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Krebs von den Lungen-6 as biomarker of the new progressive fibrotic phenotype of Interstitial Lung Disease

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1. Interstitial Lung Diseases (ILD)

Interstitial lung diseases (ILD) are an heterogenous group of nosological and non-neoplastic entities (1). They are diffuse infiltrative lung disease caused by a variable degree of pulmonary inflammation and epithelial dysfunction that can evolve into irreversible fibrosis responsible for respiratory failure (2). There are four distinct groups of interstitial lung diseases based on etiopathogenesis in the Consensus Statement of the American Thoracic Society and European Respiratory Society (ATS/ERS) (figure 1): known cause: secondary to collagenopathies, exposure to drugs, irritants, environmental or professional ; interstitial idiopathic pneumonia (IIP); granulomatous diseases of lung ; others form characterized by specific morphology such as lymphangioleiomyomatosis (LAM), Langherans cell histiocytosis and eosinophilic pneumonia (1).

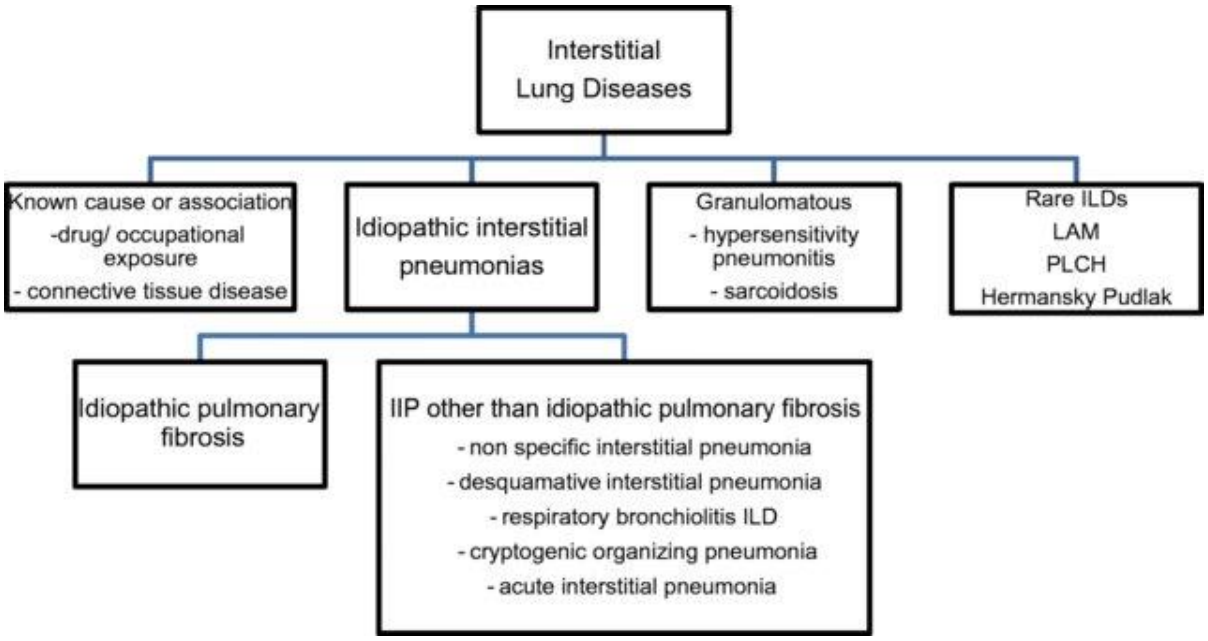


Figure 1. Interstitial lung diseases classification

In 2013, the update of the multidisciplinary classification of ILDs proposed by the ATS/ERS focused attention on the revision of idiopathic forms that were divided according to the frequency of manifestation in: major, rare and unclassifiable (2). The major ones, in turn, were divided according to the clinic into: chronic, acute/subacute and smoke-related.

TABLE 1. REVISED AMERICAN THORACIC SOCIETY/EUROPEAN RESPIRATORY SOCIETY CLASSIFICATION OF IDIOPATHIC INTERSTITIAL PNEUMONIAS: MULTIDISCIPLINARY DIAGNOSES

Major idiopathic interstitial pneumonias
Idiopathic pulmonary fibrosis
Idiopathic nonspecific interstitial pneumonia
Respiratory bronchiolitis–interstitial lung disease
Desquamative interstitial pneumonia
Cryptogenic organizing pneumonia
Acute interstitial pneumonia
Rare idiopathic interstitial pneumonias
Idiopathic lymphoid interstitial pneumonia
Idiopathic pleuroparenchymal fibroelastosis
Unclassifiable idiopathic interstitial pneumonias*

*Causes of unclassifiable idiopathic interstitial pneumonia include (*1*) inadequate clinical, radiologic, or pathologic data and (*2*) major discordance between clinical, radiologic, and pathologic findings that may occur in the following situations: (*a*) previous therapy resulting in substantial alteration of radiologic or histologic findings (e.g., biopsy of desquamative interstitial pneumonia after steroid therapy, which shows only residual nonspecific interstitial pneumonia [153]); (*b*) new entity, or unusual variant of recognized entity, not adequately characterized by the current American Thoracic Society/European Respiratory Society classification (e.g., variant of organizing pneumonia with supervening fibrosis) (79); and (*c*) multiple high-resolution computed tomography and/or pathologic patterns that may be encountered in patients with idiopathic interstitial pneumonia.

TABLE 2. CATEGORIZATION OF MAJOR IDIOPATHIC INTERSTITIAL PNEUMONIAS

Category	Clinical–Radiologic–Pathologic Diagnoses	Associated Radiologic and/or Pathologic–Morphologic Patterns
Chronic fibrosing IP	Idiopathic pulmonary fibrosis	Usual interstitial pneumonia
	Idiopathic nonspecific interstitial pneumonia	Nonspecific interstitial pneumonia
Smoking-related IP*	Respiratory bronchiolitis-interstitial lung disease	Respiratory bronchiolitis
	Desquamative interstitial pneumonia	Desquamative interstitial pneumonia
Acute/subacute IP	Cryptogenic organizing pneumonia	Organizing pneumonia
	Acute interstitial pneumonia	Diffuse alveolar damage

Definition of abbreviation: IP = interstitial pneumonia.
*Desquamative interstitial pneumonia can occasionally occur in nonsmokers.

Figure 2 Update of classification ILD ATS/ERS 2013

There are a variety of different types of idiopathic interstitial pneumonias (IIPs) and they all affect the pulmonary interstitium, the space between the epithelium and the endothelium of the lungs' basal membranes (3). The pulmonary functional alterations demonstrate a restrictive impairment due to the reduced expansion of total pulmonary capacity (4). Patients usually refer progressive exertional dyspnoea, tachypnea and chronic dry cough, while the thoracic examination is characterized by bibasal Velcro-type crackles with no wheezing and other evidence of obstruction. Other characteristics are the reduction of carbon monoxide diffusion capacity (DLCO), lung volume and compliance during respiratory function tests (1).

Radiologically, ILD pattern can be really heterogeneous: the main pattern may be characterized by nodules, reticulation, ground glass opacities, or interstitial thickening of inter and intralobular septa that may lead to the development of honeycombing areas on high resolution computed tomography (HRCT) scan of chest (5).

