**Expression profiling of the developing rabbit lung**

|  |  |
| --- | --- |
| *PhD Student:* | *Supervisor:* |
| Francesca Ricci | Dr. Gino Villetti |
|  |  |

The present PhD project is part of a more comprehensive study in collaboration with Chiesi Farmaceutici S.p.A.: “Transcriptome profiling of hyperoxia-exposed preterm rabbit model of bronchopulmonary dysplasia (BPD)”.

BPD is the most common respiratory morbidity in preterm neonates with an incidence of 5–68%, which increases significantly with declining gestational age (GA) (1). Despite advances in neonatal care have resulted in improved survival rates of premature infants, limited progress has been made in reducing BPD rates (2).

The clinical BPD phenotype is the result of a complex multifactorial process in which various pre- and postnatal factors compromise normal development of the immature lung (3). Understanding how the alveoli and capillary network develop and how these mechanisms are disrupted in BPD is critical for developing efficient therapies. In this scenario, animal models are important tools for the preclinical development of pharmacological treatments. Recently, in Chiesi laboratories an hyperoxia-exposed preterm rabbit model of BPD has been validated. This model represents a suitable lab preclinical tool for mimicking the clinical BPD phenotype to test new pharmacological treatments (4).

The aim of the study is the identification of the main gene/protein-sets involved in the development of the BPD phenotype. based on a biomolecular and histopathological characterization of the preterm rabbit model at multiple time points.

**Object:**

The activities of this PhD project are focused on the characterization of the genome-wide gene expression profile of thedeveloping rabbit lung. The study includes a full characterization of multiple time points during pre- and post-natal stages of rabbit lung development. The developing lung transcriptomics data will be a unique resource for identifying key transcriptional programs required for normal rabbit lung development, comparing genomics of lung development in rabbit and human (in comparison with other animal species), and providing insights into the relationship of normal lung development with respiratory disease processes.

The research activities of this first year of PhD were related to the collection and processing of the different biological samples, the elaboration of analytical data and the evaluation of physiological development pathways.

In vivo activities: Preterm rabbits were delivered through a C-section and lungs were collected at different GA time points corresponding to different lung developmental stages: 25GA, 27GA, 28GA, 29GA, 31GA, and 35GA. For each time point we collected three samples from three different experimental sessions to take into account the animal variability of the model. The whole lungs were removed, washed in saline solution, and weighed. After this step, right lungs were separated and stored for gene expression analysis, while left lungs were collected for histology and undergone a fixation process (4 ml 10% formalin buffer).

RNA analysis: Right lungs were isolated from animals and immediately stored in RNAlater™. Tissue samples were then homogenized in QIAzol® Lysis Reagent. After this process, phenol/chloroform RNA extraction was performed with the help of QIAGEN spin-column kits. After a quality and quantity control of the extracted RNA, a transcriptome analysis was conducted by RNA sequencing using an Illumina NexSeq500 platform (external laboratory).

Tissue analysis: The left lungs, conserved in formalin solution, were processed and analysed by an external laboratory.

In addition, we collected for each time point additional animals (n=3) for proteomic analysis that will be performed by the Chiesi Corporate R&D Preclinical Analytic & Formulation (A&F) group.

## Results

Optimized RNA extraction protocol allowed to obtain high RNA concentration avoiding protein and phenol contamination. Denaturating agarose gel electrophoresis showed 28S and 18S ribosomal RNA (rRNA) gel bands at an approximate mass ratio of 2:1, indicating high RNA integrity suitable for high-throughput RNA-seq. The transcriptome analysis identified 8425 protein-coding genes, 86% of which have a gene description and orthologues in human. In addition, Principal Component analysis highlighted three clusters representing the three developmental lung stages: Canalicular, Saccular and Alveolar.

12 modules of co-expressed genes have been identified using weighted correlation network analysis (WCNA), but only 8 modules contain genes related to processes and pathways involved in the lung development.

In order to fully analyze the data obtained, we compared expression profiling of the developing rabbit lung with literature genomic data on other animal species and human.

In particular, the preliminary results showed a significant similarity of our data with the main gene/protein-set involved in the normal lung development of humans.

In parallel, an external group is analyzing the lung histological samples evaluating several parameters to measure the progressive lung development. Currently, the Radial alveolar count (RAC) and the tissue density have been evaluated and the preliminary results showed a significant correlation of these parameters with the rabbit lung development in accordance with the literature data.

## Regarding the proteomic analysis that will be perform by A&F Chiesi group, the samples have been processed and they will be analysed by an external group. The preliminary results will be available by the end of the 2019.

