjects containing information on disease severity, age, gender and smoking behavior and freezer storage location was generated. Per subject the availability of frozen peripheral lung tissue was checked. If available the tissues were critically monitored for amount of tissue and blood. In this project only peripheral lung tissues are included, since here the amount of large bronchi is lower. Before cutting the lung tissues for protein isolation, a slice of tissue is cut and put on a glass for an HE-staining (HE before). Then the determined amount tissue is cut for protein isolation and is put directly in bead-bead tubes. Hereafter again a slice of tissue is selected for HE-staining (HE after). The stained HE's were checked by the pathologist for abnormalities. When excessive inflammation, fibrosis, tumor cells or large airways were noticed the t-number was excluded from further processing. In some cases the abnormalities were located at the outer side of the tissue block. Then this part of the tissue was cut out and the remaining part of the tissue was again sliced and checked by the pathologist. After these steps a list of lung tissue samples of 123 subjects was composed. For the protein and peptide isolation the samples were divided in three batches, each with approximately the same distribution of subjects from each experimental group. Each subject was blinded with a letter and number (MS-number). In each batch a control sample was included as a reproducibility control, to be able to correct for possible 'batch-effects'.

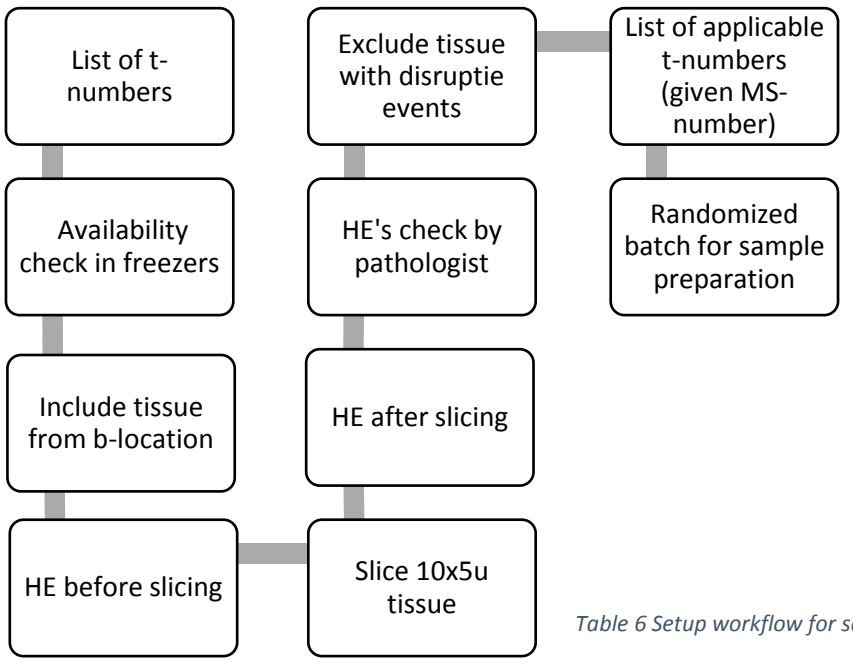

Table 6 Setup workflow for sample selection.

II LC-MS method setup and optimization

To be able to measure our peptides of interest, a specific LC-MS method for these peptides has to be developed. For this method several features had to be checked and optimized. The targeted proteomics software Skyline[29] was used to predict the values of transitions, collision energy and retention time for each peptide.

Since the peptides of interest were already selected based on unique sequence, specificity to the protein and optimal properties for mass spectrometric detection (see Table 3), only the target proteins and peptides were put into the software. In the first measurement, using a sample containing only all isotopically labeled peptides, seven transitions were measured per peptide. These seven transitions were the top seven of the predicted ranking done by Skyline. The results of the first measurement were used to select the top 3 transitions per peptide. During the selection, transitions with a disturbed peak or a lot of background (interference) were excluded. Transitions with the highest intensity, without disruptive events, were included in the top 3 (Figure 8). Skyline also predicted the collision energy (CE) for each peptide. In most cases the prediction was already in a good range and had to be adjusted with 1 or 2 steps (Figure 9A). In some cases the optimum CE was not in the window and measurement had to be redone (Figure 9B). Of each peptide the optimal CE was determined and is shown in Table 7. The retention time (RT) of each peptide was determined for TSQ1 and TSQ2, started with a broad window and then assessed the retention time (in minutes) (Table 7).