Complications in the advanced stage are the secondary pulmonary hypertension and right-sided heart failure with cor pulmonare. The advanced stages are difficult to distinguished, due to scars and massive destruction of the lung, with end-stage terminal fibrosis (6).

2. Idiopathic pulmonary fibrosis (IPF)

2.1 Definition

Chronic IIP characterized by progressive lung disease occurring in older adults. IPF is a chronic form of IIP. It is not fully understood why it occurs.

The histopathologic pattern of IPF is typical of interstitial pneumonia (UIP), a condition characterized by progressive worsening dyspnea and decreased lung function with a poor prognosis. A series of guidelines for the diagnosis and treatment of IPF were developed in 2011 by the American Thoracic Society, European Respiratory Society, Japanese Respiratory Society, and Latin American Thoracic Society (ALAT) (7). As a consequence of limitations in the clinical practice of these guidelines, the diagnostic criteria for IPF have been updated based on radiologic and histologic findings (6).

Patients with interstitial lung disease are usually diagnosed with IPF in 20-30% of cases. According to the latest incidence estimates, it is the most common idiopathic interstitial pneumonia in Asia-Pacific countries, 0.09 to 0.49 in Europe, and 0.75 to 0.93 in North America (4). As a result, the number of patients is increasing globally, due to an aging population, an increased awareness of the disease, the development of more accurate diagnostic instruments, and other factors. IPF most commonly affects males between the ages of 65 and 70 years, and smokers are more likely to suffer from the disease (4).

IPF usually has a sporadic occurrence, but occasionally can be occur in familial form. About 5% of IPF patients have familial IPF(f-IPF) (8). Both forms, sporadic and familial, are clinically and histologically indistinguishable. The sporadic IPF (s-IPF) is characterized by defined clinical features and complication, otherwise the outcome of familial IPF (f-IPF) are still undefined. The f-IPF tends to occur early, about 55 year. It is usually defined as the “presence of pulmonary fibrosis realized in at least two relatives within the same family”. It has a frequency between 0.5 to 9.5% of all IPF patients (8). The genetic association between IPF and shelterin or telomerase complex mutations has been studied extensively. It has been found that telomerase length plays a significant role in the development of IPF, a factor that is influenced by advancing age. This shortening appears to be present in 15% of patients with f-IPF and 10% of patients with s-IPF. Additionally, the most common mutated gene identified in patients with a f-IPF is TERT (telomerase reverse transcriptase) gene, which encodes the portion of telomerase. The surfactant protein C gene (SFTPC) as well as the surfactant protein A2 gene

(SFTPA) are other genes responsible for the transmission of f-IPF (9). These genetic predispositions, associated with other risk factors, such as outdoor pollution and cigarette smoke, could have an important impact on the risk to develop lung-cancer. Alveolar epithelial cells can also be affected by senescence processes and genetic predisposition, in an analogous process to the process of cancer (10).

2.2 Etiopathogenesis

IPF's etiopathogenesis remains unclear. Inflammatory cells were seen in bronchoalveolar lavages of IPF patients, triggering the initial pathogenic hypothesis (11). After abandoning this hypothesis, it was assumed that an external agent could be to blame for the development of final fibrosis, causing damage to epithelial cells and causing abnormal restorative processes to occur on the alveolar walls (12). A persistent or repeated harmful stimulus is believed to be the cause of IPF even though the exact etiopathogenic mechanism is unclear.

A plausible sequence of events leading to pulmonary fibrosis can be proposed based on recent advances in mechanical understanding of IPF pathogenesis. Pathophysiologically, these events can be divided into three stages (fig.3). A predisposition to lung fibrosis is influenced by genetic mutations, environmental exposures, and old age. In the initiation stage, profibrotic processes are activated, fibrocytes are recruited, epithelial-to-mesenchymal transitions (EMT) are completed, and unfolded protein responses are activated. The final progression stage includes (13):

- pathologic fibroblasts are a molecular process that causes fibrosis
- Degeneration, matrix deposition, matrix remodeling, stiffening of the matrix, and differentiation
- fibroblasts and epithelial cells undergo profibrotic epigenetic changes.

Figure 3. Three pathophysiologic stages of IPF

In the last years, it was demonstrated the association with different gene polymorphism, such as the polymorphism of gene that encodes TNF, for the IL-1 receptor antagonist, for surfactant proteins A and B, and for the receptor 1. Another important role in its pathogenesis is played by environmental factors (14). Several studies were performed about various infectious agents including Epstein-Barr virus, influenza A virus, HCV, parainfluenza viruses 1 and 3, HIV and HSV6, but without obtaining any confirmation. The role of non-infectious environmental factors, for example, inhalation of organic and inorganic dust (metals, wood, solvents, etc.) has also been hypothesized. There was an increase in the number of alveolar epithelial cells (AECs) in the lung as a result of these nonspecific insults. These insults have altered the epithelial barrier as well as the parenchyma, making them more susceptible to microinjuries. The alveolar epithelial cells type II (AEC2s) contribute to regeneration of alveolar epithelial cells type I (AEC1s), and in the homeostatic process or as a results of lung damage. In addition to chemoattractant factors, activated AEC release growth factors that promote epithelization. In addition to transforming growth factor, platelet-derived growth factor (PDGF), insulin-like growth factor (ILGF)-1, and endothelin (ET)-1, there are also other growth factors. In IPF, there is a duplication and fragmentation of the basal membrane that appears to not be reepithelializing the alveolar space (15,16).

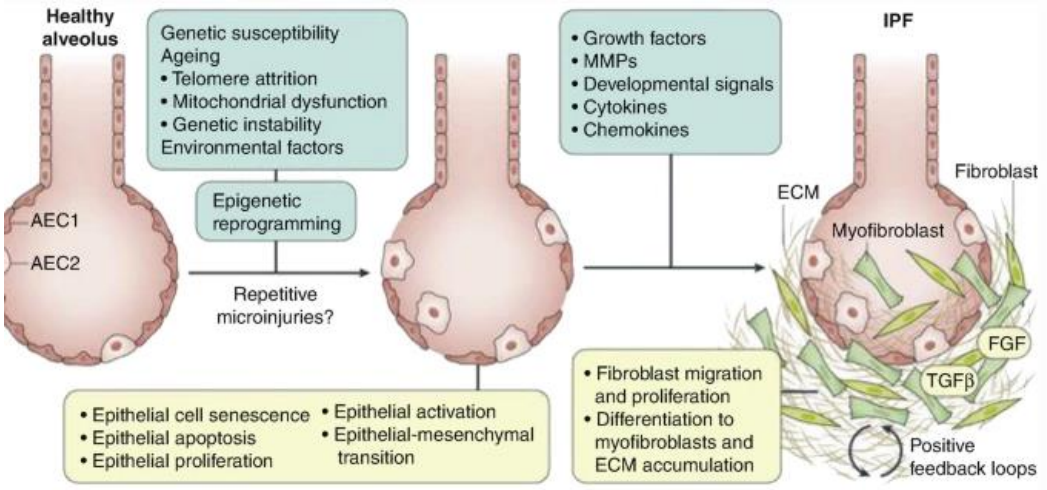


Figure 4. Pathogenic model of IPF

When epithelial cells are attached to the epithelium, they do not separate and leave the epithelium. The epithelial-mesenchymal transition (EMT) can upset this arrangement following

an injury. A mesenchymal cell's characteristics differ from those of an epithelial cell during EMT. Their apical-basal polarity is lost, they become migratory, and they express mesenchymal molecules, including fibronectin and N-cadherin (17), rather than cell adhesion molecules (17).

