Ultrastructure analysis of macrophages as a tool to shed light on Hypersensitivity Pneumonitis pathogenesis

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You can only truly shine when you are surrounded by the right people

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Resumo

As doenças pulmonares intersticiais (DPI) são um grupo de mais de 200 doenças que afetam o interstício pulmonar, muitas vezes classificadas como raras, o que dificulta um diagnóstico preciso. Estas doenças podem apresentar fenótipos diversos, desde predominantemente inflamatórias e potencialmente reversíveis até apresentarem uma fibrose estabelecida e irreversível. Curiosamente, na nossa coorte de doentes, a pneumonite de hipersensibilidade (PH) é a DPI fibrótica mais prevalente, caracterizada por uma elevada morbilidade e mortalidade. Recentemente, o polimorfismo de nucleótido único rs35705950 do gene *MUC5B* foi associado à PH fibrótica, indicando uma progressão mais grave da doença e uma resposta reduzida às terapias imunossupressoras. Estudos anteriores do grupo identificaram que os doentes com PH fibrótica têm uma percentagem aumentada de macrófagos no lavado broncoalveolar e uma acumulação significativa de MUC5B no parênquima pulmonar e no citoplasma dos macrófagos alveolares, o que levou a uma análise detalhada deste tipo de células. Neste estudo, analisámos a ultraestrutura dos macrófagos alveolares em amostras de tecido pulmonar de doentes com PH fibrótica e, como controlo, indivíduos sem DPI, tendo identificado diferenças significativas nos organelos e morfologia destas células. Os macrófagos de pacientes com PH fibrótica apresentaram um elevado número de organelos com dupla membrana de difícil identificação e com morfologia disfuncional, bem como uma diminuição de mitocôndrias preservadas face aos controlos. Ensaios funcionais realizados para melhor compreender a natureza destes organelos, revelaram uma diminuição das mitocôndrias funcionais combinada com uma acumulação de mitocôndrias danificadas, mas sem impacto significativo na fagocitose. Adicionalmente, a estratificação dos macrófagos de pacientes com PH fibrótica de acordo com o genótipo *MUC5B* rs35705950 revelou uma tendência para um aumento da presença de organelos disfuncionais com dupla membrana em pacientes portadores do alelo T do polimorfismo *MUC5B* rs35705950. O estudo dos macrófagos alveolares na PH pode fornecer informações importantes sobre os mecanismos da doença, facilitando futuramente o diagnóstico e orientando terapias direcionadas para melhorar os resultados nos doentes.

Palavras-chave: doença pulmonar intersticial, pneumonite de hipersensibilidade, macrófagos alveolares, microscopia eletrónica de transmissão.

Abstract

Interstitial lung diseases (ILD) are a group of more than 200 disorders that affect the pulmonary interstitium, many of which are classified as rare, making accurate diagnosis challenging. These diseases can exhibit diverse phenotypes, ranging from predominantly inflammatory and potentially reversible conditions to established, irreversible fibrosis. Interestingly, in our patient cohort, hypersensitivity pneumonitis (HP) is the most prevalent fibrotic ILD, characterized by high morbidity and mortality. Recently, the single nucleotide polymorphism rs35705950 of the *MUC5B* gene was associated with fibrotic HP, indicating a more severe disease progression and a reduced response to immunosuppressive therapy. Previous research from our group had shown that patients with fibrotic HP have an increased percentage of macrophages in bronchoalveolar lavage and a significant accumulation of MUC5B in lung parenchyma and in alveolar macrophages cytoplasm which led to a detailed analysis of this cell type. In this study, we analyzed the ultrastructure of alveolar macrophages in lung tissue from fibrotic HP patients, and no ILD as a control, which revealed significant differences. Macrophages from fibrotic HP patients displayed distinct dysfunctional double membrane organelles together with a decrease of preserved mitochondrias. Functional assays were conducted to understand the nature of these organelles and showed a decrease in functional mitochondria combined with the accumulation of damaged mitochondria, while no impact in the phagocytic capacity. Additionally, the stratification of macrophages from fibrotic HP patients according to the *MUC5B* rs35705950 genotype revealed an increase in the presence of dysfunctional double membrane organelles in patients carrying the *MUC5B* T-minor allele. This study of macrophages in fibrotic HP can provide key insights into disease mechanisms, facilitating future diagnosis and also guiding targeted therapies to improve patient outcomes.

Keywords: Interstitial lung disease, hypersensitivity pneumonitis, alveolar macrophages, transmission electron microscopy

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Abbreviation List

AEC – Alveolar epithelial cells

ALAT – *Latin American Thoracic Association*

APC – antigen-presenting cell

ATI – alveolar epithelial type I cell

ATII – alveolar epithelial type II cell

ATS – *American Thoracic Society*

BAL – Bronchoalveolar lavage

BASC – bronchioalveolar stem cell

EDTA – Ethylenediamine tetraacetic acid

ECM – extracellular matrix

ERS – *European Respiratory Society*

fHP – Fibrotic Hypersensitivity Pneumonitis

HP – Hypersensitivity pneumonitis

HRCT – High-Resolution Computed Tomography

IFN – Interferon

IL – Interleukin

ILD – Interstitial Lung Disease

IPF – Idiopathic Pulmonary Fibrosis

HX – Histiocytosis X

JRS – *Japanese Respiratory Society*

LAM – Lymphangiomyomatosis

MUC5B – Mucin 5B

RBC – Red blood cell

SD – Standard deviation

SNP – Single Nucleotide Polymorphism

TBS – Tris-buffered saline

TEM – Transmission Electron Microscopy

TH – T helper

ATS – *American Thoracic Society*

1. Introduction

1.1. Interstitial Lung Diseases

Interstitial lung diseases (ILD) encompass a heterogeneous group of more than 200 diseases that are characterized by lung damage involving inflammation and, eventually, fibrosis (Mueller-Mang et al., 2017). These diseases can develop due to a combination of factors, with cigarette smoking being a well-established trigger, particularly in the case of Idiopathic Pulmonary Fibrosis (IPF) (Baumgartner et al., 1997). Environmental exposures also have an impact, including prolonged contact with metals, agricultural activities, and exposure to birds and microbial agents. Additionally, gastroesophageal reflux and genetic predispositions have been associated as additional contributing factors (Raghu et al., 2011).

Several studies have identified different genetic variants associated with the development of ILDs, particularly IPF. Although some of these variants are rare, they seem to be associated with disease susceptibility. Rare variants, defined by an allele frequency of less than 0.1%, associated with ILD include genes such as SFTPC, SFTPA1, SFTPA2, ATP-binding cassette-type 3 (ABCA3), and NAF1, most of them associated with surfactant processing. Additionally, five genes related to telomere biology, TERT, TERC, DKC1, TINF2, RTEL1, and PARN, are also linked to disease susceptibility and/or severity. Inside the common variants group, defined by an allele frequency of less than 5%, the single nucleotide polymorphism (SNP) rs35705950 in the promoter region of the *MUC5B* gene is the most strongly associated with IPF (Kaur et al., 2017).

The clinical presentation of these diseases is nonspecific since different ILDs can have overlapping symptoms and morphologic features making accurate ILD diagnoses complex and challenging (Guler & Corte, 2021). Generally, the manifestations of ILDs are vague, however, the most common clinical presentation includes dyspnea and cough with a significant impact on daily functioning and well-being (Flaherty et al., 2017). Studies have demonstrated that these manifestations impact patient's life quality, restricting physical activities, social interactions, travel, and other everyday tasks

(Swigris et al., 2005). This is particularly true for IPF, which has a poor prognosis with a mean survival rate of three to five years upon diagnosis (Podolanczuk et al., 2021).

Achieving an accurate diagnosis relies on the involvement of a multidisciplinary team, which includes the analysis of clinical data, physical evaluation, lung function tests, blood tests, chest X-rays, and high-resolution computed tomography (HRCT). However, invasive procedures may be required in some cases, such as bronchoalveolar lavage (BAL) and lung cryo-biopsy which provides information on alveolar cell composition and tissue organization (Meyer, 2014).

In 2002, a classification of ILDs was proposed by the *European Respiratory Society* (ERS) and *American Thoracic Society* (ATS) divided them into four main categories as outlined in Figure 1 (Behr, 2012).

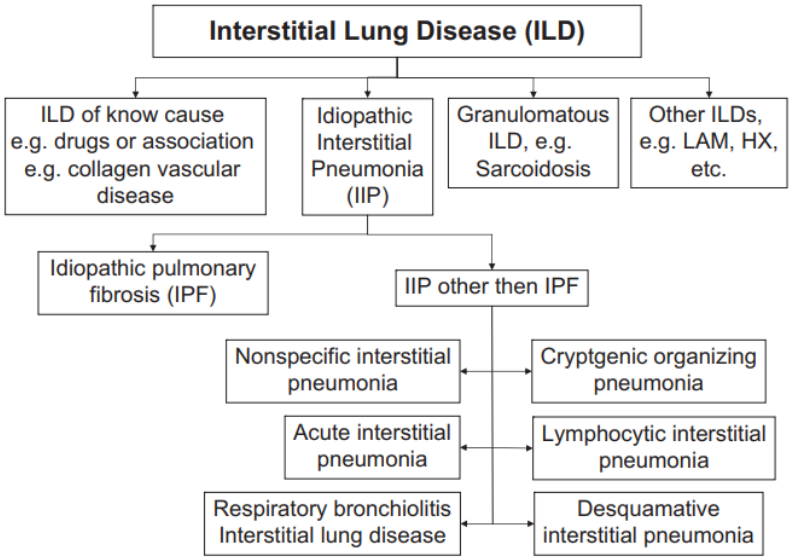

Figure 1. Classification of Interstitial Lung Diseases proposed by the *European Respiratory Society* (ERS) and *American Thoracic Society* (ATS). HX: histiocytosis X; LAM: lymphangiomyomatosis. From: (Behr, 2012)

The prevalence of ILDs is not yet thoroughly studied due to the heterogeneity of the diagnosis, however, estimates suggest that the global prevalence of ILDs is approximately 76.0 cases per 100,000 individuals in Europe and 74.3 cases per 100,000 individuals in the United States, with IPF, sarcoidosis, hypersensitivity pneumonitis (HP) and connective tissue disease-associated ILD being the most frequent among these diseases (Alhamad & Cosgrove, 2011; Wijsenbeek & Cottin, 2020).

Most ILDs are characterized by inflammation, fibrosis, or a combination of both, with inflammation typically progressing to fibrosis over time if no intervention occurs. Their clinical course can range from mild and at least partly reversible; to severe, progressive, and irreversible (Wijsenbeek et al., 2022).

Some cases can develop a progressive-fibrosing phenotype associated with worsening symptoms, decreased lung function, and early mortality (Brown et al., 2020; Nasser et al., 2021). Idiopathic pulmonary fibrosis is the prototype of a progressive pulmonary fibrotic disease remaining by far the most well-studied ILD due to the severe disease manifestation. However, other progressive fibrosing ILDs can share similarities with IPF shedding light on putative common fibroproliferative routes (Cottin et al., 2018).

Besides IPF, the most probable ILDs that can also develop a progressive phenotype are idiopathic nonspecific interstitial pneumonia (NSIP), unclassifiable idiopathic interstitial pneumonia, autoimmune ILDs, fibrotic sarcoidosis, fibrotic hypersensitivity pneumonitis, as illustrated in Figure 2 (Cottin et al., 2019).

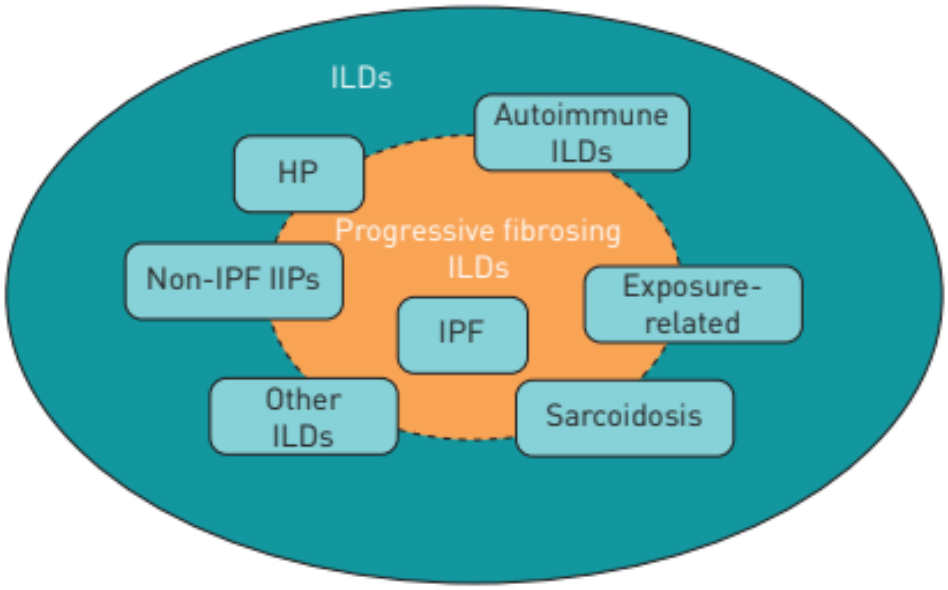

Figure 2. Illustration of interstitial lung diseases (ILDs) that may be associated with a progressive fibrosing phenotype. Interstitial lung diseases that can exhibit a progressive fibrosing phenotype are indicated by the dotted line encompassing all interstitial lung diseases. At the centre is idiopathic pulmonary fibrosis (IPF), which is the prototype of fibrotic diseases, while in the surroundings are present those that may or may not develop a progressive fibrosing phenotype. HP: hypersensitivity pneumonitis; IIPs: idiopathic interstitial pneumonia. From: (Cottin et al., 2019).

