Evolution of the bleomycin-induced lung fibrosis model
Understanding the pathogenesis and the mechanisms involved in Idiopathic Pulmonary Fibrosis

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The present PhD project is part of a more comprehensive study called: "Identification of molecular alterations involved in bleomycin-induced pulmonary fibrosis in rodents with translational relevance for human Idiopathic Pulmonary Fibrosis (IPF), and investigation of a new approach of target validation in IPF" in collaboration with Chiesi Farmaceutici S.p.A.

Idiopathic Pulmonary Fibrosis (IPF) is a multifactorial disease still poorly understood and difficult to diagnose. It is considered a specific form of chronic fibrosing interstitial pneumonia that leads, in a few years, to the progressive and irreversible loss of pulmonary architecture until death caused by respiratory failure or complicating comorbidities. The aetiology and exact pathophysiological mechanism of the disease are still unknown and until recently, no proven effective therapy was available other than lung transplantation[1].

The study of a complex disease such as IPF and the development of a new therapeutic approach critically rely on the availability of an optimized and translationally relevant animal model to be used for preclinical studies[2]. Unfortunately, a fully comprehensive IPF animal model does not exist yet, but many models of pulmonary fibrosis are available. One of these is the bleomycin (BLM)-induced lung fibrosis model in rodents.

The present work consists in characterizing, under a biomolecular and histopathological profile, a time course study of a BLM-induced lung fibrosis rat model, with the aim to acquire a deeper and comprehensive view of the mechanisms underlying the development of lung fibrosis. The multidisciplinary analyses performed on the considered BLM model will allow identifying the most informative genes/proteins involved in the fibrotic processes that will lay the groundwork for the improvement of the animal model to be used in drug-response studies and for the identification of new therapeutic targets and diagnostic biomarkers for the IPF treatment.

Previous year PhD activities were related to the setting-up of the time course study with the completion of all the in-vivo and ex-vivo activities (collection and processing of the different biological samples, as well as the elaboration of all the histopathological data and the protein analysis).

The activities of this second year of PhD were related to:
- The analysis of the whole time-dependent transcriptome profiling of the model;
- The validation of the trascriptomic data (creation of an easy-to-assay qPCR Array);
- The improvement of the animal model for the setting of a therapeutic protocol that will be used next year for the experimental validation of the model using reference drugs (Nintedanib and Pirfenidone).

▪ *In-silico* activities (analysis and validation of the transcriptomic data): the mRNA molecules extracted in the previous year from the fresh right lungs at all the analysed time points were subjected to an RNAsequencing process with an Illumina NexSeq500 platform (external laboratory) as mentioned in the previous report. The transcripts were aligned on the *Rattus norvegicus* genome and counts were determined using the latest Ensembl annotation. Counts retrieved were normalized and the Differentially Expressed Genes (DEGs) between BLM-treated animals and their controls (saline-treated animals, vehicle (VEH) group) were identified for each time-point, taking into account only genes with at least 10 counts in at least 25 samples. Genes were deemed as differentially expressed if the fold-change (FC) was >2 or <0.5 and the adjusted *p*-value <0.05. Modules of co-expressed genes were identified using the "weighted gene co-expression network analysis (WGCNA)". The gene expression module profiles were correlated with some phenotypic traits (time, body-weight and histological parameters) using Pearson correlation. Heat maps, similarity matrix and PCA data were generated.

A selection of a set of 92 genes was performed in order to create an easy-to-assay qPCR Array to test and validate experiments in the context of BLM-induced lung fibrosis. The set of genes and thus the qPCR array were selected based on the data obtained during the whole transcriptome profile analysis and on data published in the literature. The selection has been articulated in several points by applying increasingly stringent conditions (transcripts abundance, statistical significance, Log2 values ecc.).