When epithelial cells are injured (by oxidative stress, Fas activation, and angiotensin II), some of the products (such as TGF-1, oxidative stress, and Fas activation) stimulate fibroblasts to make extracellular membrane components, notably collagen, which may affect the epithelial/endothelial barrier's repair. When epithelial cells are injured (by oxidative stress, Fas activation, and angiotensin II), some of the products (such as TGF-1, oxidative stress, and Fas activation) stimulate fibroblasts to make extracellular membrane components, notably collagen, which may affect the epithelial/endothelial barrier's repair. TGF is produced as a result of cytoskeletal contractions induced by unfolded protein response (UPR) following αvβ6 integrin activation. It is considered as a strong proapoptotic mediator able to promote both the fibroblast activation, proliferation and transformation into myofibroblasts. Activated fibroblasts and myofibroblasts in fibroblastic foci serve as the major effector cells in fibrogenesis. Following the damaged area, mesenchymal cells infiltrate wounds, differentiate in myofibroblast. It is the highly contractile myofibroblasts that become resistant to apoptosis which cause the lung interstitium to accumulate ECM components such as fibrillar collagens, fibronectin, tenascin, and proteoglycans (18).

Providing cellular structure and a dynamic role in signaling, the ECM is a highly organized structure. Lung fibroblasts are affected by the alteration in transcriptional regulation of COLA1, COLA2, COL3A1, COL5A2, COL4A2, MMP2, MMP3, MMP10, and TIMP2. A structural deregulation of the ECM caused fibrous and connective tissue growth due to unregulated production of growth factors, cytokines, and secretion of atypical matrix components. Desmoplasia (growth of fibrous and connective tissue) is the result of this structural deregulation. By damaging epithelial, endothelial, and smooth muscle cells, an inflammatory mediator cascade is released. The mediators promote platelet formation, vasodilation, stimulation of collagen-producing fibroblasts, and metalloproteinases that degrade and reorganize the ECM. Cells can detect the mechanical characteristics of external tissues by contracting bundles of F-actin (stress fibers) through myosin-motor activity (19).

Fibrotic circuits are characterized by the ability of cells to sense forces outside their own tissues and apply corresponding forces to the extracellular matrix to stiffen it.

2.3 Risk factors

Although IPF is a disease with unknown aetiology, a number of potential risk factors have been described by the guidelines ATS/ERS/JRS/ALAT 2011:

- A stronger association can be observed in smokers, particularly those with a smoking history of more than 20 pack-years. Smoking: the association is stronger in smokers and those who have smoked for a long period of time. Due to the increased state of inflammation, IPF patients who were smokers had lower total cell counts, but higher and more severe alveolar space counts. Thus, macrophages accumulate in response to these factors. The rate at which lung function declines over the course of a lifetime is also associated with tobacco smoking (20).

The mechanisms by which tobacco smoking influences IPF onset and progression are not fully understood.

- There has been evidence of an increased risk of cancer following exposure to metal ducts (brass, lead, and steel) and wood dust (pine). Metallurgy, farming, textile work, welding, veterinarians, and others are some of the professions in which such pollutants are commonly found. These findings have been supported by the detection of organic particles in lymph nodes of patients with pulmonary fibrosis. As a consequence of the organic constituents in polluted air, abnormal immune responses can occur that can trigger inflammation, epithelial damage, and eventually, fibrosis. Depending on the amount, size, and concentration of crystalline silica inhaled and the individual's susceptibility to the particles, the disease risk varies over a lifetime (21).

- Age-related factors contribute significantly to IPF incidence and severity, suggesting aging is a major risk factor. A high mortality rate is also positively correlated with the older age of the patient at diagnosis as well as the increased number of people over 65 over the past few decades. Even though the mechanism responsible for ageing-susceptibly remains unclear, cellular senescence, a permanent halt to cell growth, has been shown to play an increasingly important role in IPF development. (18).

- Gender: The prevalence and incidence of this disease are higher in the male sex, whereas several studies suggest that the disease progresses more slowly and that the female sex has a better chance of survival (20).

• Microbial agents: The possible role of chronic viral infection and other microbes in the etiology of IPF has been investigated. However the role of virus infections, caused by EBV, CMV, HCV, HS, was not achieving conclusive results (21).

Nevertheless, numerous studies have demonstrated that bacteria can cause lung epithelial cell injuries by causing chronic, low-level antigenic stimulation to induce a host immune response or activate phlogosis mediators. As a result of this persistent host response, fibroblast responsiveness may be affected, suggesting that lung microbiota may contribute to repetitive alveolar injury in IPF.

• There is a possibility that gastroesophageal reflux contributes to IPF through silent aspiration of refluxate into the airway or microaspiration. In a meta-analysis of eight observational studies, acid suppression therapy was found to decrease the mortality rate associated with IPF by causing injury, triggering inflammatory cascades, and ultimately resulting in fibrotic changes to the lungs (22).

• Genetic factors: The various genetic polymorphisms identified for sporadic IPF have not been validated as predictors of the disease. In spite of this, the most consistently associated gene variant in the promoter of the MUC5B (mucin 5B) gene has been found to increase the risk of developing IPF by up to 30% (23).

2.4 Clinical manifestation and prognosis

The clinical course of IPF is sneaky, characterized by a rapid progression, sometimes by a slow onset of symptoms that become progressively worse over time, or also by episodes of sudden worsening following periods of relative stability (Figure 5) (24).

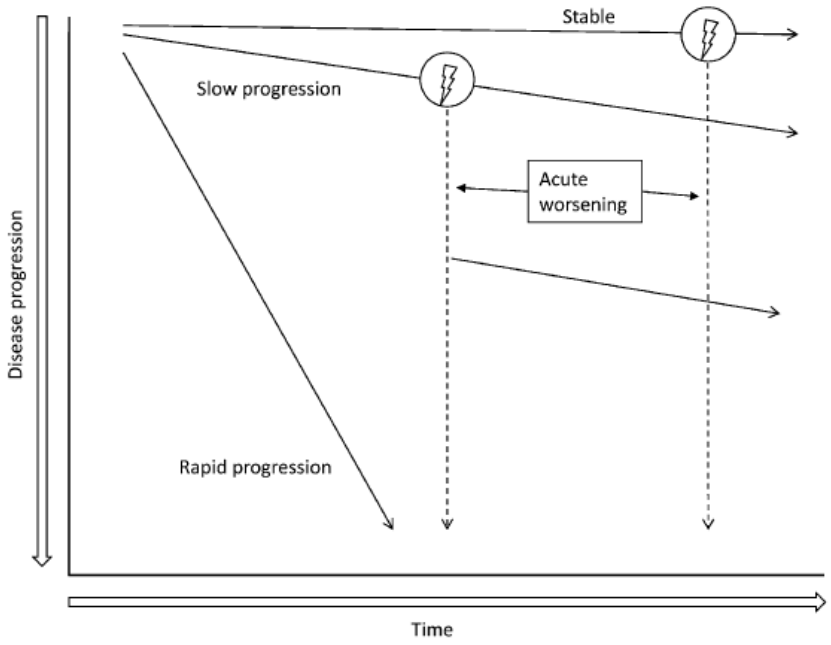


Figure 5. Disease progression

Some of these comorbidities have a negative prognostic impact as demonstrated by pulmonary hypertension and lung cancer, as the natural history of the disease is highly heterogeneous, influenced by clinical or subclinical manifestations and comorbidities. Currently, the IPF guidelines recommend several prognostic factors associated with increased mortality risk and useful to track the progression of the disease (POD):

Baseline	Longitudinal
Dyspnea §	Worsenig of dyspnea§
DLCO ≤ 40% of theorical	FVC ≤ 10%, absolute value*
Spo02≤ 88% a7 6MWT	DCO ≤ 15%, absolute valute*

Extending fibrosis at HRCT	Greater extension of fibrosis at HRCT*
pulmonary hypertension and lung cancer	

Table 1. Values of prognostic factors.