1.1.1. Fibrotic ILDs Physiopathogenesis

Understanding the fibrotic process is crucial to identify potential therapeutic targets and strategies to attenuate disease progression (Ku et al., 2024). The mechanism driving this process is initiated by a damage in the lung epithelial cells, which activates a repair mechanism associated with an inflammatory response marked by the recruitment and activation of different immune cells, namely lymphocytes and macrophages. If this process continues to progress it will culminate into the release of pro-fibrotic mediators and, consequently, fibroblasts will be activated, moved to the injured area, and will differentiate into myofibroblasts (Distler et al., 2019; Puglisi et al., 2016). A dysregulation in the myofibroblasts promotes their accumulation leading to a remodelling of the extracellular matrix within lung tissue, resulting in a decline in alveolar function, which in turn activates additional myofibroblasts and sustains the fibrotic process (Distler et al., 2019; Phan, 2008). The pulmonary fibrotic process is summarized in Figure 3 (Pokhreal et al., 2023).

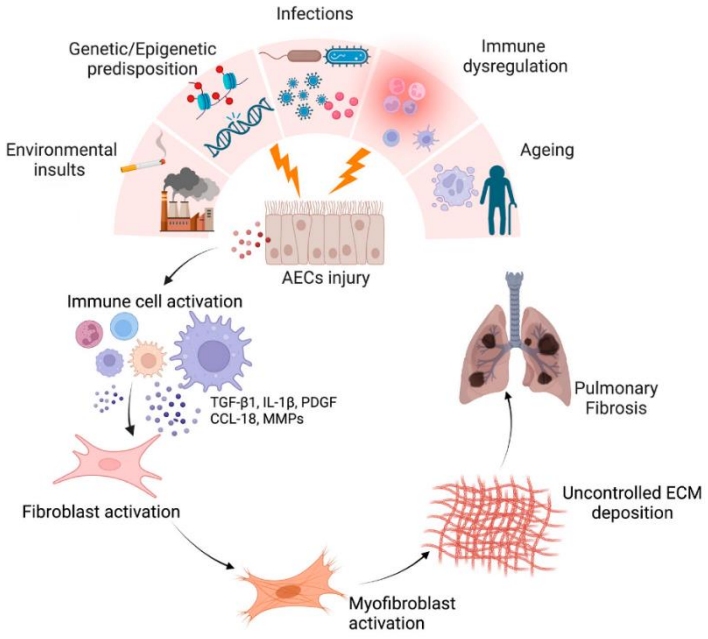

Figure 3. Auto-sustained fibrotic process. Alveolar epithelial cells (AECs) exposed to repeated injury from various causative agents will be dysregulated. This cumulative damage leads to the release of pro-inflammatory and pro-fibrotic mediators, which activate immune cells, including macrophages. In response, these macrophages release other mediators (TGF-β, IL-1β, PDGF, and CCL18) that promote the activation and proliferation of fibroblasts, driving their differentiation into myofibroblasts. The persistent activation of these pathways, combined with dysregulated wound healing processes, result in excessive deposition of extracellular matrix (ECM) leading to progressive fibrosis. If this process persist gas exchange will be compromised, impacting the overall respiratory function. From: (Pokhreal et al., 2023)

Currently, the treatment for ILDs involves the use of immunosuppressive drugs, including corticosteroids, mycophenolate mofetil, azathioprine, methotrexate, cyclophosphamide, and rituximab (Van Den Bosch et al., 2022). Therapies targeting inflammation early in the process can be effective, but if fibrosis continues to advance, a progressive fibrotic phenotype may develop, leading to a need for alternative treatment with antifibrotic agents (Cottin, 2019). Antifibrotic therapies currently in use include nintedanib and pirfenidone, which have demonstrated good results in slowing the disease progression rate (Behr et al., 2021; Flaherty et al., 2019).

Fibrotic hypersensitivity pneumonitis, a type of ILD with a low global prevalence but a higher occurrence in Portugal (Fernández Pérez et al., 2018; Santos et al., 2020; Vasakova et al., 2017) will be further detailed since it is the focus of the current study.

1.2. Hypersensitivity Pneumonitis

Hypersensitivity pneumonitis is an ILD caused by an exaggerated immune response to inhaled antigens in individuals who are genetically predisposed or susceptible (Vasakova et al., 2017).

Incidence and prevalence are difficult to assess, as this disease often goes unrecognized, and is consequently underdiagnosed. However, HP is generally considered a rare disease. Globally, studies have estimated the incidence of HP at 1.3 to 1.9 cases per 100,000 individuals in the US and between 0.3 and 0.9 cases per 100,000 in Europe (Fernández Pérez et al., 2018; Vasakova et al., 2017).

Interestingly, a previous study from our centre, revealed that out of 634 ILD-diagnosed patients from the northern region, 123 (19.4%) were HP cases (Santos et al., 2020). These results contrast with what is described for other European countries: in Greece 2.6% of ILD causes where HP and in Denmark the HP frequency reach 7.0% (Hyldgaard et al., 2014; Karakatsani et al., 2009). However, in some regions, such as India, hypersensitivity pneumonitis accounts for a significantly larger proportion, representing 47.3% of all diagnosed ILDs (Singh et al., 2019). This increased prevalence in Portugal may be linked to prolonged exposure to mold due to the high humidity levels in certain regions, as well as exposure to birds (Nogueira et al., 2019; Santos et al., 2020).

The clinical symptoms of HP are normally unspecific, and usually comprise cough, dyspnea, fatigue, weight loss, and chest tightness (Raghu et al., 2020; Santos et al., 2020). Similar to other ILDs, an accurate diagnosis requires a deeper evaluation of clinical, radiological, and, when needed, histopathological data (Raghu et al., 2020). Nevertheless, since it is an ILD triggered by an environmental antigen exposure, identifying the causative antigen is key to an accurate diagnosis and disease management, especially to establishing a prognosis and implementing preventive measures to avoid exposure (Wuyts et al., 2015) – antigen avoidance is the frontline treatment for HP. The current guidelines for diagnosing hypersensitivity pneumonitis indicate that a highly confident diagnosis can be achieved using less invasive methods when the identification of the causative antigen is possible, as demonstrated in Figure 4 (Raghu et al., 2020).

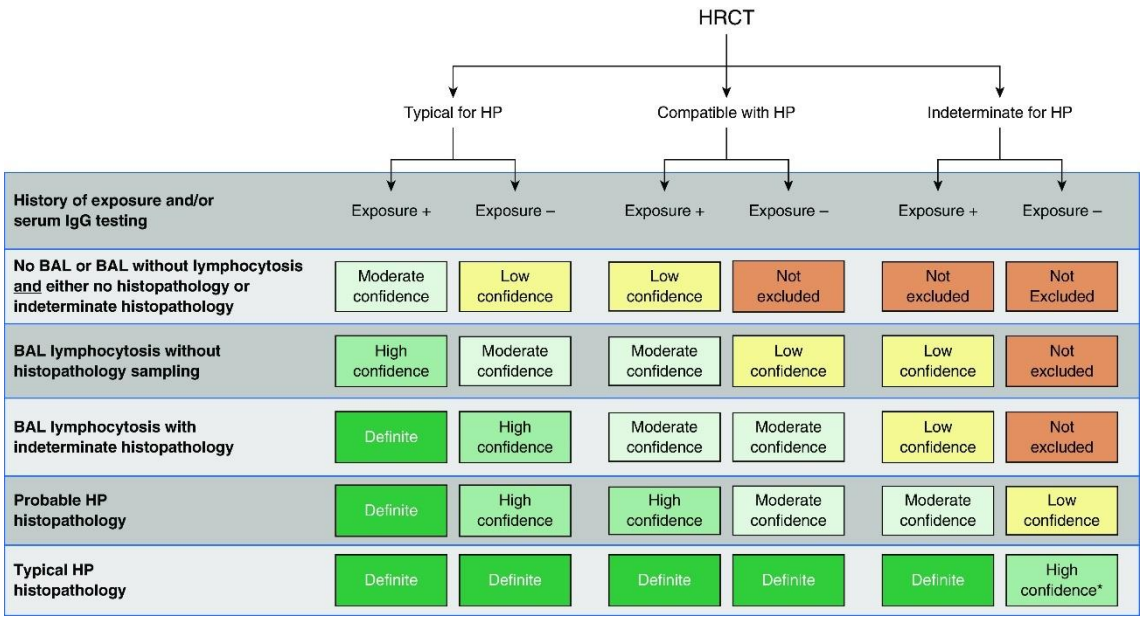

Figure 4. Guidelines for hypersensitivity pneumonitis diagnosis. Diagnostic criteria for hypersensitivity pneumonitis (HP) are based on High-Resolution Computed Tomography (HRCT) imaging patterns, antigen identification, bronchoalveolar lavage (BAL) cell counts, and histopathological findings, categorized by diagnostic confidence: definite (≥90%), high confidence (80–89%), moderate confidence (70–79%), and low confidence (51–69%). From: (Raghu et al., 2020)

The antigens most linked with HP include bacteria, proteins, fungi, or mycobacteria found in the environment. Although less frequent, chemicals can also be associated (Nogueira et al., 2019). When antigens are inhaled, they are detected by antigen-presenting cells, such as macrophages and dendritic cells. These cells capture and present the antigens to T cells present in the lung, triggering a T cell-mediated response (Kawasaki et al., 2022). In turn, the activated T cells secrete interferon-gamma (IFN-γ) and interleukin-12 (IL-12), promoting differentiation of naïve T cells into the Th1 subtype. This process generates the accumulation of lymphocytes and supports the formation of granulomas, which can still be reversible in terms of prognosis (Vasakova et al., 2019).

The progression of fibrosis and the sustained inflammatory response in HP appears to be linked to a transition from a Th1 to a Th2 phenotype, although the involved mechanisms are not yet fully understood. The Th2 response is characterized by the secretion of cytokines such as IL-4 and IL-3, which promote fibroblast proliferation and the accumulation of extracellular matrix components reflecting the main features of a progressive-fibrosing phenotype of the disease (Barnes et al., 2022; Barrera et al., 2008).

Recently, the *American Thoracic Society* (ATS), *Japanese Respiratory Society* (JRS), and *Latin American Thoracic Association* (ALAT) stratified HP according to the presence of fibrosis as non-fibrotic, and fibrotic (Fernández Pérez et al., 2021; Raghu et al., 2020). Non-fibrotic HP is associated with a good prognosis and it might be reversible. If the disease continues to progress will reach the fibrotic stage which has been described as a strong predictor of lower survival rates and is closely linked to a worse prognosis in patients. (Vourlekis et al., 2004). Fibrotic HP can be treated to stabilize progression but remains irreversible and is associated with a higher morbidity and mortality (Fernández Pérez et al., 2018). Therefore, it is important to find strategies to improve the disease diagnosis and accurately predict the progression, allowing personalized treatment adjustments to improve patient outcomes. The mechanisms driving the progression of HP are illustrated in Figure 5 (Costabel et al., 2020).