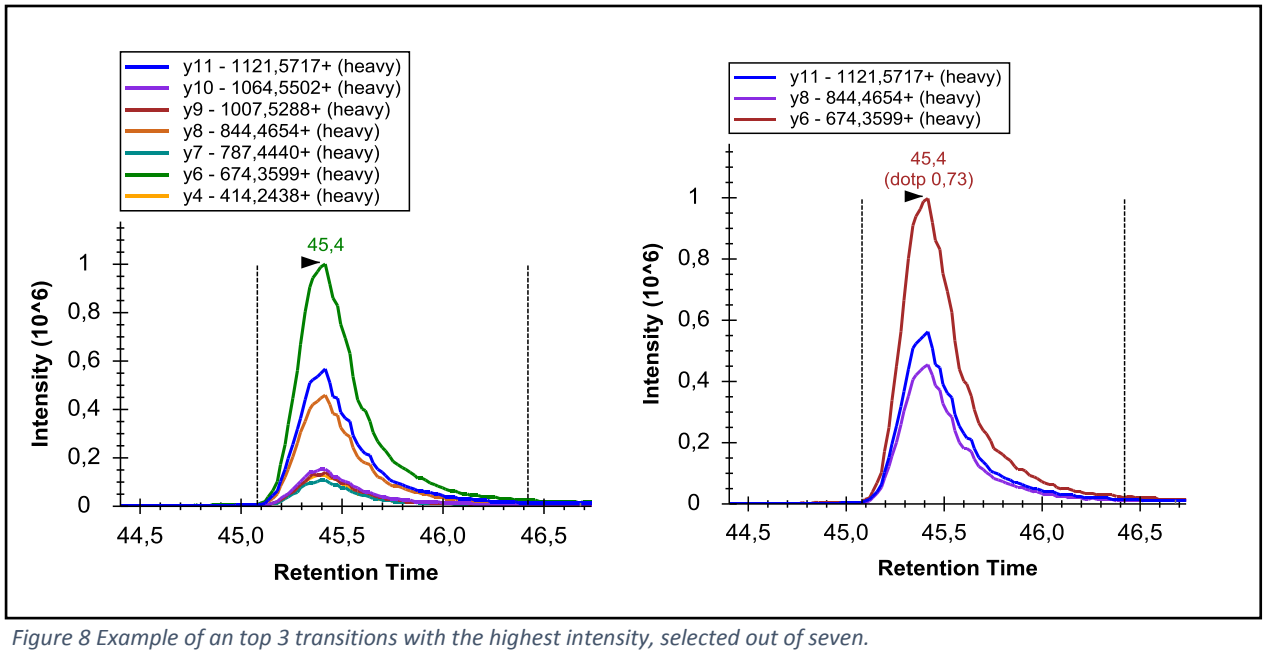

Figure 8 Example of an top 3 transitions with the highest intensity, selected out of seven.