It is common to observe progressive dyspnea, dry coughing, inspiratory crackles, and digital clubbing on physical examination as the clinical presentation of IPF. IPF patients usually report dyspnea as their first symptom at their initial appointment. When patients first present with breathlessness, patients who have never smoked as well as those who have more advanced disease are more likely to cough. Typically, this results in a delay in diagnosis, because it is commonly attributed to aging, cardiac disease, or emphysema. A common disabling phenotypic component of the disease, IPF cough has been identified as an independent predictor of disease progression and severity. In a retrospective study of patients with IPF, symptoms typically precede diagnosis by six months to two years if the minor allele of the MUC5B promoter polymorphism is present. (25). It is unusual for pulmonary fibrosis patients to experience symptoms such as weight loss, fever, or arthralgias, which should prompt an investigation to determine whether there may be secondary causes. Nearly 90% of patients with pulmonary fibrosis suffer from gastro-esophageal acid reflux, but these symptoms often do not manifest themselves.

The respiratory function tests show a reduction in force vital capacity (FVC) and a reduction in respiratory gas exchange capacity, measured as a reduction in the lung diffusion capacity of carbon monoxide (DLco). Isolated impairment of diffusion capacity can be found during the early stages of IPF (26).

Considering that IPF has a variable course, it is not possible to predict the outcome of the disease. The development of risk models incorporating the Gender, Age, Physiology (GAP) index has been attempted in some studies as a tool to predict both disease progression and mortality risk. It is evident that these scoring systems cannot predict disease behavior, highlighting the need for additional and more reliable non-invasive methods to improve risk stratification and outcome prediction in IPF.

2.5 Diagnosis

As a result of an unknown underlying cause and a variety of clinical manifestations, IPF can be challenging to diagnose. The diagnostic algorithm for IPF outlines the steps necessary to make the correct diagnosis and outlines the steps that must be followed. Therefore, the diagnosis of IPF requires multidisciplinary discussion (MDD) (Figure 6) (27).

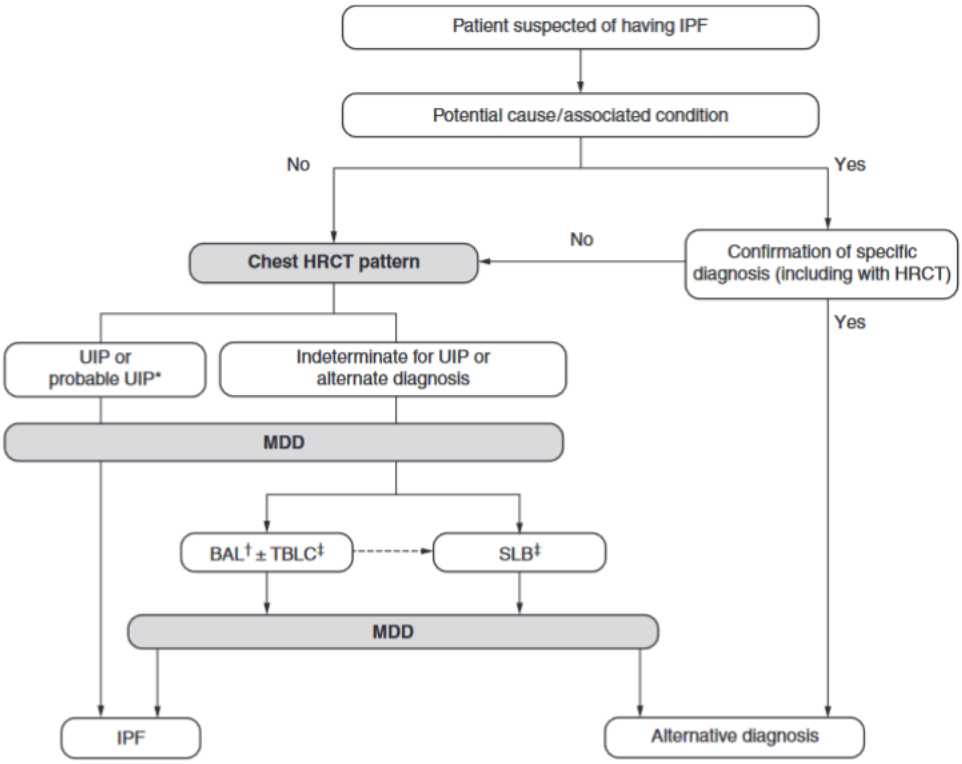


Figure 6. diagnostic algorithm ILD ATS/ERS 2018

A comprehensive clinical evaluation includes assessing clinical presentation, specific history, smoking status, pulmonary function evolution, serological tests, imaging, and, if necessary, pulmonary biopsy. High-resolution computed tomography (HRCT) is almost always used as the primary diagnostic tool. HRCT (particularly the fibrosis score) may also be used to determine ILD prognosis, whereas quantitative computed tomography (CT) may provide valuable information about disease progression. It is sufficient to use a UIP patterner to diagnose IPF when it is in the appropriate clinical setting. A key feature of UIP pattern is the honeycombing, identify on HRCT of the thorax. According to the Fleischner Society: "honeycombing is a CT feature of established pulmonary fibrosis and is defined as clustered thick-walled cystic air spaces, typically of comparable diameters on the order of 3–10 mm but occasionally as large as 2.5 cm" (28). However, in case where the HRCT lacks honeycombing

and/or contains features that are not characteristic of UIP, a histologic diagnosis may be required. The histologic diagnosis involves the surgical biopsy (29).

It is also important to exclude other causes of ILD as part of the diagnosis of IPF. When IPF is distinguished from other types of ILD, bronchoalveolar lavage (BAL) is helpful. An occult hypersensitivity pneumonitis diagnosis can be supported by a lymphocytosis of 40% or more, which prompts further investigations for environmental exposures, as well as a surgical lung biopsy (30).

The most common functional alteration in IPF/UIP is the restrictive ventilator deficit: there is a reduction in total lung capacity (TLC) with concomitant and harmonic reduction of all both static and dynamic lung volumes. The FEV1/FVC ratio or FEV1/VC (volume capacity) ratio is generally preserved or increased. The first functional parameter that is alternated is the DLCO (31).

A 6-minute walk caused by abnormal gas exchange during exercise results in significant decreases in arterial haemoglobin saturation in patients with mild to moderate IPF and normal or near-normal resting PaO2. IPF suffers from exertional arterial hypoxaemia for a variety of reasons, including changes in V′/Q′ ratios as well as diffusion.