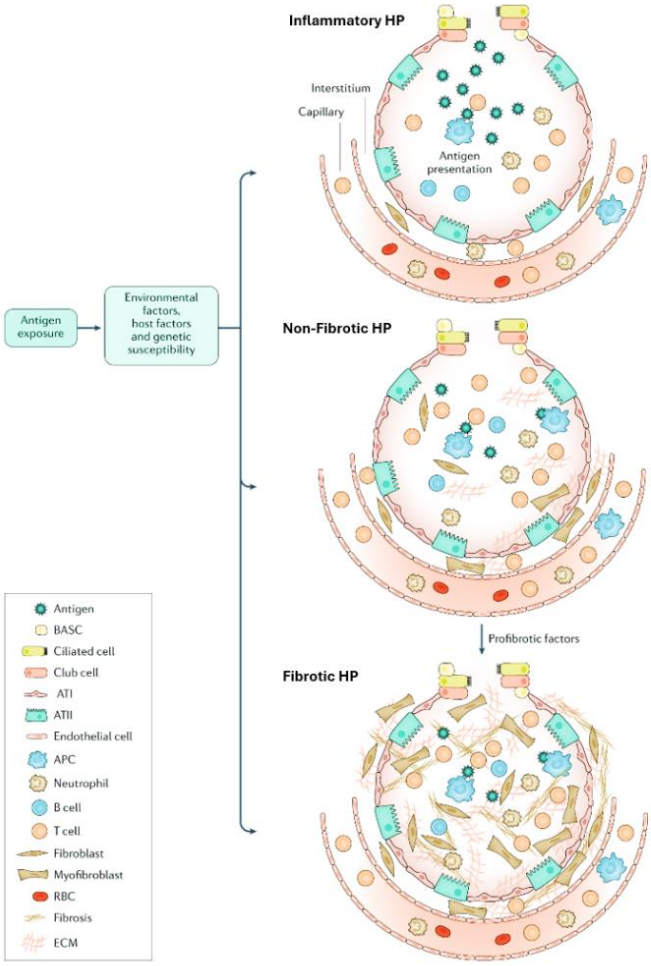

Figure 5. Pathogenesis of inflammatory, non-fibrotic, and fibrotic hypersensitivity pneumonitis (HP). Exposure to antigens triggers an exaggerated immune response that can result in inflammatory lung conditions, marked by the recruitment of antigen-presenting immune cells (top). This immune reaction can lead to non-fibrotic hypersensitivity pneumonitis, which promotes myofibroblast activation and extracellular matrix deposition (middle). In the presence of pro-fibrotic factors, activation of fibroblast and myofibroblast populations occurs leading to an excessive extracellular matrix accumulation (bottom). This process results in fibrosis which is associated with disruption of normal lung function. APC: antigen-presenting cell; ATI: alveolar epithelial type I cell; ATII: alveolar epithelial type II cell; BASC: bronchioalveolar stem cell; RBC: red blood cell. Adapted from: (Costabel et al., 2020)

1.2.1. *MUC5B* impact in fibrotic hypersensitivity pneumonitis

The *MUC5B* gene, already mentioned above as one of the strongest genetic associations for IPF susceptibility, is located at chromosome locus 11p15.5 and encodes mucin 5B, a gel-forming mucin that seems crucial in maintaining mucociliary clearance, lung homeostasis, and controlling infections (Roy et al., 2014).

Several studies have identified the *MUC5B* promoter variant rs35705950, a G/T transversion, as being strongly associated with the development of ILDs, especially IPF (Hunninghake et al., 2013; Seibold et al., 2011). MUC5B expression has also been described to be overexpressed in lung tissue from patients with IPF, suggesting a potential association between mucociliary dysfunction and fibrotic ILDs disease progression (Yang et al., 2015).

The role of genetic factors in HP remains poorly understood, however, recent studies have shown that the minor allele of rs35705950 *MUC5B* SNP was also associated with fibrotic HP frequency and prognosis (Ley et al., 2017; Raghu et al., 2020).

In our cohort, a recent study confirmed the association between the *MUC5B* rs35705950 minor T allele and several *TOLLIP* SNPs (rs3750920, rs111521887, and rs5743894) with increased susceptibility to develop fibrotic HP. The identification of these genetic variants are important to understand the disease's incidence but also can help to predict the disease progression (Mota et al., 2024).

1.2.2. Macrophages as key players in the fibrotic cascade process

The lungs are constantly exposed to various agents that can damage lung tissue. To protect against these agents, the lungs rely on a defense system made up of epithelial cells and fibroblasts. When an injury occurs, a range of immune cells including neutrophils, monocytes/macrophages, mast cells, dendritic cells, and lymphocytes migrate to the lungs to help repair damage as reviewed by Suzuki et al. 2008.

The epithelial cells of the lungs comprise type I pneumocytes, which cover most of the alveolar surface area and, in contrast, type II pneumocytes, which only occupy around 3-5% of the surface area and remain intercalated among the type I cells. These type II cells play a crucial role in secreting surfactant, a substance that reduces surface tension within the alveoli, preventing their collapse during exhalation (Khan & Lynch, 2023).

The presence of immune cells in the alveolar space is commonly assessed by bronchoalveolar lavage. The presence of alterations in the number or proportion of a specific immune cell type in BAL can point towards a specific ILD diagnosis (Meyer et

al., 2012), if the clinical and radiological features are in accordance. In the case of HP, several studies have shown an elevated number of lymphocytes in BAL, being these finding included in HP diagnosis guidelines and are associated with a higher confidence diagnosis (Raghu et al., 2020). However, these studies did not have in consideration the disease status: non fibrotic *versus* fibrotic HP. Previous analysis in our cohort had shown that in fibrotic HP the most abundant immune cell population present in BAL samples were macrophages, similar to what was described for IPF (Gonçalves, 2023).

Macrophages are cells of the innate immune system found in nearly every organ, including the lungs, where they play crucial roles. Lung macrophages contribute to maintaining homeostasis, conducting immune surveillance, clearing cellular debris, repairing tissue, eliminating pathogens, and resolving inflammation as reviewed by Shi et al. 2021.

Lung macrophages are categorized into two main types based on their tissue location: alveolar macrophages, which reside in the airspaces, and interstitial macrophages, found within lung tissue (Evren et al., 2020).

Alveolar macrophages are known to originate from fetal progenitors and to have the ability to self-renew. Under healthy conditions, they maintain their population independently, without recruiting the circulating adult monocytes (Guilliams et al., 2013; Hashimoto et al., 2013; Yona et al., 2013). However, in response to injury, circulating monocytes are recruited to the lung tissue, where they differentiate into monocyte-derived alveolar macrophages, contributing to the immune response and maintaining the inflammatory and fibrotic processes (Misharin et al., 2017).

These immune cells represent a heterogeneous and plastic population, capable of altering their phenotype according to their surrounding microenvironment. Broadly, macrophage phenotypes are classified into two functional groups: M1 macrophages, which are classically activated and pro-inflammatory, and M2 macrophages, which are alternatively activated and play anti-inflammatory and tissue-repair roles (Murray, 2017). Both phenotypes are involved in pulmonary fibrosis, although, M1 macrophages play a key role in the recovery following damage to the alveolar epithelium, whereas M2 macrophages are responsible for resolving the healing process and ending inflammatory

responses. A disruption in this process can be associated with pathological outcomes (Zhang et al., 2018). In the fibrotic process, M2 can be dysregulated leading to a permanent activity of these cells secreting cytokines that promote proliferation of fibroblasts and extracellular matrix deposition inducing a pro-fibrotic process (Braga et al., 2015; Conway & Hughes, 2012).

The involvement of macrophages in fibrosis has been extensively studied in diseases such as idiopathic pulmonary fibrosis, and for instance, M2 macrophages have an important role in the progression of pulmonary fibrosis (Pokhreal et al., 2023). Since IPF is the most extensively researched ILD, this knowledge can provide valuable insights into the pathogenesis of other ILDs characterized by a progressive fibrosing phenotype, including HP. Although, the involvement of alveolar macrophages and/or monocyte derived macrophages in the development of a progressive fibrotic phenotype in ILDs is still poorly understood.

1.3. Project Contextualization and Goals

Hypersensitivity pneumonitis is an interstitial lung disease triggered by an immune response to inhaled environmental antigens, including bacteria, proteins, fungi, mycobacteria, and chemicals, especially in genetically predisposed individuals. HP can present in both non-fibrotic and fibrotic forms, with the fibrotic form being more severe and progressive. Our previous studies revealed that HP is the most common ILD in our cohort, accounting for over 45% of all diagnosed fibrotic ILDs. Moreover, we identified a strong association between fibrotic HP and the presence of the T-minor allele of the *MUC5B* rs35705950 polymorphism.

Our prior research also demonstrated that patients with fibrotic HP have an increased percentage of macrophages in bronchoalveolar lavage and show a significant accumulation of MUC5B in lung parenchyma. Notably, intense MUC5B staining was observed in the cytoplasm of alveolar macrophages — an intriguing finding given that these cells are not typically known to produce MUC5B. This observation prompted further investigation into the potential role of macrophages in the pathogenesis of fibrotic HP.

The present study builds on these previous findings and aims to explore whether macrophages in fibrotic HP patients exhibit ultrastructural alterations that could help explain their involvement in the disease process. Specifically, this research will investigate:

- Ultrastructural differences in lung tissue between fibrotic HP patients and controls without interstitial lung disease;
- Quantification of cellular structures potentially linked to the pathogenesis of fibrotic HP;
- The effect of the T-minor allele from the rs35705950 *MUC5B* SNP on cellular structures in fibrotic HP;
- Analysis of mitochondrial functionality and phagocytic capacity in alveolar macrophages from fibrotic HP patients.

The insights gained from this study could significantly advance our understanding of the cellular mechanisms driving fibrotic HP and clarify the contribution of alveolar macrophages to the fibrotic process.

2. Methodology

2.1. Study design and participants

The current study included patients encompassed in the FIBRALUNG Portuguese cohort study (ClinicalTrials.gov Identifier: NCT05635032), who meet the following criteria:

- Aged between 18-80 years;
- Treatment-naive for disease-modifying drugs;
- Undergoing lung biopsy and/or bronchoalveolar lavage (BAL) as part of diagnostic and follow-up procedures;
- Have undergone a high-resolution computed tomography scan within the past 12 months;
- Willing and able to provide informed consent.

Only patients with a final diagnosis validated in a multidisciplinary team meeting were included. Final diagnosis was achieved based on the clinical data recorded which includes symptoms, environmental exposures, together with lung function tests, radiological findings, and, when available, data from BAL composition and lung histology.

The no ILD control group was composed by individuals who underwent pulmonary resection due to lung cancer at the cardiothoracic department of the São João University Hospital Centre. For the purpose, lung health parenchyma was collected as far as possible from the lesion.

The study protocol was reviewed and approved by the Ethics Committee of the Institution and performed following the Helsinki Declaration. Written informed consent was obtained from all participants and samples were codified to maintain patient anonymity.

2.2. DNA extraction and *MUC5B* single nucleotide polymorphism rs35705950 genotyping

Genomic DNA was extracted from peripheral blood samples collected into ethylenediamine tetraacetic acid (EDTA)-treated tubes (BD Vacutainer®) by phlebotomists. Samples were stored at – 70ºC until further DNA extraction. The extraction was done using a commercial kit (QIamp DNA Blood purification kit from

Qiagen), following the manufacturer's instructions, and the resulting DNA was quantified with Nanodrop One ™.

MUC5B single nucleotide polymorphism (SNP) rs35705950 was genotyped using TaqMan SNP Genotyping Assay (C_1582254_20). In summary, two allele-specific probes labelled with fluorescent dyes (FAM and VIC) were used, as well as a primer pair specifically aligned with the region of interest ([VIC/FAM]): CCTTCCTTTATCTTCTGTTTTCAGC[G/T]CCTTCAACTGTGAAGAGGTGAACT. DNA polymerase was used to cleave the fluorescence probe (FAM or VIC) from the hybridized probe of the DNA strand that produced fluorescence. This reaction was carried out on CFX96 from BioRad according to the following protocol: initial denaturation at 95ºC for 10 minutes followed by 40 cycles of denaturation at 95ºC for 15 seconds and paring and extension at 60ºC for 1 minute. The obtained results were analyzed using CFX software (BioRad).

2.3. Transmission Electron Microscopy

Lung tissue samples obtained through cryo-biopsies (ILD patients) or lung resection (no ILD controls) and BAL cells were fixed with 2% formaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, overnight and for one hour respectively. After fixation, samples underwent through a washing process in 0.1 M sodium cacodylate buffer and were subsequently incubated with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for one hour. After further washing in distilled water, samples were stained with 1% uranyl acetate for 30 minutes. Dehydration was carried out with increasing series of graduated ethanol (50%, 70%, 80%, and 100%), followed by propylene oxide. Mixtures of increasing concentrations of Embed-812 epoxy resin with propylene oxide (25%, 50%, 75%, and 100%) were used to infiltrate the samples until the embedded in 100% Embed-812 epoxy resin and left for 48 hours at 60ºC to polymerize.

