**DEPARTMENT OF CHEMICAL, LIFE SCIENCES AND ENVIRONMENTAL SUSTAINABILITY**

**Ph.D. in Biotechnology and Biosciences – XXXIV Cycle**

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**LC-MS based proteomic characterization of animal models for respiratory diseases**

The main goal of this research program is to identify proteins involved in the progression or establishment of pathology through analysis of Bleomycin model and other animal model in order to investigate organ and system-level responses.

The analytical platform starts from the analysis of whole lung proteome and goes down into more specific molecular details of proteins belonging to the different cellular compartments and into the quantitative analysis of relevant post-translational modification of potential target proteins.

LC-MS based proteomic has as starting point the protein mixture digestion, bottom-up proteomic approach, followed by LC separation and MS-MS analysis.

Each peptide has specific characteristic (retention time, m/z value, fragment mass) which is defined as peptide fingerprint. This fingerprint could be used to identify and quantify different peptides, and then different proteins, in complex biological sample.

Two main LC-MS platforms could be used: high-resolution MS and low-resolution MS. Both are useful to investigate proteins in biological samples with different experiments.

TMT (tandem mass tag) analysis is based on high resolution mass spectrometry: it is a proteomic technique highly sensitive, quantitative, and high-throughput which allows to analyze global protein dynamics within tissues or cells.

MRM (multiple reaction monitoring) analysis is based on low resolution mass spectrometry: it is a quantitative method useful to detect specific peptides, belonging to target proteins, based on their specific fragments (fingerprint). This approach allows to quantify a protein of interest in a complex sample, such as tissue or cell lysate.

In this first PhD year, both approaches have been used to investigate animal models of a respiratory disease:

*Bleomycin-induced lung fibrosis rat model*: to investigate pulmonary fibrosis, a chronic, progressive lung disease characterized by progressive lung scarring and the histological picture of usual interstitial pneumonia (UIP). The biological processes reflect an anomalous reparative response to repetitive alveolar epithelial injury leading to exaggerated accumulation of extracellular matrix and, consequently, of fibrotic foci. [[1]](#endnote-1)

**High resolution application**

***Improvement of sample preparation for LC-MS analysis***

Pharmacological treatment and sample collection will be performed in the laboratory of the Target Validation Unit, Chiesi Farmaceutici S.p.A.

To select the best way to study homogenate of fibrotic lung (rich of extracellular matrix proteins), I performed different test for sample preparation:

-Fractionation at subcellular level with different buffer for five compartments (cytosol, membrane, nucleus, chromatin binding and ECM), useful to have a higher identification into each subcellular proteome;

-Fractionation of peptide before LC separation, based on characteristic hydrophobicity of peptide (C18 separation) to improve LC separation and peptide identification;

LC conditions: four columns (bioZen peptide XB-C18, XSelect Peptide CSH C18, Aeris peptide XB-C18, Aeris WIDEPORE XB-C18) and three gradients (110, 120 and 170 minutes)

ESI source and chromatographic system: Vanquish, ESI, and Ultimate3000, nanoESI

***Data mining of high-throughput proteomic quantitative data (TMT analysis) obtained with untargeted analysis performed with nanoLC-MS analysis with Dionex Ultimate 3000 nano RSLC/Lumos***

**Activity and results**

Pharmacological treatment and sample collection will be performed in the laboratory of the Target Validation Unit, Chiesi Farmaceutici S.p.A.

Three rat samples of IPF bleomycin-induced model (vehicle, Bleomycin and Nintedanib, sacrified at day 28) were treated with TMT method, after tryptic digestion and the analysis was performed with nanoLC-MS analysis with Dionex Ultimate 3000 nano RSLC/Lumos at IRB in Barcelona.

For each sample, lists of proteins derived from three analytical replicates were compared, and only proteins in common were kept for Ingenuity pathway analysis.

Protein candidates will be organized in pathways to understand which signalling pathways are involved in the progression of lung damage. When possible, upstream regulators and downstream effectors were highlighted.