The gold standard of diffusion testing is the single breath CO test. A low dosage gas is used, which has an affinity for haemoglobin about 200 higher than that of oxygen, such as carbon monoxide. Some of the CO diffuses from the alveolar gas to the blood, and higher is the diffusion capacity, greater is the amount of CO transferred.

2.6 Treatment

The only therapeutic strategy for IPF until 2011 was a lung transplantation, in a subset of selected patients only (7). As a result of lung transplantation, quality of life can be improved and survival can be extended, with a survival rate of approximately 50% after five years. Since the surgery and post-surgical care are medically complex, and the availability of donor organs is limited, it is not uncommon for only a few patients to benefit from this treatment.

A number of putative therapies have been demonstrated to be ineffective or harmful in randomised controlled trials (e.g., prednisolone and azathioprine, acetylcysteine, and warfarin). These randomised controlled trials were conducted by two large phase 3 development programs in order to determine the first effective disease-modifying therapies for IPF. Following the

positive outcomes of nintedanib and pirfenidone in clinical trials, the treatment guidelines were revised in 2015.

Several animal models of pulmonary fibrosis were screened for a combination of anti-inflammatory, antioxidant and anti-fibrotic properties of pirfenidone, an orally administered pyridine. Pirfenidone acts by regulating TGF expression and inhibiting fibroblast and collagen synthesis. Pirfenidone has been approved as a treatment for IPF in Europe since 2011 and in the USA since 2014. However, it remains unclear what the exact mechanism of action is. A feasibility study conducted earlier proved that pirfenidone reduces IPF patients' forced vital capacity (FVC) decline. In post hoc analysis of pivotal international phase III studies, the FVC decline, death, progression of disease, 6-min walk distance (6MWD) and dyspnea rates of patients treated with pirfenidone were significantly reduced in comparison to patients treated with placebo. Additionally, posthoc analyses found that pirfenidone significantly reduced respiratory-related hospitalizations and had consistent effects across all subgroups.

Post-authorization data from international open-label extension studies have confirmed pirfenidone's favorable safety profile and good tolerability.

In addition to inhibiting multiple tyrosine kinases involved in lung fibrosis pathogenesis, nintedanib has been shown to protect mules from lung fibrosis when bleomycin was administered, as it is an inhibitor of multiple tyrosine kinases, including the PDGF, VEGF, and FGF receptors. During phase 2 and 3 trials, nintedanib, given twice daily at 150 mg, improved functional loss, resulting in its approval for use in patients with mild-to-moderate IPF.

As part of phase III trials involving nintedanib, patients with IPF were randomized, double-blind, placebo-controlled, multinational studies conducted. As part of the extension study (INPULSIS-ON), nintedanib's efficacy was further evaluated. The percent predicted forced vital capacity (FVC% predicted) declined significantly slower with nintedanib than with placebo after 52 weeks of treatment. The percentage of patients with no decline in the predicted FVC% increased from baseline to week 52 in those treated with Nintedanib compared with placebo, while a smaller percentage had a decline in the predicted FVC% of patients treated with Nintedanib by 5% or 10%. Thus, Nintedanib patients were more likely to maintain lung function (32).

Although significant progress has been achieved in treating IPF, a cure is still elusive, requiring new research findings in the treatment field. New strategies include therapeutic biomarkers

guiding therapy, combinations of therapies, novel drugs targeting new pathways in fibrosis, lung microbiome therapies, nonpharmacological therapies as part of lung transplants, palliative care approaches that are more efficient, and better ways to manage comorbidities.

3. Hypersensitivity pneumonitis

3.1 Definition

Defining hypersensitivity pneumonitis as an interstitial lung disease (ILD) as the result of an inflammatory and immunological response following repeated inhalations by sensitized individuals of organic compounds and chemical compounds capable of reaching alveoli (33). The disease may be caused by a number of organic antigens (thermophilic actinomycetes, pigeon and parrot breeders, isocyanates, and fungi such as Aspergillus Fumigatus). The changing working conditions have resulted in some HP-related causes declining significantly, whereas new exposures, including metalworking fluid, are emerging that have been implicated as causative agents for HP among mechanical workers (34). The development of AAE would derive from the combination of a humoral and a cell-mediated reaction that would act consequentially: in the initial phase of exposure to the antigen, the humoral reaction predominates with induction, in predisposed subjects, of acute immune complex alveolitis; later, when exposure to the antigen ends, the cell-mediated reaction follows with the characteristic formation of granulomas (35). A characteristic of the disease is the presence in the serum of precipitating antibodies (IgG serum precipitins) towards the various antigens, which in any case can also be only an index of exposure and not of disease.

3.2 Clinic and diagnosis

The disease occurs in three clinical stages based on onset and symptoms (36):

• Acute HP: The acute form of HP is characterized by episodic respiratory symptoms and a systemic response within hours of exposure (e.g. fever, general malaise). Healing occurs spontaneously, however the symptoms recur each time the antigen is inhaled. Inhaled antigen concentration, individual predisposition and exposure time determine the severity of acute crises.

• Chronic HP: Usually, patients develop dyspnea and cough in a very subtle manner for months or even years (repeated low-dose antigen inhalation) and do not have a clear temporal relationship to the antigen that caused the symptoms, so they are difficult to identify. Identifying the responsible antigens and diagnosing chronic hypertension.

It is useful to look for evidence of bronchoalveolar lavage lymphocytosis (prevalence of CD8+ lymphocytes, with a reversed CD4+/CD8+ ratio) for differential diagnosis (37). Interstitial

infiltrate consists primarily of lymphocytes, while alveolar cells contain foamy macrophages (38). The etiology of the disease must, however, be determined by reviewing the medical history, since antigen avoidance significantly reduces mortality. Prognosis depends on exposure duration and intensity, as well as stage of disease when the diagnosis is made. When only a few acute episodes have occurred and exposure can be stopped, it is extremely good, but the prognosis is dependent on the duration and intensity of exposure. In order to confirm HP exposure and HP risk, a serum specific IgG test may be used.

During diagnosis and follow-up, pulmonary function tests are typically performed to evaluate functional impairment. The most frequently observed alteration is a restrictive pattern. The reduction in lung diffusion capacity of carbon monoxide (Dlco) occurs earlier than the reduction in lung volume.

In order to determine survival, the presence and extent of pulmonary fibrosis have been classified according to histology or high-resolution computed tomography (HRCT) specimens, based on their fibrotic features. HP cannot be diagnosed early or prognostic using specific biomarkers, nor can it be assessed based on treatment response or prognosis in advance of diagnosis (39).

Between April and August 2017, 45 interstitial lung disease experts from 14 countries participated in a three-round Delphi International Modified Survey to identify the most accurate clinical, radiological, laboratory, and pathological diagnostic criteria for HP (40). Experts also discovered that there is a temporal relationship between symptoms and exposure when there is a positive history of HP, mosaic attenuation HRCT, centrilobular nodules (CLN), or ground glass opacities (GGO). A CLN is typically associated with chronic obstructive pulmonary disease, but they can also be associated with interstitial and airway diseases, as well as varying histological correlations.