For section overview and pre sample localization for electron microscopy, semi-thin sections were cut using an RMC Ultramicrotome (PowerTome) with a Diamond knife at 500 nm and stained with Richardson. Ultrathin sections were cut at 60 nm, picked up on 200 mesh copper grids, and stained with UranyLess (Electron Microscopy Sciences) and

3% lead citrate (Electron Microscopy Sciences) for 5 minutes each. Samples were visualized in JEOL JEM 1400 transmission electron microscope operating at 80 kV. Digital images of five to ten cells per condition were captured with a PHURONA camera. Quantitative analysis was carried out using Empanada Software (Conrad & Narayan, 2023) and ImageJ software (Schneider et al., 2012).

2.4. Data quantification

For cell analysis and ultrastructure quantification, five to ten macrophages were acquired in each sample. Based on specific features outlined in the literature (Laskin et al., 2015), macrophages identification was based on the following criteria:

- Cell size and abundant cytoplasm;
- Irregular and acentrically located indented nuclei;
- Several spherical mitochondria;
- Abundant vacuoles, lipid droplets, phagocytic vesicles, and lysosomes.

The quantitative analysis was focused on understanding the ultrastructural differences between macrophages from fibrotic HP and no ILD controls. Two groups of organelles were selected:

Preserved mitochondria: mitochondria were identified following the features previously described in the literature (Alberts B, 2002; Hossler, 2014; Palade, 1953), namely double membrane organelles located in the cell's cytoplasm with inner membrane folds known as cristae.

Dysfunctional double membrane organelles: organelles within this group were characterized by being enclosed within a double membrane and containing non-identified amorphous content inside without visible cristae.

The preserved mitochondria and the dysfunctional double membrane organelles were identified using Empanada software, which creates a 2D segmentation of the mitochondria and allows the identification of the other organelles manually. The final segmentations were saved in a TIFF file, loaded onto ImageJ, and converted to an 8-bit mask while the threshold was removed to perform the "analyse particles" function to

obtain the following measurements: area and number of mitochondria and dysfunctional double membrane organelles. The Image J was also used to measure the cell area using the "Measure" function after outlining the area.

2.5. Mitochondrial content and phagocytic assay

Bronchoalveolar lavage (BAL) cells were collected for diagnostic purposes, and the remaining material was used for *ex vivo* assays. Mitochondrial mass and membrane potential were measured by flow cytometry. For this, 0.5×10^6 BAL cells were labeled with 50 nM MitoTracker™ Green and 50 nM MitoTracker™ Red (both from Thermo Fisher Scientific). The MitoTracker™ probes were diluted in pre-warmed media without serum, and cells were incubated for 30 minutes at 37ºC. Following incubation, cells were washed twice with FACS buffer, blocked using TrueStain Monocyte (BioLegend) and stained with anti-human HLA-DR APC-Cy7 (clone L243, BioLegend).

For phagocytosis assays, 2 mg of pHrodo™ Red E. coli BioParticles™ (Thermo Fisher) were reconstituted with 500 µl of TBS containing 5 mM $CaCl_2$ and sonicated for 5 minutes. A total of 0.5×10^6 BAL cells were incubated with 1 µl of pHrodo™ Red E. coli BioParticles™ for 6 hours to allow phagocytosis. After incubation, cells were washed twice with FACS buffer, blocked with TrueStain Monocyte (BioLegend), and stained with anti-human HLA-DR APC-Cy7 (clone L243, BioLegend).

All samples were analyzed by flow cytometry using an LSR Fortessa (BD Biosciences), and data analysis was performed with FlowJo™ v10.10 software (BD Biosciences).

2.6. Statistical Analysis

Quantitative data was analyzed with GraphPad Prism™ v8 software (GraphPad Software®). Results are expressed as means ± standard deviation (SD). Specific statistical tests are detailed in the legend of each figure. Statistical significance is considered when $p < 0.05$.

3. Results and Discussion

The FIBRALUNG cohort, which aims to study the pathogenesis of ILDs, includes more than 800 ILDs patients from the north and central region of Portugal. So far, almost 34% are cases of hypersensitivity pneumonitis, either fibrotic or non-fibrotic, being the most prevalent ILD in our setting. This high prevalence of HP cases exacerbates the need to further study this disease. Previous unpublished data from the group (+++Gonçalves, 2023), had shown that most of the fibrotic HP (fHP) patients (~ 75%) from our cohort are *MUC5B* rs35705950 T-carriers, in contrast with controls where T allele is only observed in around 25% of the individuals. We showed that *MUC5B* rs35705950 T allele is associated with increasing MUC5B expression in lung tissue, namely in honey combing cists, but also, surprisingly, in macrophages cytoplasm. Since this cell type in not known to be MUC5B producers, this observation led us to a more detailed investigation of the ultrastructure of these cells to clarify if any ultrastructural feature could shed light to the putative expression/uptake of MUC5B by these cells.

3.1. Patient's Demographics

To understand the main characteristics of the macrophages and their role in the HP pathogenesis, we analysed lung tissue from a total of 14 individuals composed by 8 individuals diagnosed with fibrotic HP, part of the FIBRALUNG Portuguese cohort study, and 6 individuals with no ILD as a control group.

The participants ranged in age from 51 to 81, with a mean age of 67,0 years for fHP patients and 67,8 years for no ILD control group, demonstrating that the age of individuals in both groups was similar and reflects the prevalence of fHP in older individuals (65 years or older) (Dabiri et al., 2022) (Table 1). The gender distribution, while similar between the two groups, showed a slightly lower number of females than males in both groups. Specifically, males comprised approximately 66,7% of the no ILD control group and 62,5% of the fHP group, while females constituted 33,3% and 37,5%, respectively.

Table 1. Patients Demographics

Variable	fHP patients	No ILD control
Age (years),		
Mean ± SD (min-max)	67,0 ± 7,0 (51-75)	67,8 ± 7,6 (59-81)
Gender, n (%)		
Female	3 (37,5)	2 (33,3)
Male	5 (62,5)	4 (66,7)
Smoking status, n (%)		
Non-smokers	5 (62,5)	-
Smokers	1 (12,5)	3 (50,0)
Ex-smokers	2 (25,0)	3 (50,0)
Types of Exposure		
Organic	6 (75,0)	-
Inorganic	2 (25,0)	1 (16,7)
Not Reported	-	5 (83,3)

fHP: Fibrotic Hypersensitivity Pneumonitis; ILD: Interstitial Lung Disease

Importantly, as previously anticipated, the smoking status of the two groups showed some discrepancies. The no ILD control group is consisted by individuals who had undergone pulmonary lobectomy associated with lung cancer, and it is already well established that smoking habits and tobacco exposure are strongly linked to cancer (Sasco et al., 2004), especially lung cancer. In accordance, all the elements of this control group had contact with tobacco: 50% are active smokers and the others 50% are ex-smokers. Controversy, the fHP patients are mainly non-smoking individuals (62,5%) and the smokers and ex-smokers are only 25,0% and 12,5% respectively. This fact may be explained by the lower prevalence of HP in smokers (McSharry et al., 1985), while the reason for this remains partially unclear it is thought to be associated with the effect of nicotine in impairing lymphocyte and macrophage function, refraining the immune response associated with HP development (Blanchet et al., 2004). Although, smokers who develop HP usually present a worse clinical phenotype and prognosis (Ohtsuka et al., 1995). Since HP is

associated with an exaggerated immune response triggered by the inhalation of environmental antigens, more than 200 antigens have already been identified as being linked with this ILD. The most prevalent antigens are found in the environment (fungi, bacteria, proteins) but exposure to chemicals also appears to be associated, although less prevalent (Nogueira et al., 2019). In this study, we decided to categorize the type of known exposure into three main groups: organic, inorganic, and not reported. Organic exposures included biological antigens such as fungi, bacteria, and animals (avian exposure) and were the most associated with fHP, representing 75,0% of the group. Inorganic exposures were associated with non-biological antigens, normally occupational exposures, such as chemicals, metals, and textiles which represented 25,0% of the fHP patients. Regarding the no ILD control group only one participant reported exposure to inorganic antigens, all the other (83,3%) did not describe any type of relevant exposure.

The small sample size of this study is associated with the difficulty in obtaining biological samples from patients since it is an invasive procedure and only performed, when it is not possible to reach a reliable diagnosis by other complementary techniques. Although, the patient demographics included a diverse cohort represented by different factors that can influence the progression of HP, such as smoking status, and various types of exposure allowing the identification of potential correlations between these factors and the differences in the ultrastructural analysis.

3.2. Ultrastructural analysis reveals differences in lung tissue between fibrotic hypersensitivity pneumonitis patients and controls without interstitial lung disease

Based on the earlier analysis, discrepancies were noted in tissue organization and MUC5B expression in particular cells, such as alveolar macrophages, in lung samples from fibrotic HP patients (Gonçalves, 2023). To understand if these cells from fibrotic HP patients display an alteration in the clearance process that can be responsible for the increased accumulation of MUC5B in their cytoplasm, we perform ultrastructural analysis using TEM having no ILD lung tissue as a control.

After sample collection, by cryo-biopsy as part of the diagnostic workup for the fHP patients, or by surgical resection in the no ILD participants, lung tissue was processed for TEM analysis. No significant differences regarding tissue preservation were observed between the two groups.

Even in low magnification (Figure 6, first row) is possible to observe remarkable differences between both groups. Alveolar space appears clean in the no ILD control (left image), in contrast, with a compact and crowdy alveolar space with inflammatory-like content in fHP patients (right image), including red blood cells (rbc) and fibrin (fbn) accumulation around the immune cells. While red blood cells accumulation might be a consequence of the cryo-biopsy procedure, the fibrin deposition is already described as a characteristic of several ILDs. It seems to be involved in the pathogenesis of these diseases influencing the expression of inflammatory mediators and changing the proliferation rate and migration of various types of cells, such as fibroblasts (Jablonska et al., 2008), leading to the replacement of healthy lung tissue by fibrotic component impairing the air exchange and the patient's life quality.

The type II pneumocytes (Figure 6, second row) were found preserved with many microvilli and lamellar bodies (lb) both in fHP patients and in the control group, revealing no ultrastructural differences in this type of cells. However, several studies reported an increase in the type II pneumocytes cells and the number of lamellar bodies present, associated with HP (Quezada et al., 1987). These specific alterations could only be confirmed when analyzed quantitatively, which is beyond this project's scope.

On the other hand, alveolar macrophages (Figure 6, third and fourth row) found in the lung samples demonstrated notable alterations between patients and controls. Since all the no ILD control participants were exposed to tobacco (as smokers or ex-smokers, Table 1), alveolar macrophages had several inclusions of black pigment (bp), most probably associated with smoking habits. Exposure to tobacco is linked with the accumulation of black-pigmented materials in human lungs, such as carbon particles, especially in phagocytic cells, and it seems to be associated with the presence of substances in cigarettes that are not easily digested by the phagocytic cells (You et al., 2015). In these cells from the no ILD control group, as expected, was also possible to observe preserved organelles, as mitochondria, in their cytoplasm.

In fHP patients, macrophages presented more pseudopodia (ps) compared to no ILD control cells, and it was possible to observe fibrin (fbn) accumulation close to the immune cell's membrane. Interestingly, engulfment of extracellular material by the macrophages pseudopods is observed in some cells suggesting active phagocytosis (phag) in fibrotic HP patients. These observations were expected since it's already described fibrin deposition in lung tissue from IPF patients (Imokawa et al., 1997), also macrophages can play a role in the clearance of debris depending on the environment where they reside (Bain & MacDonald, 2022).