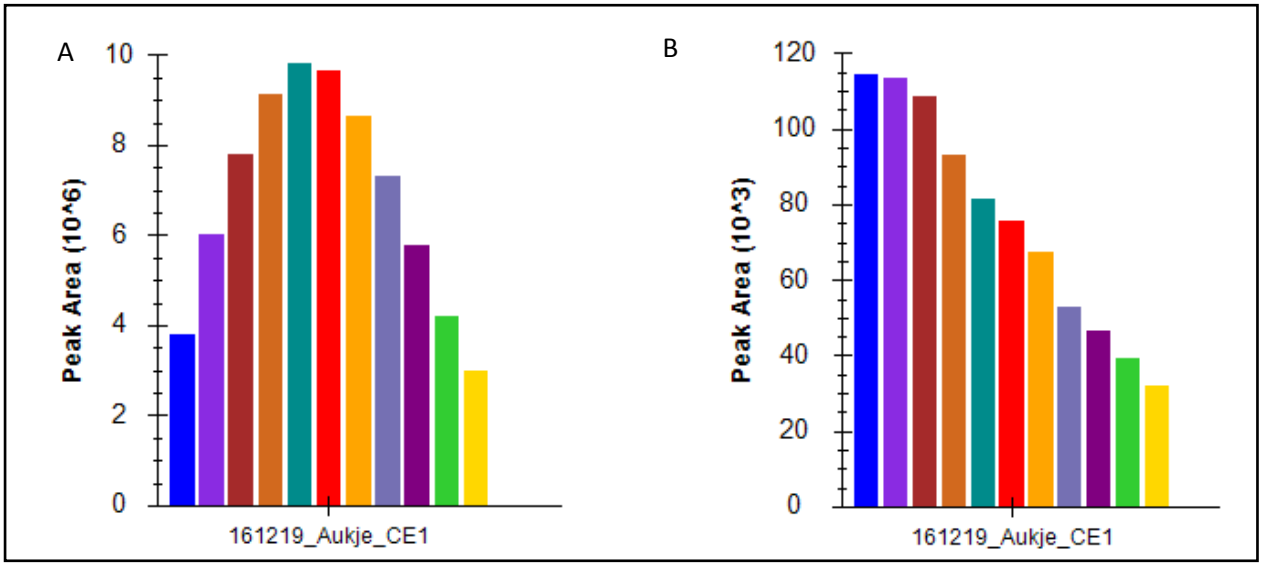

Figure 9 Transported from Skyline. A: Skyline's predicted CE in right range. B: Predicted CE out of window.

Protein	Peptide	Top 3 transitions	Optimum CE	Optimum RT TSQ1	Optimum RT TSQ2
MFAP4	GWNDYK.L	y3+, y4+, y5++	13.7	34	30
	VDLEDFENNTAYAK.Y	y7+, y8+, y10+	26.4	51	50
LTBP2	QSTFTLPLSNQLASVNPSLSVK.V	y6+, y9+, y10+	21.7	61	59
ELN	LPGGYGLPYTTGK.L	y6+, y11+, y12++	27.8	47	45
	LPYGYGPGGVAGAAGK.A	y10+, y11+, y15++	26.4	43	40
FBLN5	QSGQCLDIDECR.T	y4+, y5+ ,y6+	25.1	35	33.3
	TIPEACR.G	y4+, y5+, y5++	14.6	25	24
	CIDPIR.C	b3+, y3+, y4+	13.5	31	28.5
	YPGAYYIFQIK.S	y7+, y9+, y10++	23.4	57	59

Table 7 Overview of optimum CE, RT TSQ1 and TSQ2 per selected peptide.

Preliminary results

Once the sample preparations were performed and the method for the LC-MS were set-up and optimized, mass spectrometric measurement were performed on the lung tissue samples. In this project we aimed to measure FBLN5 concentration in lung tissue by a proteomics approach. Here we show that we are able to measure each selected FBLN5 peptide in our samples (Figure 10). Also both ELN peptides were measured, both the endogenous as the synthetic. The GWNDY(K)-peptide of the MFAP4 protein was measured, but for the other MFAP4-peptide, VDLEDFENNTAYA(K), we were not able to measure the endogenous peptide in all samples. We were able to measure the selected peptide for LTBP2, but during the project both endogenous and synthetic peptide were not consistently measured. Therefore, we selected another peptide for LTBP2 during the project. The peptides of GAPDH and HPT were able to be measured both endogenous and synthetic.