▪ *In-vivo* and *in-vitro* activities (improvement of the model): Male Sprague-Dawley (SD) rats were injected with BLM (1 U/kg – Baxter batch, a more pure and controlled BLM) or saline solution on day 0 and day 4 and sacrificed at day 21 and 28 after first BLM dose. The solutions were instilled intratracheally, with the same procedure and device of the time course study. For each time-point, two groups of animals ("Bleomycin" and "Vehicle") were included. The whole blood was collected and analysed with an automated cell counting system (DASIT) for the differential blood cells count. The plasma was obtained, aliquoted and frozen for further analysis. The whole lungs were removed and weighed. After this step, left lungs were collected in formalin solution, processed and analysed for the histological analysis (performed by an external laboratory), while right lungs were stored for extraction of RNA. The RNA was extracted using the same procedure performed in the time course study. After a quality and quantity control of the extracted RNA, a transcriptome analysis was conducted by RNA sequencing with the same procedures of the previous year.

Results

▪ Transcriptomic data: The PCA calculated on the dataset revealed a clear separation on the x-axis between vehicle-treated animals, which clustered with the T0 control condition, and BLM-treated animals. Samples were also distributed on the *y*-axis according to the different time-points. PCA also revealed strongly coherent transcription profiles among biological replicates. The number of DEGs ranged from 892 (T14) to 1700 (T07), summing up to a total of 3768 differentially expressed genes for the five different time points. The number of BLM up-regulated genes was higher than (T14), or equal to (T07), the number of down-regulated genes at early time-points, whereas the opposite situation was observed at later time-points (T21, T28, T56). DEG analysis did not give information on the time-course dependence of the measured transcription profiles. In order to investigate the transcription profile as a function of time from BLM treatment, a weighted gene co-expression network analysis was applied to the entire set of expressed genes derived from all time-points. This analysis allowed the identification of 19 modules of co-regulated (or anti-regulated) genes. The first five modules accounted for 66% of all the analyzed genes, the other modules contained less than 5% of genes each. Module 1 (M1) was enriched in genes coding for nuclear proteins with a marked down-regulation at late time-points (T21 to T56), while M2, M3 and M5 were characterized by a strong up-regulation at early time-points (M3) or a persistent up-regulation up to 28 days (M5) or until day 56 (M2). The WGCNA also allowed to compare the identified modules with phenotypic traits and biomarkers measured during the experiment. The modules M2 and M5 significantly correlated with the weight of the animals and the histological parameters. Pathway analysis performed on modules, revealed that these coordinately expressed genes were involved in different biological processes and many of them were related to extracellular matrix repair.

▪ Validation of the transcriptomic data: the qPCR Array realized to test and validate the experiments in the context of BLM-induced lung fibrosis produced results in agreement with the transcriptomic data with very few exceptions.

▪ Improvement of the model: the multidisciplinary data originated in a second experiment using a more controlled and pure bleomycin, only focused on the central time point (T21-T28), were in agreement with the data originating during the time course experiment.

Conclusions and Next steps

The biomolecular and histopathological analysis performed in this two years on the time course study showed that in this BLM-induced lung fibrosis rat model the animals developed a pathological condition that appeared to be more stable and consistent between 21 and 28 days post-BLM, where the initial acute inflammatory reaction, left the place to a different type of fibrosis. The data originated from the second experiment focused on the most relevant time points (T21-T28), confirmed and validated the development of a pathologic condition useful for evaluating targets and drugs.

Therefore, in the next year, the model will be used to evaluate the efficacy, both at the biomolecular and histoptological level, of the two commercially available drugs for the treatment of IPF (Nintedanib e Pirfenidone). Moreover, in the next year, the multidisciplinary analysis used in these two years will be performed on human IPF samples (if available) to compare the animal model to the human pathology. The data originated from this comparative analysis will allow us to evaluate the translational potential of the bleomycin-induced lung fibrosis rat model for the IPF disease.

References

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2. Ahluwalia N, Shea BS, Tager AM. New therapeutic targets in idiopathic pulmonary fibrosis. Am J Respir Crit Care Med. 2014; 90(8):867-878.