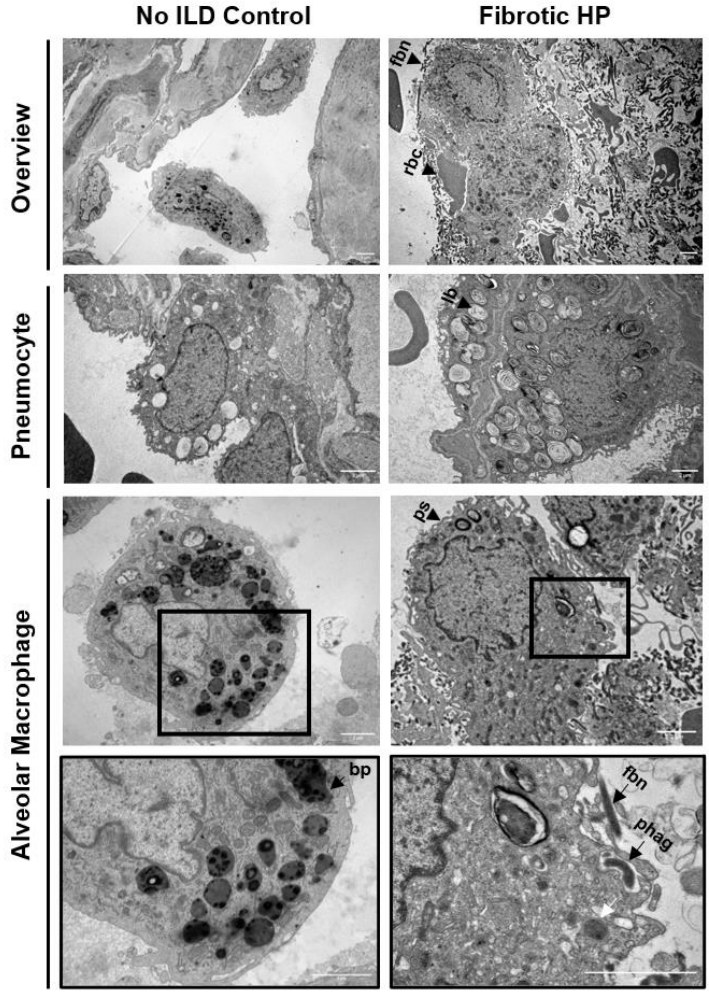

Figure 6. Transmission Electron Microscopy (TEM) analysis reveals ultrastructural differences in lung tissue between fibrotic hypersensitivity pneumonitis (HP) patients and controls without interstitial lung disease (ILD). Lung tissue was obtained via lung cryo-biopsies from fibrotic HP patients and surgical resections from no ILD controls, then processed for TEM analysis. The overview (first row) highlights an increase in inflammatory material, including red blood cells and fibrin, in the fibrotic HP samples compared to the no ILD controls. Pneumocytes (second row) exhibit no significant ultrastructural differences between patients and controls. Alveolar macrophages (third and fourth rows), however, demonstrate notable alterations. In fibrotic HP patients, alveolar macrophages displayed increased pseudopodia (ps), fibrin (fbn) accumulation in their proximity, multiple areas of engulfment of extracellular material indicative of active phagocytosis (phag) and the presence of double membrane organelles (white arrow). Additionally, black pigment (bp), associated with smoking habits, was observed in macrophages of smokers. Scale bar: 2 µm.

Additionally, in fHP samples numerous round or oval double-membrane organelles with amorphous content occupying most of the macrophage's cytoplasm are observed (fourth row, right picture, white arrow). In the overview analysis, the same structures seem to be almost absent in the control group.

3.3. Fibrotic Hypersensitivity Pneumonitis is linked to the presence of dysfunctional double membrane organelles in lung tissue

Considering the abundance of unidentified double membrane organelles in the fHP patients' cells, as shown in Figure 7A, and given the complexity in categorizing these structures based on already described features for healthy/preserved organelles, we decided to perform a deeper analysis and generally classify them as "dysfunctional double membrane organelles" (Fig. 7A, right picture, black arrow). These double membrane organelles, that occupy most of the cell cytoplasm in fHP patients, resemble mitochondrias regarding size and shape but no cristae were visible. To perform an unbiased analysis and understand if there was a significant difference in dysfunctional double membrane organelles but also preserved mitochondria (black arrowhead) in the macrophages from fHP and from no ILD control participants we decided to quantify the area and number of these structures in both groups. For quantification, four cases from fHP patients and no ILD control were selected. The cases were chosen to have representativity of the different smoking habits, types of exposure, and *MUC5B* genotypes.

As summarized in Figure 7B, lung tissue samples were processed for electron microscopy, and in the semi-thin sections stained with Richardson's macrophages were co-localized using an optical microscope. The areas with more macrophages were elected, and thin sections were collected into grids for further observation using the transmission electron microscope. Images of 5 to 10 cells were acquired per case and analysed using Empanada software (Conrad & Narayan, 2023) to segment the preserved mitochondria and dysfunctional double membrane organelles. Then, ImageJ was used to create a mask and access the area of each organelle individually, as well as, to measure the total cell area.

The quantitative analysis of these organelles shown in Figure 7C and 7D supported what we previously observed, confirming differences between fHP and control cells. The results from preserved mitochondria quantification (left graphs) revealed differences between fibrotic HP patients (pink circles) and control group (blue circles), showing a decreased area of these organelles in the fHP patients' macrophages (Figure 7C, left). Additionally, it is also possible to observe a marked decrease in the number of preserved mitochondria per cell in fibrotic HP patients (Figure 7D, left). The presence of dysfunctional double membrane organelles (right graphs) was significantly higher in fHP patients than in no ILD control cells in both percentage of the total cell area (Figure 7C, right) and number per cell (Figure 7D, right).

Whereas we validated a significant difference in the presence of dysfunctional double membrane organelles between fHP and no ILD control cells, the exact classification of these organelles remains unclear. Based on our observations during the analysis, we began to hypothesize that these structures could potentially be categorized as damaged mitochondria or autophagic organelles.

The most well-described double membrane organelles in cells are mitochondria, and the organelles we observed resembled mitochondria in both shape and size, as well as in their double membrane structure. Considering the reduction in the number of preserved mitochondria, the accumulation of damaged mitochondria in the macrophages could be a hypothesis, as it was already described for other ILDs, such as IPF, and is thought to be linked to the disease progression (Tsitoura et al., 2019).

However, the presence of intense pseudopodia in the macrophages from fHP patients suggests that the cells can be in active phagocytosis and trying to improve the lung environment clearance. Additionally, a previous study revealed high levels of autophagy markers present in macrophages from lung tissue of HP patients and they hypothesized that this can be associated with active or defective autophagy during the development of the disease (Cabrera et al., 2020), therefore the presence of autophagic organelles is also a possibility in these cells.

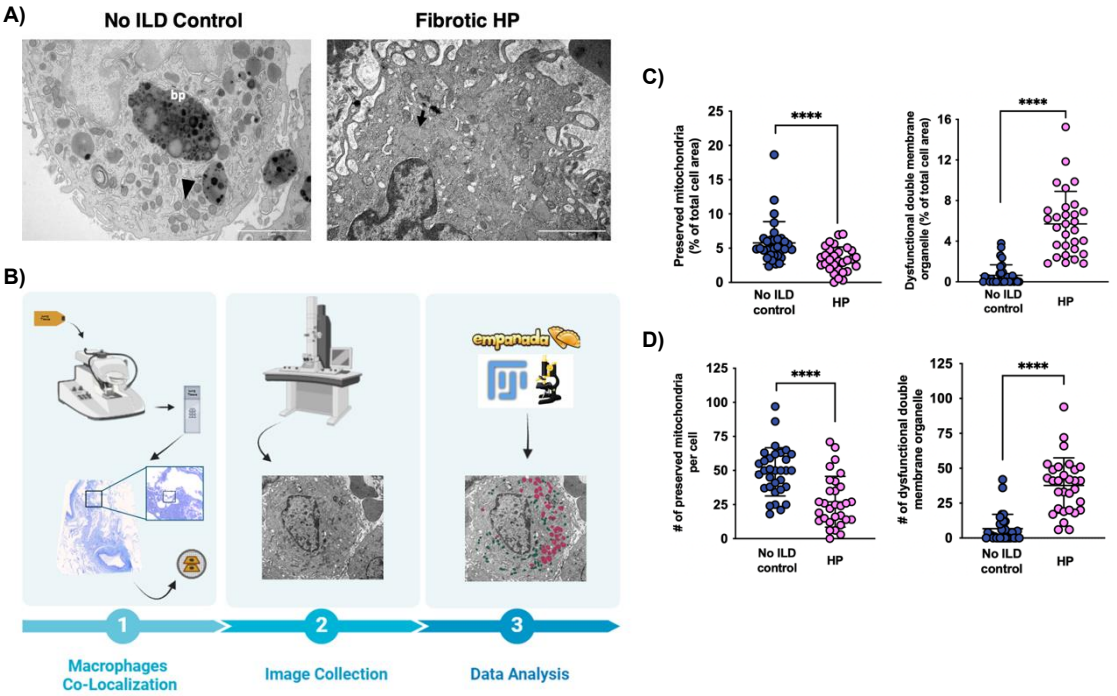

Figure 7. Fibrotic Hypersensitivity Pneumonitis (HP) is linked to the presence of dysfunctional double membrane organelles in lung tissue. Lung tissue obtained through lung cryo-biopsies (fibrotic HP) and surgical resections (no interstitial lung disease (ILD) controls) was analyzed using transmission electron microscopy. **A)** Representative images depict preserved mitochondria (black arrowhead) and black pigment (bp) in the no ILD control sample, in contrast to dysfunctional double membrane organelles (black arrow) in fibrotic HP patients. Scale bar: 2 µm. **B)** Workflow illustrating the collection of images and data analysis to quantify organelles within cells. Initially, tissue samples were processed for transmission electron microscopy (TEM) to co-localize the macrophages. A semi-thin section was collected and stained with Richardson's stain. Macrophages were localized in the semi-thin section using an optical microscope, and a thin section from the same area was selected for grid preparation and TEM analysis. Images of 5 to 10 cells per case were acquired. The acquired pictures were analyzed using Empanada to segment the organelles and ImageJ to create a mask and calculate the area of each organelle individually. **C)** Quantitative analysis showing the percentage of the total cell area corresponding to preserved mitochondria (left graph) and dysfunctional double membrane organelles (right graph) in no ILD controls (blue circles) and fibrotic HP patients (pink circles). **D)** Number of the same structures per cell. Each dot represents one cell (5 to 10 cells per case) from a total of 4 no ILD controls and 4 fibrotic HP individuals. Values are shown as mean ± SD. Data were analyzed using the nonparametric Mann-Whitney test comparing no ILD controls and fibrotic HP patients, ***P < 0.005, ****P < 0.001.

3.4. Bronchoalveolar lavage (BAL) cells from interstitial lung diseases patients showed a decrease in functional mitochondria in HLA-DR$^+$ cells, combined with an accumulation of damaged mitochondria

To understand the macrophage functionality and gain insights into the categorization of these structures, we conducted assays to evaluate phagocytosis and mitochondria function with live cells from the remaining BAL samples collected for diagnosis. This included five samples from patients with suspected fHP, at the time of the procedure, in which two were then confirmed fHP and the others were diagnosed with other fibrotic ILD. In the impossibility of having a healthy control associated with the complexity and risks of the procedure, a participant who performed a BAL for other respiratory complication was included as a no ILD control.

In parallel with the live cell assays, aliquots from the BAL samples were processed for TEM and no significant differences regarding ultrastructure preservation were observed comparing the tissue samples from cryo-biopsies, as well as the dysfunctional double membrane organelles that remain present in the macrophages as illustrated in Figure 8A. This fact confirms that the structures that are seen in the cells from cryo-biopsies are not artifacts generated by the procedure, because they keep appearing on cells collected with other procedures.

The phagocytic capacity was evaluated by incubation of BAL cells with pHrodo™ Red E. coli BioParticles for 6 hours and further analysed by flow cytometry. The particles used are fluorescent under acidic conditions, meaning that when cells internalize the particles they are enclosed within phagolysosomes, resulting in red fluorescence that can be detected and measured. By analysing the percentage of HLA-DR$^+$ cells that were positive for the presence of the particles by flow cytometry, it was possible to observe that no ILD control (blue circles), fHP (pink circles), and other fibrotic ILD (green circles) were all capable of performing phagocytosis as shown in the graph of Figure 8B, not revealing significant differences between them. However, cells from fHP patients (pink circles) presented a slightly lower percentage when compared to the others. Illustrative contour plots for no ILD control and fHP are shown in Figure 8B (right side).

To access the functionality of the mitochondria, we used a flow cytometry staining already established by Monteiro et al, 2020, using a combination of MitoTracker Green and Red. The MitoTracker Green gives information about the mitochondria mass, while MitoTracker Red binds the mitochondria that were negatively charged, indicating functionality. In summary, the presence of a higher quantity of both stains is associated with functional mitochondria, while a slight decrease in MitoTracker Red intensity suggests loss of membrane potential associated with the dysfunctional mitochondria (Monteiro et al., 2020).

When evaluating mitochondrial functionality, a reduction in functional mitochondria was observed in HLA-DR$^+$ cells from ILD patients (both fibrotic HP or other fibrotic ILDs), accompanied by an accumulation of damaged mitochondria, as compared to controls without ILD (Figure 8C). Illustrative contour plots for no ILD control and fHP are shown in Figure 8C (right side). This finding aligns with previous quantitative analyses, which also demonstrated a decrease in both the number and area of preserved mitochondria.