As defined by the American Thorological Society, they are opacities that are separated by a few millimeters from pleural surfaces, ranging from a few millimeters to one centimeter, and may be dense or appear as ground glass. A HRCT clearly demonstrates centrolobular nodules that correspond to granulation tissue within the alveoli and bronchioles of an adjacent lung. The HRCT also detects air trapped in the lungs distal to obstructions, which are usually found at the level of secondary lung lobules, which can also be detected with CT imaging. Known as ground-glass HRCT, evidence of partial filling of air spaces, as well as partial collapse of

alveoli, can be indicative of a wide range of conditions, such as inflammatory and infectious conditions as well as neoplastic conditions; it is also possible to experience this radiological sign as a result of interstitial thickening, particularly thickening of the interlobular septa, caused by fluid and cells as well as fibrosis. Therefore, there are many chronic infiltrative lung diseases that appear like ground glass, such as some interstitial pneumonias that are both idiopathic and have known etiologies (41). There are a number of radiological features and lobular distributions associated with GGO that can assist in diagnosis; in the case of HP, for instance, it is envisaged that the ground glass will have a centrilobular distribution. Figure 7 schematizes the diagnostic algorithm for chronic hypersensitivity pneumonitis. In the advanced stages of fibrosis with honey-combing at HRCT it is essential to make a differential diagnosis with idiopathic pulmonary fibrosis (IPF).

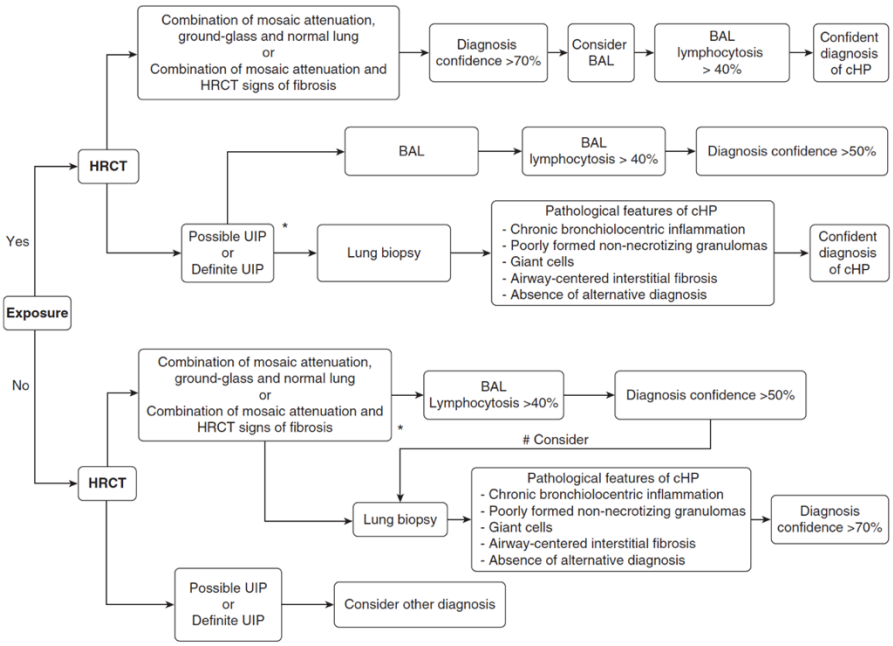


Figure 7: Diagnostic algorithm for chronic hypersensitivity pneumonitis

3.3 Prognosis and therapy

The prognosis basically depends on the duration and intensity of exposure, and even more on the stage of the disease in which the diagnosis is made. This depends on the intensity of the symptoms and the subjective tolerance of the patients. The prognosis is excellent when only a

few acute episodes have occurred and exposure can subsequently be stopped. A history of repeated acute episodes carries a much less favorable prognosis. The presence of pulmonary fibrosis is evidently a sign of an irreversible alteration. According to literature data, survival for the majority of patients with extrinsic allergic alveolitis is about 10 years from diagnosis. In chronic forms, with evident signs of pulmonary fibrosis, survival is similar to that of other types of fibrosis and is linked to the functional alterations of the lung typical of this anatomical situation.

The prognosis is worse if the patient is a topic: while this condition, in fact, does not have a favorable action on the development of the disease, it involves, through the bronchial obstruction to which it itself predisposes, a worse functional evolution from the disease itself. Cigarette smoke, which paradoxically seems to carry out a protective action against sensitization (by decreasing, through the reduction induced on the bronchial caliber, the penetration of the antigens towards the lung periphery and immunologically through the stimulus induced on the macrophages, which therefore would be more active also in the purification action towards the inhaled antigens) once the disease is established, on the other hand, it determines a worse evolution, due to the chronic and emphysematous bronchitic component. The negativization of serum precipitins in subjects removed from exposure represents a favorable element in the prognostic evaluation. Essential for the improvement of the disease is the removal from the allergen. This fact, however, implies a change or reorganization of the profession which is not always possible.

Corticosteroids, administered systemically, are the only effective therapy and should be used when environmental prophylaxis is ineffective. Treatment of the acute phase involves the administration of prednisone at doses of 40-60 mg / day, which can be progressively reduced after two weeks. In conclusion, it should be emphasized that, since AAE determines irreversible lung damage, it is necessary to formulate the diagnosis as early as possible and set up a consequent therapy, even aggressive, when the mere removal from the antigen is not able to control the disease (42).

4. Histiocytosis

4.1 Definition and classification

The term histiocytosis defines a group of rare diseases that have in common the proliferation and accumulation of dendritic or macrophage-derived cells in different organs (43). Most histiocytes derive from the CD34 + stem cell which, conditioned by the cytokines of the cellular microenvironment, continues its development along two main pathways, characterized respectively by CD14 + and CD14- cells (Figure 8).

Group	Entities
L	Langerhans cell histiocytosis (LCH) Indeterminate cell histiocytosis Erdheim-Chester disease (ECD) Mixed ECD and LCH
C	Cutaneous non-LCH histiocytoses Cutaneous non-LCH histiocytoses with a major systemic component
M	Primary malignant histiocytosis Secondary malignant histiocytosis
R	Familial Rosai-Dorfman disease (RDD) Classical (nodal) RDD Extranodal RDD Neoplasia-associated RDD Immune disease–associated RDD Other non-C, non-L, non-M, and non-H histiocytoses
H	Primary HLH: Mendelian-inherited conditions leading to HLH Secondary HLH (apparently non-Mendelian HLH) HLH of unknown/uncertain origin

Figure 8. Schematic representation of the pathologies included in the 5 different groups into which histiocytosis and neoplasms of the dendritic and macrophage lines are divided (Frater JL, 2016).

According to the Working Group of the Istiocyte Society, the first classification of histiocytosis was published in 1987 and classified the disease into three categories based on clinical features and immunohistochemical markers (44):

• Langerhans cell histiocytosis (ICL): derived from dendritic cells

• non-Langerhans cell histiocytosis: deriving from the macrophage lineage

• malignant histiocytosis.

There are Langerhans cells (CL) present in ICL, as well as Langerin + / CD207 Langerin + / S100 + histiocytes. In contrast, non-Langerhans cell histiocytosis, also known as juvenile xanthogranuloma, Rosai-Dorfman disease, histiocytosis of the breast accompanied by massive

lymphadenopathy, and Erdheim-Chester disease, is characterized by the accumulation of histiocytes with immunophenotypic characteristics that are distinct from CL.