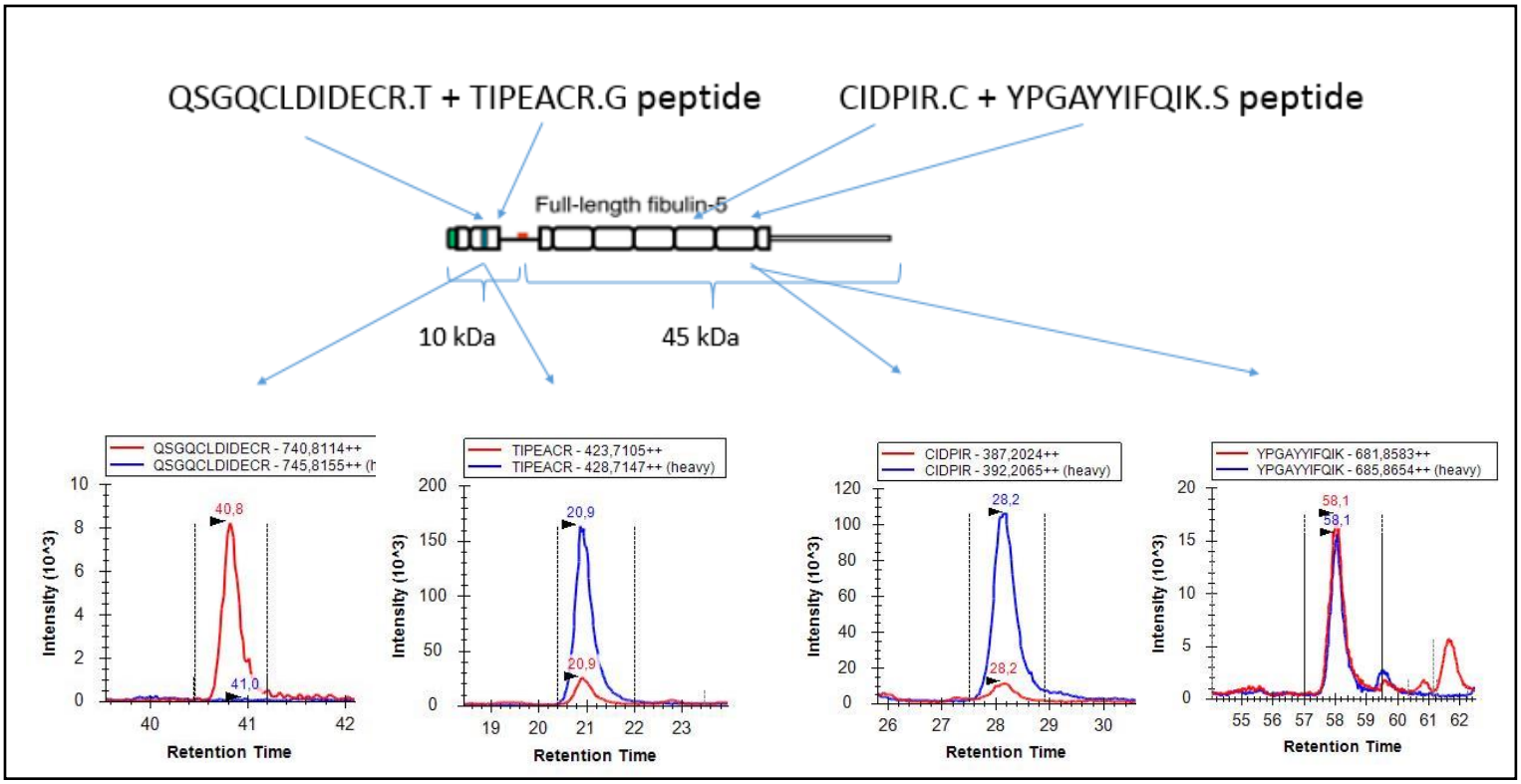

Figure 10 Measurement of the selected peptides of FBLN-5.

Discussion

The overall aim of this study is to measure the levels of (cleaved) FBLN5 protein in a large set of COPD and control lung tissue samples using a targeted quantitative mass spectrometry analysis. The initial aim of this study was to setup a workflow to select lung tissue samples. Furthermore, a LC-MS method to measure the proteins of interest had to be created. Optimization had to be performed during the workflow- and method setup. After the optimization procedure, the selected tissue samples were prepared for LC-MS measurements.

In this project a procedure had to be setup for lung tissue preparation. Test experiments were performed and resulted in a lung tissue preparation workflow, which is shown in Table 6 Setup workflow for sample selection. Our decision to put lung tissue slices directly in screw cap tubes was based on the fact that lung tissue rapidly thaws when it is transported into a new cup. Given that thawing of tissue contribute to changes in the molecular profile of the tissue and the integrity of nucleic acids[30,31], excluding the transport of tissue into new cups will reduce the chance of thawing and its effects.

Our decision on the amount of lung tissue used for the protein extraction was based on two test experiments with two lung tissue samples, which indicated that the amount of tissue was sufficient for our purpose. Nonetheless, additional validation and reproducibility tests would have been justified as results from 1 sample were mostly rejected due to blood contamination. Given that lung tissue consist a high density of capillary network, blood contamination is inevitable [32]. Therefore, the HPT-peptide is included to measure the level of contamination of blood in each sample. Furthermore, the size variance of lung tissue per t-number was considered. The amount of slices was adjusted to the size of the frozen tissue block. For each sample the amount of included tissue will examined by the concentration of GAPDH and p63.

The decision on the use of bead-beating to prepare the tissue was based on the results of the intensity of ELN peptides. ELN peptides were not detected when only lysis was performed, neither with sonication. ELN is an extracellular matrix protein, surrounded by glycoproteins, which are suggested to inhibit digestion by enzymes[33]. It has been found that agitating techniques are acquired to enhance tryptic fragmentation in cellular matrixes[34,35].

Furthermore, since each sample will be measured only once, we introduced two controls in each batch. One control to examine the reproducibility of the preparation of the samples in each batch, the sample control. Next to this an MS control sample was taken into account to administer the stability of the LC-MS

measurements. Therefore, from one sample, multiple aliquots were made after preparation. In every measurement series, this MS control sample will be measured to check and correct for possible variance due to the LC-MS (Appendix I[3]). The preparation of the samples before measurement required multiple pipetting steps and is therefore very prone for deviations. Sample preparation is done by one person, attempting to reduce these deviations. Next to this only Low-Bind Eppendorf cups were used to diminish loss of the content.