Since both assays were conducted in HLA-DR$^+$ cells, the results reflect the activity of a variety of antigen-presenting cells (APCs), including dendritic cells, B lymphocytes, monocytes, and macrophages. However, from the APCs mentioned above, BAL is composed mainly by macrophages. To improve these analysis in future, establishing a correlation between the number and types of cells present in each sample could help minimize variations due to differences in cell populations across samples.

Regarding the autofluorescence of the cells used in these experiments, an optimized and more complex flow cytometry staining could have given more insights into these results. However, the difficulties in predicting the diagnosis and the possibility of obtaining such time-sensitive samples, and due to the timeline of this project a better approach was not feasible.

After these experiments, the identification of these organelles remains unclear due to the small sample size and the needing of a better approach. It was not possible to exclude the possibility of autophagy defects since the phagocytosis assay only detected the presence of phagolysosomes, which are associated with an acidic pH, meaning that if the defect is in the fusion between the lysosome and the phagosome it will not be detected. In summary, the combined analysis of phagocytic activity and mitochondrial function provides crucial insights into cellular efficiency.

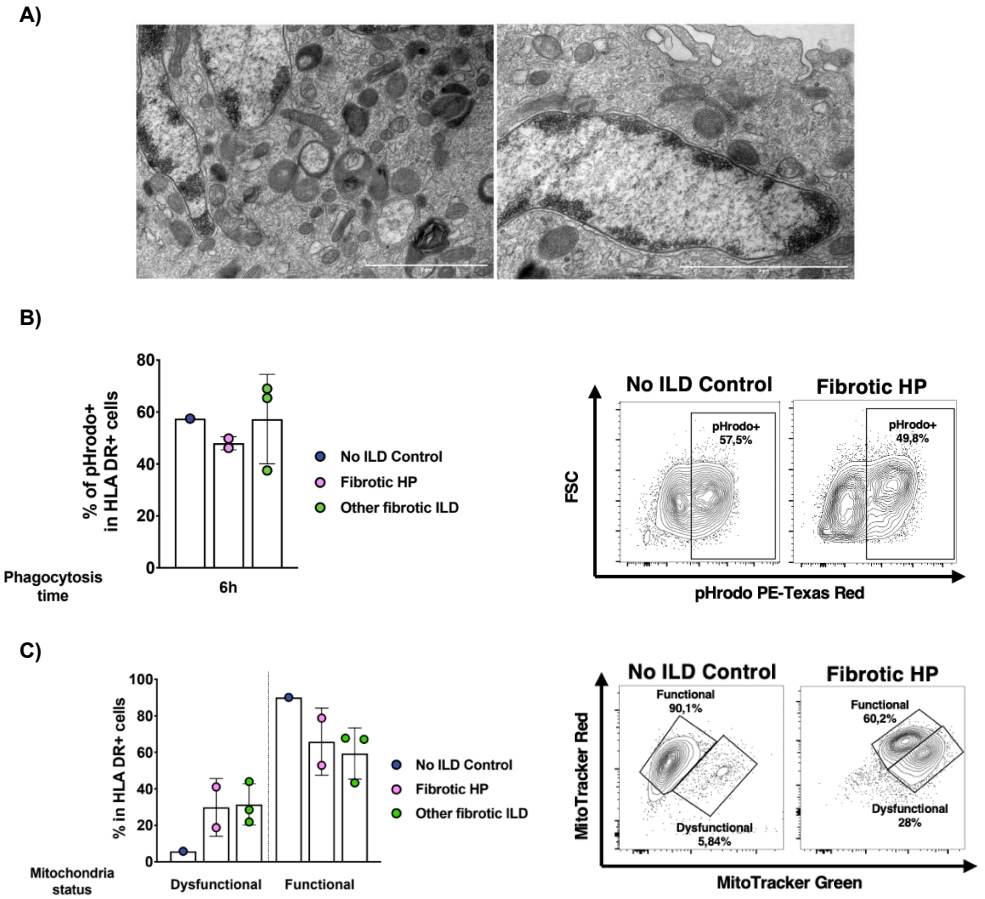

Figure 8. Bronchoalveolar lavage (BAL) cells from interstitial lung diseases (ILD) patients exhibit a reduction in functional mitochondria in HLA-DR$^+$ cells, accompanied by an accumulation of damaged mitochondria. A) Representative transmission electron microscopy images of macrophages from fibrotic hypersensitivity pneumonitis (HP) BAL fluid. Scale bar: 2 µm. **B)** Ex vivo analysis of phagocytosis using pHrodo^{TM} Red E. coli BioParticles^{TM}. BAL cells from patients with fibrotic HP (pink circles) and other fibrotic ILDs (green circles), as well as one control without ILD (blue circles), were incubated with BioParticles for 6 hours and analyzed by flow cytometry. The graph on the left shows the percentage of HLA-DR$^+$ cells that internalized the BioParticles, with the representative contour plots from no ILD control and fibrotic HP patients on the right. **C)** Total mitochondrial mass and membrane potential in BAL cells were assessed by staining with MitoTracker Green and Red. Left graph: percentage of HLA-DR$^+$ cells with functional (MitoTracker Green^{int}/MitoTracker Red^{high}) versus dysfunctional/damaged mitochondria (MitoTracker Green^{high}/MitoTracker Red^{int}). Representative contour plots from no ILD control and fibrotic HP patients (right). Each dot represents an individual. Values are shown as mean ± SD. The numbers of individuals in each group is as follows: no ILD control (n = 1), Non fibrotic ILD (n = 4); Other fibrotic ILD (n = 3), fibrotic HP (n = 2).

3.5. Stratification of fibrotic hypersensitivity pneumonitis patients by the *MUC5B* rs35705950 genotype shows a tendency toward increased dysfunctional double membrane organelles in patients carrying the *MUC5B* T-minor allele

According to what was previously described (Gonçalves, 2023), the presence of the *MUC5B* minor allele (T) was associated with a higher expression of MUC5B in the lung parenchyma, and, surprisingly, it was also visible in the alveolar macrophages cytoplasm, cells that were not related to the production of this protein. To investigate if there was any relationship between the *MUC5B* genotype and the presence of dysfunctional double membrane organelles, we decided to stratify our patients according to the genotype. The no ILD control group was mostly homozygote for the *wild-type* allele (G) which is the most prevalent genotype in healthy individuals, but we also had one heterozygous (GT) participant. The presence of homozygotic individuals for the T allele would be interesting to analyse among the control group but not expected. In our cohort, we described, previously, that the TT genotype represents only 0,90% of the control group in contrast to 10.34% in fibrotic HP patients (Gonçalves, 2023).

During the ultrastructural analysis, the differences between genotypes were not noticeable, as shown in Figure 9A, the cells from no ILD controls presented the same features already described, as well as the HP patient's cells. However, when the quantified cell structures were organized by *MUC5B* genotype a tendency for the presence of dysfunctional double membrane organelles in higher number and area in the homozygote genotype TT was observed, as shown in the graphs from Figure 9B and Figure 9C. The area of preserved mitochondria only presented differences between cases and controls, as already shown before. However, the number of mitochondria seems to be higher in fHP patients carrying the *MUC5B* minor allele (T) (Figure 9B and 9C). The presence of dysfunctional double membrane organelles seems to be associated with the presence of the *MUC5B* minor allele (T) in HP patients, however without reaching statistical significance. Bigger sample size would be required to further validate *MUC5B* genotype with any of the ultrastructural alterations described above for the fibrotic HP patients. Altogether, the quantification of preserved mitochondrias or dysfunctional double membrane organelles according to *MUC5B* genotype revealed mild differences.

However, the most pronounced disparities remained between the fHP patients and the no ILD control group.

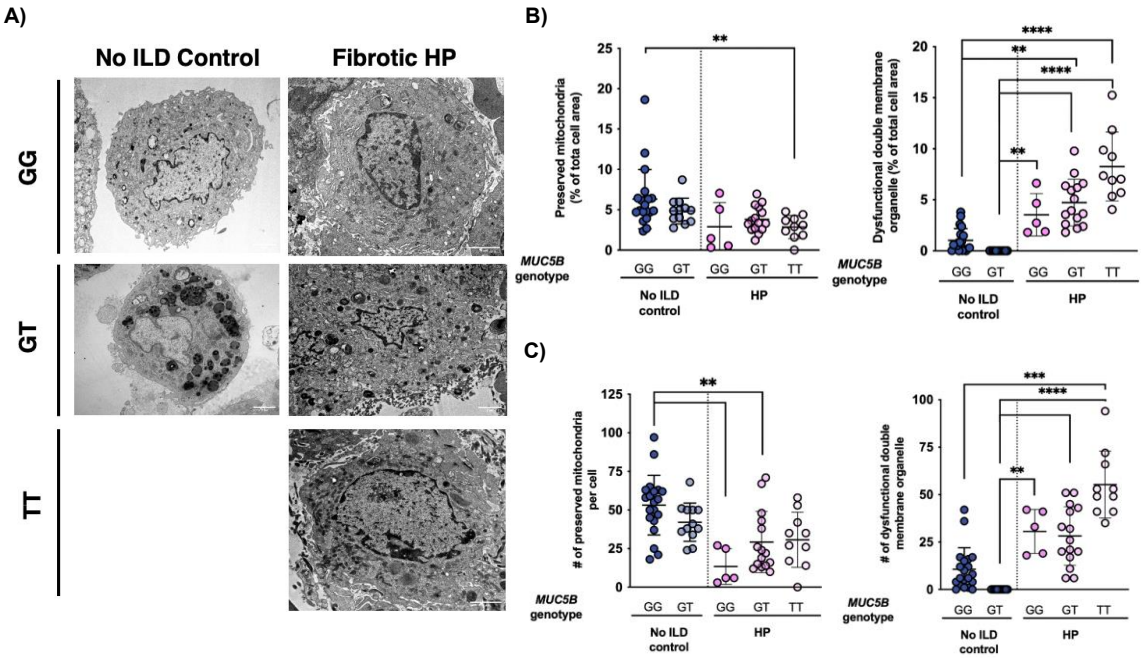

Figure 9. Stratification of fibrotic hypersensitivity pneumonitis (HP) patients by the rs35705950 genotype of the *MUC5B* gene shows a tendency toward increased dysfunctional double membrane organelles in patients carrying the *MUC5B* minor allele (T). Lung tissue obtained through lung cryo-biopsies and surgical resections was analyzed using transmission electron microscopy. **A)** Representative images of cells stratified by the rs35705950 genotype of the *MUC5B* gene (GG, GT, TT). Scale bar: 2 µm. **B)** Quantitative analysis of the percentage of the total cell area occupied by preserved mitochondria (left graph) and dysfunctional double membrane organelles (right graph) in no ILD controls (blue circles) and fibrotic HP patients (pink circles), stratified by *MUC5B* rs35705950 genotype. **C)** Number of the same structures per cell. Each dot represents one cell (5 to 10 cells per case). Values are shown as mean ± SD. Numbers of individuals in each group: no ILD control GG (n = 3), GT (n = 1); fibrotic HP GG (n = 1), GT (n = 2), TT (n = 1). Data were analyzed using the Kruskal-Wallis one-way ANOVA test comparing the different no ILD controls and fibrotic HP groups, **P < 0.01, ***P < 0.005, ****P < 0.001.

4. Conclusion and Future Perspectives

Considering the higher prevalence of HP in Portugal, especially associated with the fibrotic phenotype, which is considered the worst prognosis for the disease, previous studies have indicated that the macrophages may have an increased activity in the pathogenesis of HP disease. In this study, we aimed to deeply analyse the ultrastructure of this cell type and understand if macrophages from fibrotic HP patients display ultrastructural alterations that justify the previous results when compared to no ILD control cells.

The categorization of the dysfunctional double membrane remains unclear, so further experiments are necessary to identify them with accuracy. In the future, to explore more these results, we can analyze and quantify the number of pseudopods, optimize an immunocytochemistry or immunogold labeling technique to detect antigens associated with structures that we hypothesize and improve the flow cytometry staining in order to get more information in live cells.

The differences in the cell ultrastructure were notable between the two conditions, specifically in the mitochondria number and in the presence of dysfunctional double membrane organelles. The presence of these organelles seems to be higher in patients carrying the *MUC5B* minor allele (T), however, to confirm this tendency we need to increase the sample size in the future.

The macrophages seem to play a key role in the fibrotic HP pathogenesis, although, the mechanism and their function are not yet fully understood. Further studies of this cell type can provide valuable insights and shed light on the pathogenesis of hypersensitivity pneumonitis.