Recently, on the basis of the knowledge on pathogenetic mechanisms, clinical presentation and molecular characteristics, a new classification of histiocytosis and neoplasms of the dendritic and macrophage lines has been proposed. These disorders were divided into 5 groups:

• L (Langerhans histiocytosis),

• C (cutaneous and mucocutaneous non-Langerhans histiocytosis),

• M (malignant histiocytosis),

• R (Rosai-Dorfman disease and non-cutaneous non-Langerhans histiocytosis),

• H (haemophagocytic lymphohistiocytosis)

4.2 Langerhans cell histiocytosis

4.2.1 Definition and etiopathogenesis

Langerhans cell histiocytosis (ICL) is a rare disease and is the most frequent chronic histiocytic disorder, with an estimated annual incidence of 5.4 cases per million in children and 1-2 cases per million in adults. In pediatric age, males are affected in more than twice the proportion of females, but with increasing age the predominance of males is reduced to an almost reversal. No genetic component has been demonstrated to date. The spectrum of clinical manifestations varies from a single lesion that tends to regress spontaneously, the so-called eosinophilic granuloma, to multiple osteolytic lesions of the skull associated with exophthalmos and diabetes insipidus (DI), defined as Hand-Schüller-Christian disease, up to one very rare disseminated multiorgan form with an acute and potentially fatal course, called Letterer-Siwe disease. The term "Histiocytosis X" used by Lichtenstein in 1953 to define the three different disorders was due to the lack of knowledge on etiopathogenesis. In 1973, Nezelof demonstrated that granulomas were the result of the proliferation and spread of pathological histiocytes, characterized by the presence of intracytoplasmic Birbeck granules. The Histiocyte Society (HS) redefined these disorders in 1987 by replacing the term Histiocytosis X with ICL. There are several types of histiocytes found in the granulomas of LCH, including atypical histiocytes

(CD1a+ / CD207 Langerina+ / S100+), in addition to normal histiocytes, lymphocytes and eosinophils. Several hypotheses have been proposed regarding the etiopathogenesis of the disease, ranging from neoplastic nature to altered immune system function, viral infections, and specific changes in lymphocytes. According to recent research advances, the CL of the ICL results from somatic mutations acquired in precursors of the myeloid lineage as a result of a pathological activation of the MAPK pathway. In the category of dendritic cells, CLs are hematopoietic cells that present antigens. The presence of specific surface markers, such as CD1a and CD207, also known as langherin (type C lectin, which acts by internalizing the antigen in connection with Birbeck's granules), allow the identification of abnormal histiocytes whose finding is diagnostic for ICL. Immunohistochemical characterization with these antigens allows the differential diagnosis with indeterminate cell histiocytosis (CD207-), with Rosai-Dorfman disease (multinucleated histiocytes with evident emperipolesis, CD1a- or CD207-). LCH is distinguished from malignant histiocytosis by the presence of histiocytes with nuclear atypia and high mitotic index (45).

The variability in clinical manifestations, ranging from a single lesion that can spontaneously regress to disseminated forms with potentially fatal multiorgan involvement, has fueled doubts about the pathogenesis of this disease, causing a decades-long debate on whether LCH can be considered a dysreactive inflammatory disorder. or a neoplasm. The function of the activated langerhans cell is to collect and process the antigen, it migrates to the regional lymph nodes where it initiates an adaptive immune response, presenting the transformed antigen to the T lymphocytes. Compared to normal CL, the histiocytes of the ICL have a greater proliferative capacity and a lower ability to present the antigen. This suggests that CLs stop at a more immature state of activation. Furthermore, the simultaneous presence of pathological CL in multiple organs and tissues, including the skin and lymph nodes, as occurs in Letterer-Siwe disease, has led to the hypothesis that tissue infiltration in ICL could be due to dysregulated expression of chemokine receptors. In support of the inflammatory nature of LCH, there is also clinical evidence, in particular the possibility of spontaneous regression of the lesions and the good response to mild chemotherapy of extensive forms. CL and infiltrating cells generate a variety of cytokines, including glycoproteins like GM-CSF, interferons like IFN-γ, TNF-α, and numerous interleukins (IL) (e.g. IL-1α, IL-2 and IL-10). Recently, it has been shown that the serum levels of certain cytokines / chemokines of inflammatory lesions and activating factors of both dendritic cells and macrophages are negative prognostic markers (IL-2R, IL-12p40, IL-18 and CXCL9) (98). The increased production of some cytokines (IFN-γ, TNF-α, IL-6, GM-

CSF) by CL and associated lymphocytes could explain some clinical manifestations of the disease (the increase in TNF- α could be responsible for fever, osteolytic phenomena, haematological and hepatic changes) (46).

In LCH lesions there are also multinucleated giant cells similar to osteoclasts not only in the bone but also in skin and lymph node lesions. Enzymes derived from osteoclast-like multinucleated giant cells could play an important role in the chronic destruction of tissues observed in lesions characteristic of ICL.

The alternative model concerning the pathogenesis of LCH is based on the hypothesis of the neoplastic transformation of the epidermal Langerhans cell. Within the ICL lesions, the same pathogenetic mechanisms are observed that are associated with neoplastic pathologies, such as immune evasion caused by the increase in regulatory T lymphocytes, inflammation promoted by neoplasia with the local and systemic increase of pro cytokines. -inflammatory, the expression of metalloproteases (MMP2-MMP9) promoting invasion and metastasis. However, the fact that cell clonality is not indicative of malignancy and the persistent inability to identify specific genetic abnormalities have prevented LCH from being classified as a neoplastic disorder.

Because of the heterogeneity of the cellular components of ICL, analyzing genomic alterations has traditionally proved difficult. Genome amplification and the identification of a critical single base mutation in rare cell populations have been enhanced by the advent of new sequencing technologies. In approximately 60% of cases of ICL, a somatic point mutation, BRAF-V600E, was found for the first time. This marked an historic turning point in the field of ICL research. A large cohort of patients with LCH has been studied in the subsequent years and it has been shown that the BRAF-V600E mutation is highly prevalent. The BRAF-V600E mutation has been identified in about 8% of all human cancers, it is rarely associated with non-histiocytic malignant hemopathies, with the exception of hairy cell leukemia, of which it represents the diagnostic marker (47). BRAF V600E is specific to CL of the ICL and is absent in normal proliferating CL and in other types of histiocytosis (48). To date, the prognostic significance of this mutation is not yet clear as it has been found in both multisystem (MS) and unisystemic (SS) forms, although a greater risk of reactivation has recently been reported in patients with the mutation.

The BRAF-V600E mutation is also associated with increased resistance to chemotherapy, reactivation rates five years later, and the development of long-term permanent sequelae from the disease and/or its treatment. By activating the receptor for tyrosine kinases and phosphorylating various kinases (e.g. Ras, Raf, MEK (mitogen-activated ERK kinase), ERK (extracellular signal-regulated kinase)), BRAF contributes to the signal cascade leading to a change in gene expression., resulting in a modulation of gene expression. The LCH, therefore, could be considered as an uncontrolled proliferative disorder that originates from a somatic mutation of a gene that regulates cell proliferation, not yet identified, with the activation of accompanying inflammatory cytokines (49).

4.2.2 Clinical manifestations

The clinical picture of LCH is highly variable, conditioned by the type of tissue involved and the extent of the disease. The course of the disease is generally benign, although in children (age <2 years) it can be more aggressive (50). The disease can be:

• localized: mostly at the bone-cutaneous level

• widespread: involving multiple organs and / or systems (bone, pituitary, hematopoietic system, liver, spleen, lymph nodes, eye, intestine, heart, nervous system, lungs) with a more severe prognosis.