In this project we aimed to measure FBLN5 in lung tissue by a proteomics approach. To accomplish this goal, a sample preparation workflow was developed. We demonstrated that we are able to measure all FBLN5, ELN, GAPDH and HPT selected peptides in frozen lung tissue. One peptide of MFAP4, VDLEDFENNTAYA(K), requires some optimization, since we were not able to measure the endogenous peptide. During the project both endogenous and synthetic peptide of LTBP2 were not consistently measured. This could de due to wrongly determining the accurate concentration of pure synthetic peptide standards or uncertainty of the quality[36]. Therefore another peptide for LTBP2 was selected in the end-stadium of this project.

A large set of lung tissue samples (n=123) are prepared during this project and ready for MS-measurements. This will result in a large set of data of COPD and control samples, which could be used for further research and adjust knowledge to the new field of proteomics in COPD. Next, the developed method for lung tissue preparation for proteomics approaches could also be used for comparable projects. The method for targeted measurement of our selected peptides could be used for studies interested in the same peptides, but has to be tailored to obtain the maximal intensity of the transitions. Yet, more experiment could be performed to further optimize the methods and to increase validity. An interim test during preparation is suggested, to test the stability of the peptides. Next to this a test is required for examining the absence of the, as we assume, degraded part of the cleaved FBLN5 protein. This is an essential control that has to be performed, since our experiment is based on the assumption that the shorter part of FBLN5 protein after cleavage will not be detect by the MS. After LC-MS analysis and determination of the protein levels, the levels of cleaved FBLN5 can be correlated with the presence of COPD and degree of emphysema. Furthermore, the large set of lung tissue samples are applicable for shotgun proteomic. This could result in a protein identification of lung tissue of several stages of COPD and a step towards personalized medicine[37].

[3] See appendix I for the more detailed setup and measurement procedure.

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Appendix

Appendix I

Starting MS measurements: trial and error

To test the stability and reliability of the measurements an 'MS control sample' is taken into account and is measured after every eight samples. An MS setup is developed which will be used for each batch similar. In the starting phase several blanc test are performed, to check disruptions in the MS spectra. Also the control sample for the MS is measured several times in the starting phase to administer interferences. Once this starting phase is completed the measurement of the samples is done in a fixed order and is repeated until all the samples of the batch are measured. The MS setup for the Thermo Xcalibur is shown in Table 8 MS setup Thermo Xcalibur: sequence of measurement..

Starting phase	Blanco_Q1 (method WenYuan)
	Blanco1_a (own method)
	controlMS_a
	controlMS_b
	controlMS_c
Repeat	Blanco1_b
	4 samples
	Blanco2
	4 samples
	controleMS_d

Table 8 MS setup Thermo Xcalibur: sequence of measurement.

During measurement of batch 1, the results of the MS control samples in the starting phase showed low intensity of the synthetic peptide containing cysteines. Thereby retentions times of a several peptides were 4 to 5 minutes shifted compare to the determined retention time. Due to these deviant results we decide to measure the synthetic peptide mix without endogenous peptide. Again results showed low intensity of peptides containing cysteines. To determine which compound causes this problem, the MS or the synthetic peptide mix, the most recent sample were all synthetic peptide were detected is used for trouble shooting. Results of this measurement demonstrated the MS was working well, all synthetic peptide were detected in a high intensity and were similar to the previous measurement of the sample. We assumed the dilution of the FBLN5 synthetic peptide mix went wrong. Therefor the dilution of the synthetic peptides for FBLN5 is redone. For testing this newly diluted mix of peptide, 5 µl of sample plus 5 µl of the new peptide mix is tested. Since technical problems occurred to the TSQ2 the sample had to be put in the TSQ1 and therefor the method had to be adjusted. Next to the this sample, the other sample were al peptides still were detected (control sample) was put in for measurement. Again the synthetic peptides with cysteines were not detect in the newly diluted peptide mix, but were present in the control

sample. We decided to make a new synthetic peptide mix concerning all of the synthetic peptides, not using previously made aliquots but starting from the stock dilution. We started with the reduction and alkylation of the three peptides of FBLN5 containing cysteines (QSGQCLDIDEC(R),TIPEAC(R), CIDPI(R)) separately. Before continuing mixing all the peptides into a master mix, the reduction and alkylation of the three cysteines-containing peptide was tested. On base of the results we can conclude that the reduction and alkylation of the peptide did work. The peptides QSGQCLDIDEC(R) and CIDPI(R) are measured in high intensity, though the TIPEAC(R)-peptide is detected in a more lower intensity (Figure 11). Measurement of the TIPEAC(R)-peptide has to be redone to determine it is due to an insufficient reduction and alkylation or to a low concentration.

