Histological characterisation of a bleomycin-induced model of pulmonary fibrosis in mice

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Introduction

Pulmonary fibrosis (PF) is an ultimately fatal disease characterised by **excessive deposition of collagen** in the lung. Despite research efforts identifying several treatments with promising preclinical results, there are currently only two approved drugs for the treatment of pulmonary fibrosis. These demonstrate only a **marginal benefit** for patients, and are associated with a number of **adverse effects**[1,2].

An intratracheal administration of **bleomycin**, in mice or rats, is the most commonly used preclinical model in PF and interstitial lung disease (ILD) research, as it presents a relatively simple induction technique with reproducible results[3,4]. However, there are challenges with translation to the human clinical disease[5].

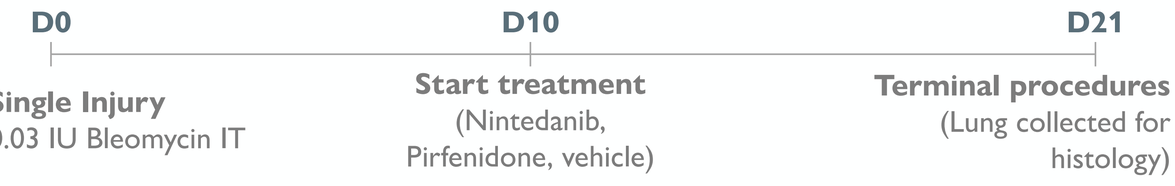
Due to the unmet clinical need, we sought to further characterise the histology of the lung tissue and cellular events taking place in the tissue using **immunohistochemistry** (IHC) and **digital image analysis**, with the aim to use this as a basis for a full characterisation of specific cellular events in the bleomycin model to uncover robust translatable biomarkers.

In-life methods

Male C57BL6/J mice were administered saline or 0.03 IU bleomycin sulphate in a volume of 50 µL under brief isoflurane/oxygen anaesthetic on day 0.

10 days post bleomycin challenge once the fibrotic phase of the model was under way, treatment was initiated with groups of animals receiving either once daily oral doses of 60 mg/kg nintedanib, twice daily oral doses of 50 mg/kg pirfenidone for a total daily dose of 100 mg/kg or twice daily oral administrations of vehicle.

21 days following the bleomycin challenge mice were sacrificed and the lungs removed. The left lung was inflated to a pressure of 20 cmH_2O with 10% neutral-buffered formalin and fixed in formalin for 24-48 hours prior to being processed for histology as described below.



Prosurfactant Protein C staining

- ProSP-C analysis can be used to determine whether alveolar epithelial type II cells (AEC2) cells are morphologically normal and cycling surfactant and can be used as an indirect measure of epithelial differentiation in injury and repair.
- Grades (0-4) were assigned according to the area of the lung tissue; in **lesional** areas scores were based on loss of signal in the lesions, and **peri-lesional** areas were scored based on signal prevalence and hyperplasia of AEC2 cells.

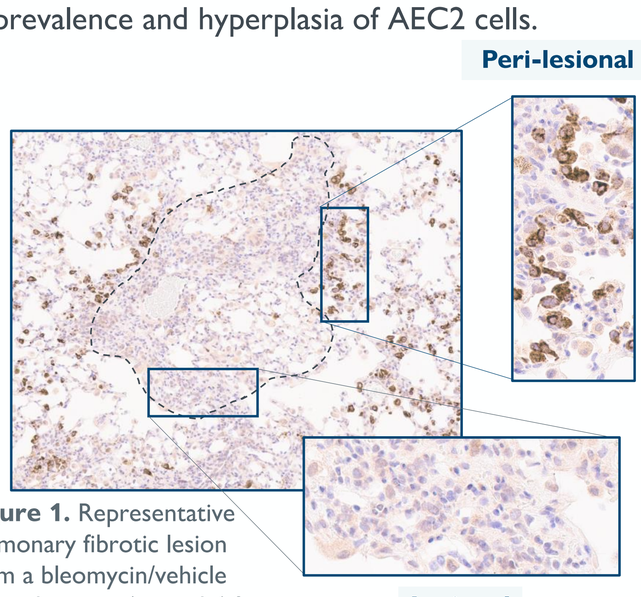


Figure 1. Representative pulmonary fibrotic lesion from a bleomycin/vehicle treated mouse (scored 4 for both lesional and peri-lesional SpC)