## Conclusions and Next steps

The characterization of the expression profile of normal lung development is an important starting point to understand how the lung development mechanisms are deregulated in BPD.

During the first year of PhD we have finalised RNA seq analysis of lung samples collected at different time points of GA that correspond to different lung developmental stages of rabbit. We identified using WCNA

8 modules contain genes related to processes and pathways involved in the lung development. The preliminary results showed a significant similarity of our data with the main gene/protein-set involved in the normal lung development of humans. These results were confirmed by preliminary histological analysis.

In the next two years a complete analysis of the lung transcriptome data in comparison with the data available in the literature will be performed. The data originated from the comparative analysis between the animal model and the human (and other animal species) data will allow us to evaluate the translational potential of the hyperoxia-exposed preterm rabbit model for the BPD disease. In order to obtain a complete biomolecular and histopathological characterization, the transcriptome results will be then correlated with the histological and proteomic analysis.

In addition, based on the preliminary data we have considered to add additional lung development time points to obtain a more comprehensive characterization of the rabbit lung development. In particular, we will insert samples collected at 21GA and 23GA (pseudo-glandular phase) and post-natal day 9, 11, 14. A 14-days old rabbit, based on literature data, corresponds to a human neonate of 36 weeks (5). The clinical guidelines consider 36 weeks after birth as the time point to access BPD diagnosis through radiography and oxygen dependency (6).

1. **The Future of Bronchopulmonary Dysplasia: Emerging Pathophysiological Concepts and Potential New Avenues of Treatment.** [Front Med (Lausanne).](https://www.ncbi.nlm.nih.gov/pubmed/28589122) 2017 May 22;4:61. doi: 10.3389/fmed.2017.00061. eCollection 2017. [Collins JJP](https://www.ncbi.nlm.nih.gov/pubmed/?term=Collins%20JJP%5BAuthor%5D&cauthor=true&cauthor_uid=28589122), [Tibboel D](https://www.ncbi.nlm.nih.gov/pubmed/?term=Tibboel%20D%5BAuthor%5D&cauthor=true&cauthor_uid=28589122), [de Kleer IM](https://www.ncbi.nlm.nih.gov/pubmed/?term=de%20Kleer%20IM%5BAuthor%5D&cauthor=true&cauthor_uid=28589122) et al.
2. **Trends in Care Practices, Morbidity, and Mortality of Extremely Preterm Neonates,** **1993-2012**. Jama 314: 1039-1051, 2015. Stoll BJ, Hansen NI, Bell EF et al.
3. **Bronchopulmonary Dysplasia: Chronic Lung Disease of Infancy and Long-Term Pulmonary Outcomes** [J Clin Med](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5294957/). 2017 Jan; 6(1): 4. [Lauren M. Davidson](https://www.ncbi.nlm.nih.gov/pubmed/?term=Davidson%20LM%5BAuthor%5D&cauthor=true&cauthor_uid=28067830) and [Sara K. Berkelhamer](https://www.ncbi.nlm.nih.gov/pubmed/?term=Berkelhamer%20SK%5BAuthor%5D&cauthor=true&cauthor_uid=28067830).
4. **Functional assessment of hyperoxia-induced lung injury after preterm birth in the rabbit.** J Physiol Lung Cell Mol Physiol 306: L277–L283, 2014. Richter J, Toelen J, Vanoirbeek J, et al.
5. **Rabbits and men: relating their ages.** [J Basic Clin Physiol Pharmacol.](https://www.ncbi.nlm.nih.gov/pubmed/29672272) 2018 Sep 25;29(5):427-435. doi: 10.1515/jbcpp-2018-0002. [Dutta S](https://www.ncbi.nlm.nih.gov/pubmed/?term=Dutta%20S%5BAuthor%5D&cauthor=true&cauthor_uid=29672272), [Sengupta P](https://www.ncbi.nlm.nih.gov/pubmed/?term=Sengupta%20P%5BAuthor%5D&cauthor=true&cauthor_uid=29672272).
6. **Bronchopulmonary dysplasia.** [Am J Respir Crit Care Med.](https://www.ncbi.nlm.nih.gov/pubmed/11401896) 2001 Jun;163(7):1723-9. [Jobe AH](https://www.ncbi.nlm.nih.gov/pubmed/?term=Jobe%20AH%5BAuthor%5D&cauthor=true&cauthor_uid=11401896), [Bancalari E](https://www.ncbi.nlm.nih.gov/pubmed/?term=Bancalari%20E%5BAuthor%5D&cauthor=true&cauthor_uid=11401896).