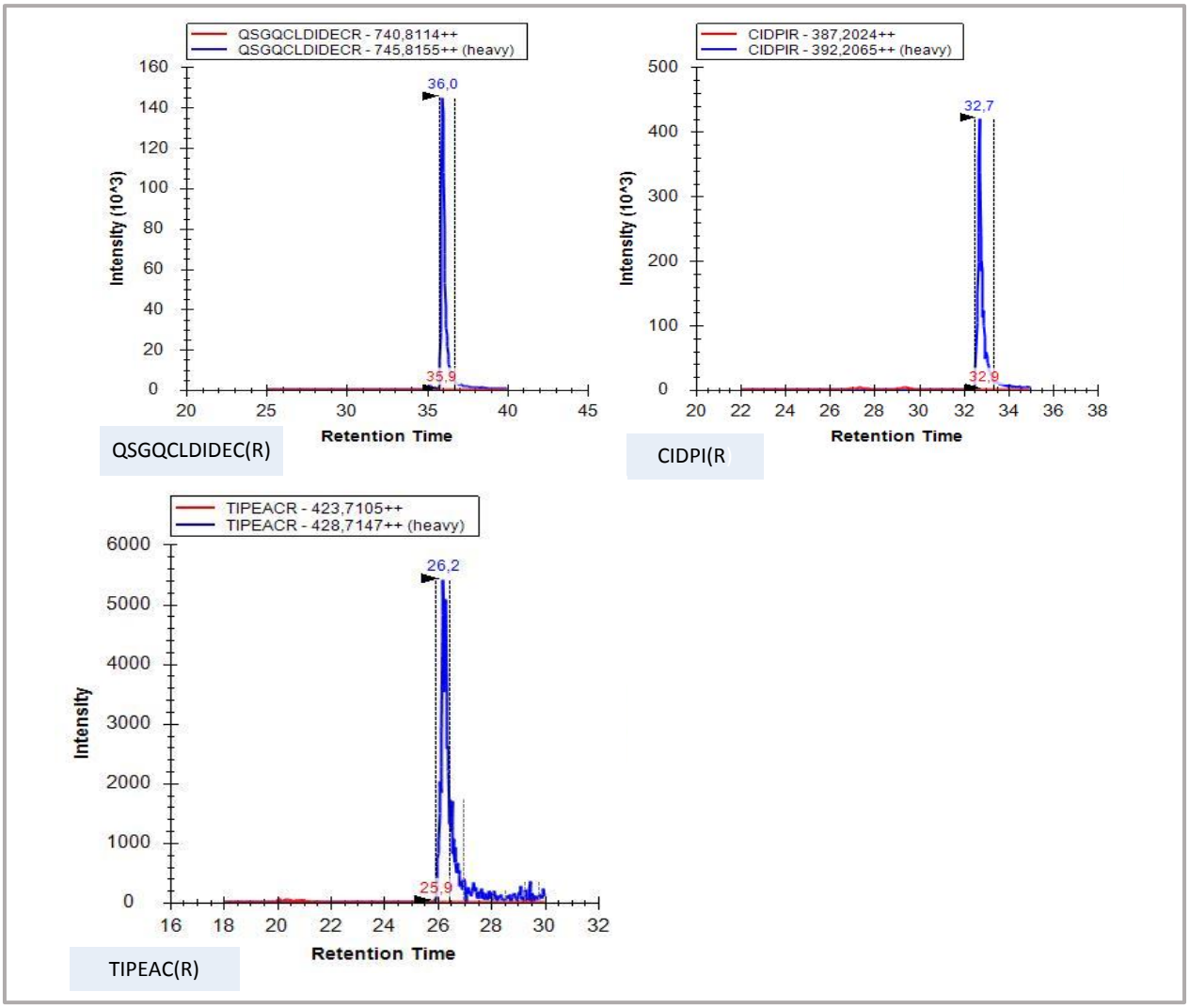

Figure 11 Graphs from Skyline showing high intensity for the peptides QSGQCLDIDEC(R) + CIDPI(R) and a lower intensity for TIPEAC(R)