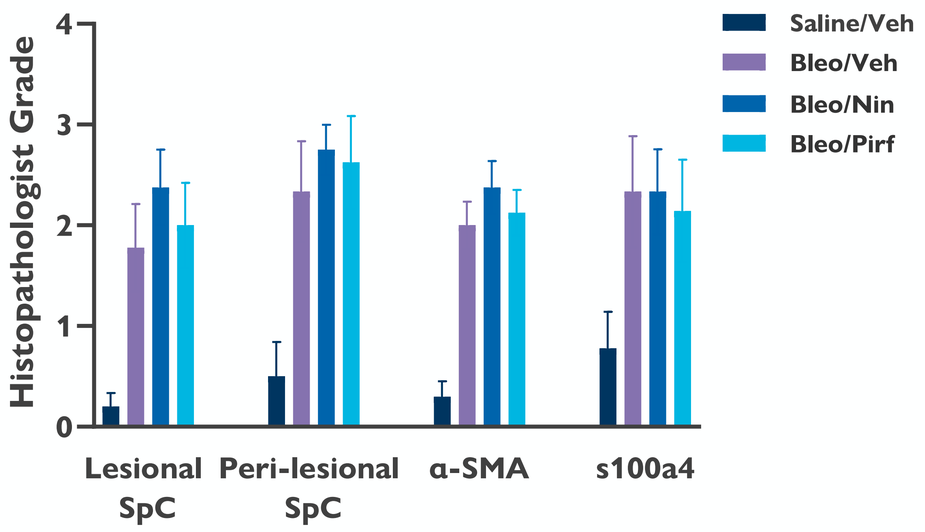
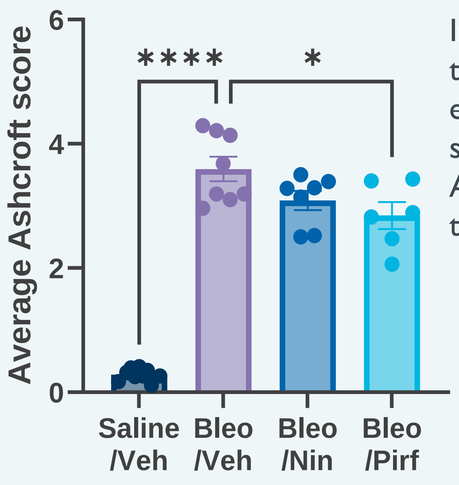


Figure 2. Average IHC marker scores for groups following single injury (bleomycin/saline) and 12-day treatment. Data are given as mean ± SEM (n=8-10). *P* <0.05 for all bleomycin groups vs saline control for all IHC markers; one-way ANOVA with Dunnett's multiple comparisons test.

Immunohistochemical analysis

Standard model: Ashcroft scoring



Individual fields of view of Masson's trichrome stained lung sections were examined at 10x magnification and scored from 0-8 using a modified Ashcroft method. FOV's were averaged to give a score per lung.

Figure 4. Average modified Ashcroft scores for groups following single injury (bleomycin/saline) and 12-day treatment. Data are presented as mean ± SEM (n=8-10). Data were analysed using one-way ANOVA with Dunnett's multiple comparisons test * *P* <0.05, **** *P* <0.0001

α-SMA and S100-A4 staining

- S100-A4 is a marker for fibroblasts derived from epithelial-mesenchymal transition and a DAMP which has been shown to have a functional role in fibroblast proliferation. α-SMA is a marker of myofibroblast formation. These signals can cluster together, as S100-A4 upregulates α-SMA expression to promote fibroblast activation[9].
- Grades (0-4) were determined based on signal prevalence and position relative to fibrotic foci and/or fibrotic transition zones.

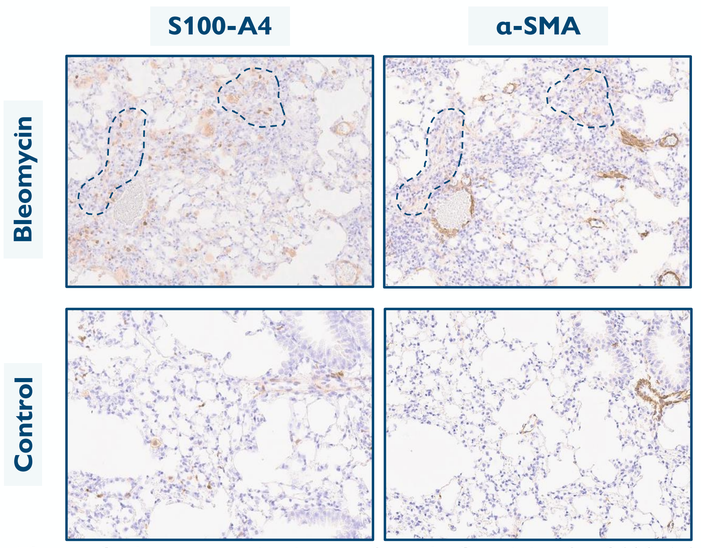


Figure 3. Representative images from IHC staining for S100-A4 and α-SMA from a saline/vehicle and bleomycin/vehicle treated mouse

- Picrosirius Red staining binds selectively to collagen fibres, providing more specificity compared to trichrome. This makes it particularly useful for collagen deposition analysis.
- Mature and newly formed fibres, can be separated based on hue and intensity[10].
- We have developed a semi-automated method for image analysis based on collagen morphology parameters (intensity, thickness, and circularity) analysed in previous publications[11,12].