5. References

Alberts B, J. A., Lewis J, et al. (2002). The Mitochondrion. In *Molecular Biology of the Cell. 4th edition.* Garland Science. https://www.ncbi.nlm.nih.gov/books/NBK26894/

Bain, C. C., & MacDonald, A. S. (2022). The impact of the lung environment on macrophage development, activation and function: diversity in the face of adversity. *Mucosal Immunology*, *15*(2), 223-234. https://doi.org/10.1038/s41385-021-00480-w

Barnes, H., Troy, L., Lee, C. T., Sperling, A., Strek, M., & Glaspole, I. (2022). Hypersensitivity pneumonitis: Current concepts in pathogenesis, diagnosis, and treatment. *Allergy*, *77*(2), 442-453. https://doi.org/10.1111/all.15017

Barrera, L., Mendoza, F., Zuñiga, J., Estrada, A., Zamora, A. C., Melendro, E. I., Ramírez, R., Pardo, A., & Selman, M. (2008). Functional Diversity of T-Cell Subpopulations in Subacute and Chronic Hypersensitivity Pneumonitis. *American Journal of Respiratory and Critical Care Medicine*, *177*(1), 44-55. https://doi.org/10.1164/rccm.200701-093oc

Baumgartner, K. B., Samet, J. M., Stidley, C. A., Colby, T. V., & Waldron, J. A. (1997). Cigarette smoking: a risk factor for idiopathic pulmonary fibrosis. *American Journal of Respiratory and Critical Care Medicine*, *155*(1), 242-248. https://doi.org/10.1164/ajrccm.155.1.9001319

Behr, J., Prasse, A., Kreuter, M., Johow, J., Rabe, K. F., Bonella, F., Bonnet, R., Grohe, C., Held, M., Wilkens, H., Hammerl, P., Koschel, D., Blaas, S., Wirtz, H., Ficker, J. H., Neumeister, W., Schönfeld, N., Claussen, M., Kneidinger, N.,…Waschki, B. (2021). Pirfenidone in patients with progressive fibrotic interstitial lung diseases other than idiopathic pulmonary fibrosis (RELIEF): a double-blind, randomised, placebo-controlled, phase 2b trial. *The Lancet Respiratory Medicine*, *9*(5), 476-486. https://doi.org/10.1016/s2213-2600(20)30554-3

Blanchet, M.-R., Israël-Assayag, E., & Cormier, Y. (2004). Inhibitory Effect of Nicotine on Experimental Hypersensitivity Pneumonitis In Vivo and In Vitro *American Journal of Respiratory and Critical Care Medicine*, *169*(8), 903-909. https://doi.org/10.1164/rccm.200210-1154oc

Braga, T. T., Agudelo, J. S. H., & Camara, N. O. S. (2015). Macrophages During the Fibrotic Process: M2 as Friend and Foe. *Frontiers in Immunology*, *6*. https://doi.org/10.3389/fimmu.2015.00602

Cabrera, S., Rodríguez-Bobadilla, C., Vázquez-Morales, D., Gaxiola, M., Maciel, M., Selman, M., & Pardo, A. (2020). Identification of Autophagy-related Proteins in Lungs From Hypersensitivity Pneumonitis Patients. *Journal of Histochemistry & Cytochemistry*, *68*(6), 365-376. https://doi.org/10.1369/0022155420932103

Conrad, R., & Narayan, K. (2023). Instance segmentation of mitochondria in electron microscopy images with a generalist deep learning model trained on a diverse dataset. *Cell Systems*, *14*(1), 58-71.e55. https://doi.org/10.1016/j.cels.2022.12.006

Conway, B., & Hughes, J. (2012). Cellular orchestrators of renal fibrosis. *QJM*, *105*(7), 611-615. https://doi.org/10.1093/qjmed/hcr235

Costabel, U., Miyazaki, Y., Pardo, A., Koschel, D., Bonella, F., Spagnolo, P., Guzman, J., Ryerson, C. J., & Selman, M. (2020). Hypersensitivity pneumonitis. *Nature Reviews Disease Primers*, *6*(1). https://doi.org/10.1038/s41572-020-0191-z

Cottin, V. (2019). Treatment of progressive fibrosing interstitial lung diseases: a milestone in the management of interstitial lung diseases. *European Respiratory Review*, *28*(153), 190109. https://doi.org/10.1183/16000617.0109-2019

Cottin, V., Wollin, L., Fischer, A., Quaresma, M., Stowasser, S., & Harari, S. (2019). Fibrosing interstitial lung diseases: knowns and unknowns. *European Respiratory Review*, *28*(151), 180100. https://doi.org/10.1183/16000617.0100-2018

Dabiri, M., Jehangir, M., Khoshpouri, P., & Chalian, H. (2022). Hypersensitivity Pneumonitis: A Pictorial Review Based on the New ATS/JRS/ALAT Clinical Practice Guideline for Radiologists and Pulmonologists. *Diagnostics*, *12*(11), 2874. https://doi.org/10.3390/diagnostics12112874

Evren, E., Ringqvist, E., & Willinger, T. (2020). Origin and ontogeny of lung macrophages: from mice to humans. *Immunology*, *160*(2), 126-138. https://doi.org/10.1111/imm.13154

Fernández Pérez, E. R., Kong, A. M., Raimundo, K., Koelsch, T. L., Kulkarni, R., & Cole, A. L. (2018). Epidemiology of Hypersensitivity Pneumonitis among an Insured Population in the United States: A Claims-based Cohort Analysis. *Annals of the*

American Thoracic Society, *15*(4), 460-469. https://doi.org/10.1513/annalsats.201704-288oc

Fernández Pérez, E. R., Travis, W. D., Lynch, D. A., Brown, K. K., Johannson, K. A., Selman, M., Ryu, J. H., Wells, A. U., Tony Huang, Y.-C., Pereira, C. A. C., Scholand, M.-B., Villar, A., Inase, N., Evans, R. B., Mette, S. A., & Frazer-Green, L. (2021). Diagnosis and Evaluation of Hypersensitivity Pneumonitis. *Chest*, *160*(2), e97-e156. https://doi.org/10.1016/j.chest.2021.03.066

Flaherty, K. R., Wells, A. U., Cottin, V., Devaraj, A., Walsh, S. L. F., Inoue, Y., Richeldi, L., Kolb, M., Tetzlaff, K., Stowasser, S., Coeck, C., Clerisme-Beaty, E., Rosenstock, B., Quaresma, M., Haeufel, T., Goeldner, R.-G., Schlenker-Herceg, R., & Brown, K. K. (2019). Nintedanib in Progressive Fibrosing Interstitial Lung Diseases. *New England Journal of Medicine*, *381*(18), 1718-1727. https://doi.org/10.1056/nejmoa1908681

Gonçalves, Mellany (2023). *Vias moleculares comuns ligadas às doenças pulmonares intersticiais com fenótipo fibrosante progressivo: o papel da variante promotora MUC5B* [Tese Mestrado, Instituto Politécnico do Porto]

Guilliams, M., De Kleer, I., Henri, S., Post, S., Vanhoutte, L., De Prijck, S., Deswarte, K., Malissen, B., Hammad, H., & Lambrecht, B. N. (2013). Alveolar macrophages develop from fetal monocytes that differentiate into long-lived cells in the first week of life via GM-CSF. *Journal of Experimental Medicine*, *210*(10), 1977-1992. https://doi.org/10.1084/jem.20131199

Hashimoto, D., Chow, A., Noizat, C., Teo, P., Mary, Leboeuf, M., Christian, See, P., Price, J., Lucas, D., Greter, M., Mortha, A., Scott, E, Tanaka, M., Nico, García-Sastre, A., E, Ginhoux, F.,…Merad, M. (2013). Tissue-Resident Macrophages Self-Maintain Locally throughout Adult Life with Minimal Contribution from Circulating Monocytes. *Immunity*, *38*(4), 792-804. https://doi.org/10.1016/j.immuni.2013.04.004

Hossler, F. E. (2014). Ultrastructure Atlas of Human Tissues. https://doi.org/10.1002/9781118284551

Hunninghake, G. M., Hatabu, H., Okajima, Y., Gao, W., Dupuis, J., Latourelle, J. C., Nishino, M., Araki, T., Zazueta, O. E., Kurugol, S., Ross, J. C., San José Estépar, R., Murphy, E., Steele, M. P., Loyd, J. E., Schwarz, M. I., Fingerlin, T. E., Rosas,

I. O., Washko, G. R.,…Schwartz, D. A. (2013). MUC5B Promoter Polymorphism and Interstitial Lung Abnormalities. *New England Journal of Medicine*, *368*(23), 2192-2200. https://doi.org/10.1056/nejmoa1216076

Hyldgaard, C., Hilberg, O., Muller, A., & Bendstrup, E. (2014). A cohort study of interstitial lung diseases in central Denmark. *Respiratory Medicine*, *108*(5), 793-799. https://doi.org/10.1016/j.rmed.2013.09.002

Imokawa, S., Sato, A., Hayakawa, H., Kotani, M., Urano, T., & Takada, A. (1997). Tissue Factor Expression and Fibrin Deposition in the Lungs of Patients with Idiopathic Pulmonary Fibrosis and Systemic Sclerosis. *American Journal of Respiratory and Critical Care Medicine*, *156*(2), 631-636. https://doi.org/10.1164/ajrccm.156.2.9608094

Jablonska, E., Guenther, A., Preissner, K., Markart, P., & Wygrecka, M. (2008). Current view on alveolar coagulation and fibrinolysis in acute inflammatory and chronic interstitial lung diseases. *Thrombosis and Haemostasis*, *99*(03), 494-501. https://doi.org/10.1160/th07-11-0666

Karakatsani, A., Papakosta, D., Rapti, A., Antoniou, K. M., Dimadi, M., Markopoulou, A., Latsi, P., Polychronopoulos, V., Birba, G., Ch, L., & Bouros, D. (2009). Epidemiology of interstitial lung diseases in Greece. *Respiratory Medicine*, *103*(8), 1122-1129. https://doi.org/10.1016/j.rmed.2009.03.001

Kaur, A., Mathai, S. K., & Schwartz, D. A. (2017). Genetics in Idiopathic Pulmonary Fibrosis Pathogenesis, Prognosis, and Treatment. *Frontiers in Medicine*, *4*. https://doi.org/10.3389/fmed.2017.00154

Kawasaki, T., Ikegawa, M., & Kawai, T. (2022). Antigen Presentation in the Lung. *Frontiers in Immunology*, *13*. https://doi.org/10.3389/fimmu.2022.860915

Khan, Y. S., & Lynch, D. T. (2023). *Histology, Lung*. StatPearls Publishing, Treasure Island (FL). http://europepmc.org/abstract/MED/30521210

Ku, J. C., Raiten, J., & Li, Y. (2024). Understanding fibrosis: Mechanisms, clinical implications, current therapies, and prospects for future interventions. *Biomedical Engineering Advances*, *7*, 100118. https://doi.org/10.1016/j.bea.2024.100118

Laskin, D. L., Malaviya, R., & Laskin, J. D. (2015). Pulmonary Macrophages. In (pp. 629-649). Elsevier. https://doi.org/10.1016/b978-0-12-404577-4.00032-1

Ley, B., Newton, C. A., Arnould, I., Elicker, B. M., Henry, T. S., Vittinghoff, E., Golden, J. A., Jones, K. D., Batra, K., Torrealba, J., Garcia, C. K., & Wolters, P. J. (2017). The MUC5B promoter polymorphism and telomere length in patients with chronic hypersensitivity pneumonitis: an observational cohort-control study. *The Lancet Respiratory Medicine*, *5*(8), 639-647. https://doi.org/10.1016/s2213-2600(17)30216-3

McSharry, C., Banham, S. W., & Boyd, G. (1985). Effect of cigarette smoking on the antibody response to inhaled antigens and the prevalence of extrinsic allergic alveolitis among pigeon breeders. *Clinical & Experimental Allergy*, *15*(5), 487-494. https://doi.org/10.1111/j.1365-2222.1985.tb02299.x

Meyer, K. C. (2014). Diagnosis and management of interstitial lung disease. *Translational Respiratory Medicine*, *2*(1), 4. https://doi.org/10.1186/2213-0802-2-4

Meyer, K. C., Raghu, G., Baughman, R. P., Brown, K. K., Costabel, U., Du Bois, R. M., Drent, M., Haslam, P. L., Kim, D. S., Nagai, S., Rottoli, P., Saltini, C., Selman, M., Strange, C., & Wood, B. (2012). An Official American Thoracic Society Clinical Practice Guideline: The Clinical Utility of Bronchoalveolar Lavage Cellular Analysis in Interstitial Lung Disease. *American Journal of Respiratory and Critical Care Medicine*, *185*(9), 1004-1014. https://doi.org/10.1164/rccm.201202-0320st