The quantitative data was submitted to Metascape software performing a custom analysis to determine the pathways involved in the process. Several processes appeared to be resolved after Nintedanib treatment, such as the collagen formation. Others, like extracellular matrix organization, are still significant in the N vs V comparison but to a much lesser extent.

The same quantitative data submitted to Metascape, were submitted to IPA software (Ingenuity Pathway Analysis, Qiagen), which allows to investigate proteins and molecules that may have a role in the regulation of gene/protein expression. The bleomycin resulted as significantly activated after Bleomycin treatment and inhibited after Nintedanib treatment. Further analyses on these upstream regulators will be conducted to get insights into their specific role and the feasibility of their use to relieve the fibrosis.

**Low resolution application**

***Targeted analysis based on LC-MS/MS (MRM mode) of specific biomarker and proteins of interest for IPF identified in untargeted analysis***

**Activity and results**

Pharmacological treatment and sample collection will be performed in the laboratory of the Target Validation Unit, Chiesi Farmaceutici S.p.A.. Ten rat lung samples, of IPF bleomycin-induced model, for each treatment (vehicle and Bleomycin sacrified at day 28) were pooled and homogenized in PBS.

Lung homogenates were digested with trypsin, after denaturation, reduction and alkylation.

After desalting, peptide mixture was analysed with LC-MRM method, with XSelect Peptide CSH C18 column and 110 minutes gradients. From the results of untargeted analysis, the proteotypic peptides of protein target were selected and an LC-MRM approach were set. The protein selected is actin aortic smooth muscle (ACTA2), known as biomarker of epithelial-mesenchymal transition, part of reparative response.

Different isoforms exist for actin family. ACTA2 is specific for smooth muscle and has a high homology with the other isoforms. The peptides selected for the target analysis (Table 1) are specific of this isoform (ACTA2, P62738) and with the MRM method is possible to identify and measure these specific sequences.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Annotated Sequence** | **Residue** | **m/z [Da]** | **Charge** | **transition** | |
| GYSFVTTAER | 199-208 | 565,77 | 2 | y5: 577,29 | y6: 676,36 |
| VAPEEHPTLLTEAPLNPK | 98-115 | 652,7 | 3 | 568,3 | 869,47 |

Table 1: ACTA2 proteoptypic peptide

Each of these peptides was detected in lung homogenates and analyzed with a relative quantitation.

The results don’t show an increment or decrement for bleomycin treatment and the peptide as the same trend. This result show that ACTA2 is not a biomarker at time point day28 for IPF bleomycin-induced, as described in recent literature.[[2]](#endnote-2)

## Conclusions and Next steps

The sample preparation of lung homogenate for proteomic characterization was improved with different type of fractionation and chromatographic separation, useful for different experiments.

High resolution mass spectrometry is a valid tool to investigate variation in proteome comparing healthy and fibrotic animals, suggesting new pathways or proteins as biomarker of pathology.

Different experiments have been done to improve proteins identification, such as subcellular fractionation and peptide fractionation, tests of different LC columns and gradients.

Low resolution mass spectrometry allows to determine and quantify target proteins, distinguishing a single amino acid in a peptide (1 Da). This methodology (MRM) have been used to investigate other interesting proteins, such as histones.

In the next year the purpose is to investigate with high resolution approach Post Translational Modifications (phosphorylation and acetylation) in rat lung proteome to evaluate the impact of PTM in different animal models.

1. Fernando J. Martinez, et al. (2017), *Idiopathic pulmonary fibrosis*, *Nature Reviews Disease Primers volume 3,* Article number: 17074 [↑](#endnote-ref-1)
2. Kai-Hui Sun et al. (2016), alpha-Smooth muscle actin is an inconsistent marker of fibroblasts responsible for force-dependent TGFbeta activation or collagen production across multiple models of organ fibrosis, Am J Physiol Lung Cell Mol Physiol 310: L824–L836 [↑](#endnote-ref-2)