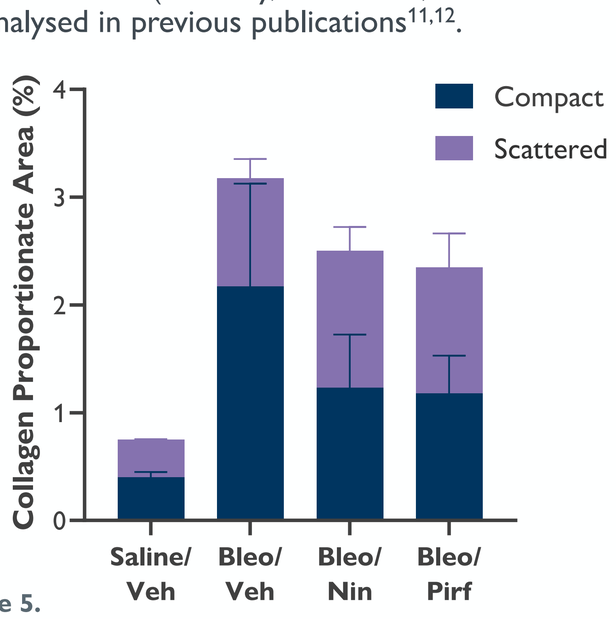
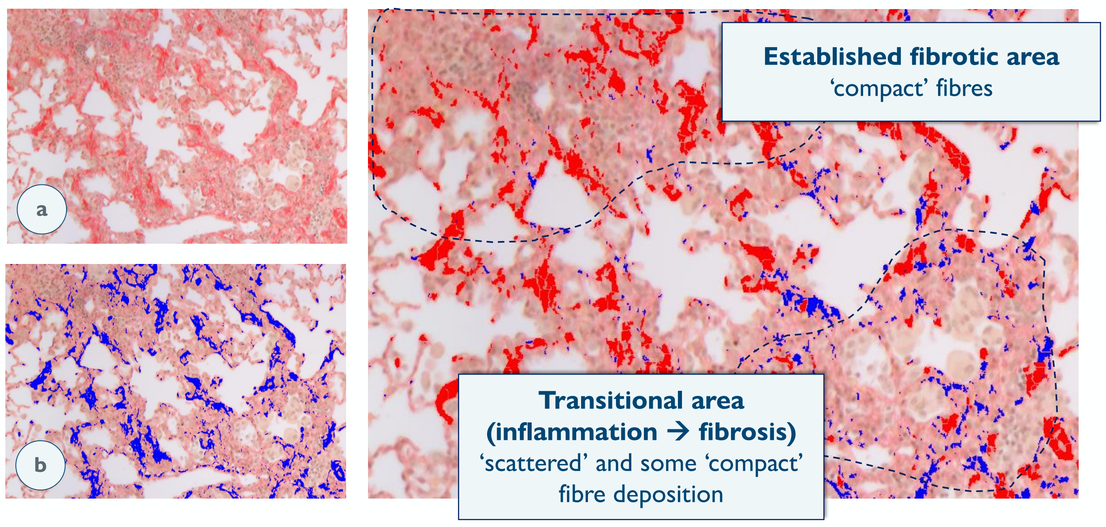


Figure 5. Average parenchymal compact and scattered fibre collagen area for groups following single injury (bleomycin/saline) and 12-day treatment. Data are presented as mean ± SEM (n=3-4).

Picrosirius red collagen analysis



- ImageJ methods previously defined by Courtoy et al.[13] were used as the basis for scripting semi-automated macros to define collagen fibres as either scattered or compact.
- First, a filter based on image staining, intensity and hue was applied to identify collagen fibres.
- A second script was run to determine fibre class based on intensity of staining, thickness and circularity.
- A compound score was then calculated giving priority to the parameters in the order described above.
- Fibres were then able to be classed as scattered or compact based on the resulting score.
- Following this, methods based on those developed by Kammerer et al.[14] were used for supplemental lung segmentation to highlight major airways.
- Finally, an expansion of the airway detection allowed for exclusion of baseline major airway collagen from the analysis.

Figure 6. Sample overlay visualisation images of steps for collagen fibre classification methodology. **a)** original sample image. **b)** overlay of collagen following first filtering, blue represents all collagen fibres. **c)** overlay following fibre classification, red represents compact collagen and blue represents scattered.

Conclusions

- Through IHC and digital image analysis we have begun to build a greater understanding of the most common *in vivo* model of lung fibrosis. Fibrotic lesions show a stable injury with compact collagen, and a loss of AEC2 cells, with surrounding peri-lesional areas showing high levels of fibroblasts resulting from epithelial to mesenchymal transition and further myofibroblast differentiation closer to lesional areas.
- These methods, will form the basis for further work that will be undertaken to further characterise specific cellular events in the model to uncover robust translatable biomarkers, and provide more translatable insights into the effects of novel treatments in this model and in other models of lung fibrosis.

References and contacts