Misharin, A. V., Morales-Nebreda, L., Reyfman, P. A., Cuda, C. M., Walter, J. M., McQuattie-Pimentel, A. C., Chen, C.-I., Anekalla, K. R., Joshi, N., Williams, K. J. N., Abdala-Valencia, H., Yacoub, T. J., Chi, M., Chiu, S., Gonzalez-Gonzalez, F. J., Gates, K., Lam, A. P., Nicholson, T. T., Homan, P. J.,…Perlman, H. (2017). Monocyte-derived alveolar macrophages drive lung fibrosis and persist in the lung over the life span. *Journal of Experimental Medicine*, *214*(8), 2387-2404. https://doi.org/10.1084/jem.20162152

Monteiro, L. D. B., Davanzo, G. G., De Aguiar, C. F., & Moraes-Vieira, P. M. M. (2020). Using flow cytometry for mitochondrial assays. *MethodsX*, *7*, 100938. https://doi.org/10.1016/j.mex.2020.100938

Mota, P. C., Soares, M. L., Ferreira, A. C., Santos, R. F., Rufo, J. C., Vasconcelos, D., Carvalho, A., Guimarães, S., Vasques-Nóvoa, F., Cardoso, C., Melo, N.,

Alexandre, A. T., Coelho, D., Novais-Bastos, H., & Morais, A. (2024). Polymorphisms and haplotypes of TOLLIP and MUC5B are associated with susceptibility and survival in patients with fibrotic hypersensitivity pneumonitis. *Pulmonology*. https://doi.org/10.1016/j.pulmoe.2024.01.002

Murray, P. J. (2017). Macrophage Polarization. *Annual Review of Physiology*, *79*(1), 541-566. https://doi.org/10.1146/annurev-physiol-022516-034339

Nogueira, R., Melo, N., Novais E Bastos, H., Martins, N., Delgado, L., Morais, A., & C. Mota, P. (2019). Hypersensitivity pneumonitis: Antigen diversity and disease implications. *Pulmonology*, *25*(2), 97-108. https://doi.org/10.1016/j.pulmoe.2018.07.003

Ohtsuka, Y., Munakata, M., Tanimura, K., Ukita, H., Kusaka, H., Masaki, Y., Doi, I., Ohe, M., Amishima, M., Homma, Y., & Kawakami, Y. (1995). Smoking Promotes Insidious and Chronic Farmer's Lung Disease, and Deteriorates the Clinical Outcome. *Internal Medicine*, *34*(10), 966-971. https://doi.org/10.2169/internalmedicine.34.966

Palade, G. E. (1953). AN ELECTRON MICROSCOPE STUDY OF THE MITOCHONDRIAL STRUCTURE. *Journal of Histochemistry & Cytochemistry*, *1*(4), 188-211. https://doi.org/10.1177/1.4.188

Podolanczuk, A. J., Wong, A. W., Saito, S., Lasky, J. A., Ryerson, C. J., & Eickelberg, O. (2021). Update in Interstitial Lung Disease 2020. *American Journal of Respiratory and Critical Care Medicine*, *203*(11), 1343-1352. https://doi.org/10.1164/rccm.202103-0559up

Pokhreal, D., Crestani, B., & Helou, D. G. (2023). Macrophage Implication in IPF: Updates on Immune, Epigenetic, and Metabolic Pathways. *Cells*, *12*(17), 2193. https://doi.org/10.3390/cells12172193

Quezada, A. L., Gimpel, S. F., Miranda, D. O., Las Heras, J. B., & Andreis, M. C. (1987). Ultrastructural Features of Alveolar Cells in Experimental Hypersensitivity Pneumonitis. *Respiration*, *51*(2), 127-136. https://doi.org/10.1159/000195179

Raghu, G., Collard, H. R., Egan, J. J., Martinez, F. J., Behr, J., Brown, K. K., Colby, T. V., Cordier, J.-F., Flaherty, K. R., Lasky, J. A., Lynch, D. A., Ryu, J. H., Swigris, J. J., Wells, A. U., Ancochea, J., Bouros, D., Carvalho, C., Costabel, U., Ebina, M.,…Schünemann, H. J. (2011). An Official ATS/ERS/JRS/ALAT Statement:

Idiopathic Pulmonary Fibrosis: Evidence-based Guidelines for Diagnosis and Management. *American Journal of Respiratory and Critical Care Medicine*, *183*(6), 788-824. https://doi.org/10.1164/rccm.2009-040gl

Raghu, G., Remy-Jardin, M., Ryerson, C. J., Myers, J. L., Kreuter, M., Vasakova, M., Bargagli, E., Chung, J. H., Collins, B. F., Bendstrup, E., Chami, H. A., Chua, A. T., Corte, T. J., Dalphin, J.-C., Danoff, S. K., Diaz-Mendoza, J., Duggal, A., Egashira, R., Ewing, T.,…Wilson, K. C. (2020). Diagnosis of Hypersensitivity Pneumonitis in Adults: An Official ATS/JRS/ALAT Clinical Practice Guideline. *American Journal of Respiratory and Critical Care Medicine*, *202*(3), e36-e69. https://doi.org/10.1164/rccm.202005-2032st

Roy, M. G., Livraghi-Butrico, A., Fletcher, A. A., McElwee, M. M., Evans, S. E., Boerner, R. M., Alexander, S. N., Bellinghausen, L. K., Song, A. S., Petrova, Y. M., Tuvim, M. J., Adachi, R., Romo, I., Bordt, A. S., Bowden, M. G., Sisson, J. H., Woodruff, P. G., Thornton, D. J., Rousseau, K.,…Evans, C. M. (2014). Muc5b is required for airway defence. *Nature*, *505*(7483), 412-416. https://doi.org/10.1038/nature12807

Santos, V., Martins, N., Sousa, C., Jacob, M., Padrão, E., Melo, N., Mota, P. C., Bastos, H. N., Guimarães, S., Moura, C. S., Sokhatska, O., Cunha, R., Pereira, J., & Morais, A. (2020). Hypersensitivity pneumonitis: Main features characterization in a Portuguese cohort. *Pulmonology*, *26*(3), 130-137. https://doi.org/10.1016/j.pulmoe.2019.09.004

Sasco, A. J., Secretan, M. B., & Straif, K. (2004). Tobacco smoking and cancer: a brief review of recent epidemiological evidence. *Lung Cancer*, *45*, S3-S9. https://doi.org/10.1016/j.lungcan.2004.07.998

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, *9*(7), 671-675. https://doi.org/10.1038/nmeth.2089

Seibold, M. A., Wise, A. L., Speer, M. C., Steele, M. P., Brown, K. K., Loyd, J. E., Fingerlin, T. E., Zhang, W., Gudmundsson, G., Groshong, S. D., Evans, C. M., Garantziotis, S., Adler, K. B., Dickey, B. F., Du Bois, R. M., Yang, I. V., Herron, A., Kervitsky, D., Talbert, J. L.,…Schwartz, D. A. (2011). A Common *MUC5B*

Promoter Polymorphism and Pulmonary Fibrosis. *New England Journal of Medicine*, *364*(16), 1503-1512. https://doi.org/10.1056/nejmoa1013660

Shi, T., Denney, L., An, H., Ho, L.-P., & Zheng, Y. (2021). Alveolar and lung interstitial macrophages: Definitions, functions, and roles in lung fibrosis. *Journal of Leukocyte Biology*, *110*(1), 107-114. https://doi.org/10.1002/jlb.3ru0720-418r

Singh, S., Collins, B., Sharma, B., Joshi, J., Talwar, D., Katiyar, S., Singh, N., Ho, L., Samaria, J., Bhattacharya, P., Chaudhari, S., Singh, T., Pilania, K., Pipavath, S., Ahuja, J., Chetambath, R., Ghoshal, A., Jain, N., Gayathri Devi, H.,…Raghu, G. (2019). Hypersensitivity pneumonitis: Clinical manifestations – Prospective data from the interstitial lung disease-India registry. *Lung India*, *36*(6), 476. https://doi.org/10.4103/lungindia.lungindia_263_19

Suzuki, T., Chow, C., & Downey, G. (2008). Role of innate immune cells and their products in lung immunopathology. *The International Journal of Biochemistry & Cell Biology*, *40*(6-7), 1348-1361. https://doi.org/10.1016/j.biocel.2008.01.003

Swigris, J. J., Stewart, A. L., Gould, M. K., & Wilson, S. R. (2005). Patients' perspectives on how idiopathic pulmonary fibrosis affects the quality of their lives. *Health and Quality of Life Outcomes*, *3*(1), 61. https://doi.org/10.1186/1477-7525-3-61

Tsitoura, E., Vasarmidi, E., Bibaki, E., Trachalaki, A., Koutoulaki, C., Papastratigakis, G., Papadogiorgaki, S., Chalepakis, G., Tzanakis, N., & Antoniou, K. M. (2019). Accumulation of damaged mitochondria in alveolar macrophages with reduced OXPHOS related gene expression in IPF. *Respiratory Research*, *20*(1). https://doi.org/10.1186/s12931-019-1196-6

Van Den Bosch, L., Luppi, F., Ferrara, G., & Mura, M. (2022). Immunomodulatory treatment of interstitial lung disease. *Therapeutic Advances in Respiratory Disease*, *16*, 175346662211170. https://doi.org/10.1177/17534666221117002

Vasakova, M., Morell, F., Walsh, S., Leslie, K., & Raghu, G. (2017). Hypersensitivity Pneumonitis: Perspectives in Diagnosis and Management. *American Journal of Respiratory and Critical Care Medicine*, *196*(6), 680-689. https://doi.org/10.1164/rccm.201611-2201pp

Vasakova, M., Selman, M., Morell, F., Sterclova, M., Molina-Molina, M., & Raghu, G. (2019). Hypersensitivity Pneumonitis: Current Concepts of Pathogenesis and

Potential Targets for Treatment. *American Journal of Respiratory and Critical Care Medicine*, *200*(3), 301-308. https://doi.org/10.1164/rccm.201903-0541pp

Vourlekis, J. S., Schwarz, M. I., Cherniack, R. M., Curran-Everett, D., Cool, C. D., Tuder, R. M., King, T. E., & Brown, K. K. (2004). The effect of pulmonary fibrosis on survival in patients with hypersensitivity pneumonitis. *The American Journal of Medicine*, *116*(10), 662-668. https://doi.org/10.1016/j.amjmed.2003.12.030

Wijsenbeek, M., Suzuki, A., & Maher, T. M. (2022). Interstitial lung diseases. *The Lancet*, *400*(10354), 769-786. https://doi.org/10.1016/s0140-6736(22)01052-2

Wuyts, W., Sterclova, M., & Vasakova, M. (2015). Pitfalls in diagnosis and management of hypersensitivity pneumonitis. *Current Opinion in Pulmonary Medicine*, *21*(5), 490-498. https://doi.org/10.1097/mcp.0000000000000199

Yang, I. V., Fingerlin, T. E., Evans, C. M., Schwarz, M. I., & Schwartz, D. A. (2015). MUC5B and Idiopathic Pulmonary Fibrosis. *Annals of the American Thoracic Society*, *12*(Supplement 2), S193-S199. https://doi.org/10.1513/annalsats.201503-110aw

Yona, S., Kim, K.-W., Wolf, Y., Mildner, A., Varol, D., Breker, M., Strauss-Ayali, D., Viukov, S., Guilliams, M., Misharin, A., David, Perlman, H., Malissen, B., Zelzer, E., & Jung, S. (2013). Fate Mapping Reveals Origins and Dynamics of Monocytes and Tissue Macrophages under Homeostasis. *Immunity*, *38*(1), 79-91. https://doi.org/10.1016/j.immuni.2012.12.001

You, R., Lu, W., Shan, M., Berlin, J. M., Samuel, E. L. G., Marcano, D. C., Sun, Z., Sikkema, W. K. A., Yuan, X., Song, L., Hendrix, A. Y., Tour, J. M., Corry, D. B., & Kheradmand, F. (2015). Nanoparticulate carbon black in cigarette smoke induces DNA cleavage and Th17-mediated emphysema. *eLife*, *4*, e09623. https://doi.org/10.7554/eLife.09623

Zhang, L., Wang, Y., Wu, G., Xiong, W., Gu, W., & Wang, C.-Y. (2018). Macrophages: friend or foe in idiopathic pulmonary fibrosis? *Respiratory Research*, *19*(1). https://doi.org/10.1186/s12931-018-0864-2