In some cases, the diagnosis may be occasional. In the disseminated forms, typical of the pediatric age, general symptoms such as fever, asthenia and reduced body-weight gain are frequent. In a minority of cases the disease can explode, very serious, already in the first months of life with a leukemic-like picture: widespread papulo-pustular, purpuric and / or necrotic skin lesions, fever, hepato-splenomegaly, adenomegaly, cytopenia. This form, formerly referred to as Letterer-Siwe disease, is burdened by a mortality of 20%, even with the most modern treatments.

4.2.3 Pulmonary impairment

Cigarette smokers (young adults aged 20 to 40, with no gender predilection) are more likely than non-smokers to be affected by pulmonary Langerhans cell histiocytosis (PLCH). An important characteristic of PLCH is that Langerhans cells and other inflammatory cells accumulate in the small airways, leading to nodular inflammatory lesions (bronchiolocentric granulomas, 1 to 10 mm in diameter) that result in the formation of the lesions. The vast

majority of patients suffering from this disease are believed to have been induced by smoking. However, the mechanism by which smoking causes it remains unclear. Langerhans cells are activated in the small airways by a number of factors. The smoking of cigarettes causes epithelial cells and macrophages to produce growth factors, resulting in the activation of Langerhans cells. In addition to stimulating macrophages, smoking also releases GM-CSF from epithelial cells and fibroblasts. In PLCH lung biopsies, epithelial cells produce growth factor TGFβ, which is overexpressed due to cigarette smoking. Among the key factors supporting Langerhans cell formation, TGF plays an essential role in fibrosis and scar formation (51).

An inflammation of the bronchiles can be associated with interstitial lung involvement or vascular involvement. A major characteristic of early disease is cellular inflammation, however, as the disease progresses, pulmonary vascular remodeling is observed along with cystic pulmonary destruction and airway scars. Respiratory function can therefore be compromised in various ways: from a clinically asymptomatic reduction in gas exchange at the alveolar level, up to a restrictive alteration of varying degrees and respiratory failure. High-resolution CT scan chest imaging may show characteristic nodular and cystic abnormalities (52).

The presence of> 5% CD1a cells in bronchoalveolar lavage fluid is highly indicative of the disease. The biopsy shows a proliferation of Langerhans cells with an occasional cluster of eosinophils (which has given rise to the obsolete definition of eosinophilic granuloma) at the center of cellular and fibrotic nodules that can assume a star-like configuration. Immunohistochemical stains are positive for CD1a, S-100 protein and HLA-DR Ag. In cases where imaging findings are highly characteristic, a lung biopsy may not be necessary for a definitive diagnosis. The most severe lesions are typically found in the upper lung fields and in the peri-hilar areas, with sparing of the bases. All smokers should be made aware of the smoking cessation program, which may result in remission of the disease and the elimination of the need to take systemic immunosuppressive medications (53). The prognosis for most patients is relatively good, in particular the functional trend is stable (median survival 12 years). Complications such as pneumothorax (spontaneous in 10 to 25% of patients, due to the formation of emphysematous bubbles and subsequent rupture) and secondary pulmonary hypertension can shorten life expectancy. The total cessation of smoking involves a regression of the lesions or a stabilization of the same and of the symptoms (dyspnoea, angry cough, haemophtoe, weight loss, pleuritic chest pain). In 15% of patients the disease is asymptomatic

and is found incidentally for a chest x-ray performed for other reasons. Patients with progressive disease may require lung transplantation, usually if associated with smoking cessation (54).

4.2.4 Diagnosis

Differential diagnosis is fundamental in the diagnostic process as LCH can be confused with various other pathologies due to the extreme variability of clinical manifestations. It is therefore necessary to carry out an accurate clinical history, a detailed assessment of the site and characteristics of the lesions and, for a correct histological definition of the pathology, a biopsy of the most accessible lesion is essential. The definitive diagnosis is based on histological and immunohistochemical examination that allows the morphological identification of CL that are positive for the S-100 protein and for the CD1a antigen. Where the biopsy is too risky for the patient due to the location of the lesion, in this case the diagnosis is radiological, as the lesion is typical of the ICL. Currently, a new specific marker for langherina is used for the diagnostic definition, CD207, which has replaced the search for Birbeck's granules since its expression fully correlates with their presence. There are, however, organs, such as the liver, in which CL do not contain Birbeck's granules and CD1a and / or langherin (CD207) may be negative.

Once the histological diagnosis has been made, it is necessary to carry out further investigations to identify any asymptomatic localizations of the disease. History should be aimed at identifying the type and duration of symptoms, such as: pain, swelling, rash, otorrhea, irritability, fever, loss of appetite, diarrhea, weight loss, growth retardation, polydipsia, polyuria, dyspnoea, exposure to smoking, lifestyle habits and neurological alterations. In adults, it is important to ascertain any comorbidities. The physical examination is aimed at an accurate evaluation of the skin and mucous membranes, of the oral cavity (gingival and palatal lesions), of the abdomen (hepato-splenomegaly), of the nervous system (papilledema, anomalies of the cranial nerves, cerebellar signs) and of the lungs. Fundamental is the physical examination to exclude a picture of hepatosplenomegaly to be confirmed with radiological investigation (abdomen ultrasound). In adult patients, it is useful to perform targeted investigations for the main target organs affected by the disease, such as the lung, pituitary and oral cavity, even in the absence of symptoms. Respiratory function tests with diffusion of carbon dioxide (Dlco) can identify early pulmonary compromise that is not radiologically detectable; MRI of the brain with gadolinium can give indications on the pituitary gland and orthopanoramic radiography with dental evaluation can highlight lesions that are not otherwise identifiable. With regard to the evaluation of any bone lesions, in vertebral involvement it is useful to investigate the spine with MRI to

exclude the presence of newly formed tissue that could affect the spinal cord. From the data available so far, PET has no indications either in the staging or in the clinical management of LCH, since despite being a highly sensitive investigation it is not very specific. Medullary bone biopsy is indicated only in the presence of anemia and / or thrombocytopenia and / or leukopenia, with no obvious cause. In the case of isolated pulmonary involvement, if broncholwashing is not diagnostic for ICL (<5% CD1a + cells in non-smokers), a lung biopsy must be performed, for a correct differential diagnosis with other pathologies, even if the examination biopsy is hampered by the multifocality of lung lesions. Alternative procedures such as cryobpiosia could be used but with a risk of pneumothorax.

4.2.5 Clinical classification

The disease is defined as uni-systemic or single system (SS-ICL) when only one organ is affected; in turn, based on the number of lesions it is defined as uni-focal (single lesion), multifocal (multiple lesions affecting the same organ or system). The disease is referred to as multi-system or multisystem (MS-ICL) when two or more organs are involved. Clinical classification is used at diagnosis to plan the therapeutic strategy.

4.2.6 Therapy

The disease has a variable and unpredictable course. Localized forms (skin, bone) have a good prognosis and can also regress spontaneously or with local therapies. The diffuse forms, on the other hand, require chemotherapy which is not always effective, especially in multi-systemic disease with organ dysfunction, frequent in children aged <2 years. The first cytotoxic drugs that have shown efficacy in the treatment of LCH, in mono-chemotherapy or in combination, were: chlorambucil, vincristine (VCR), cyclophosphamide (CTX), methotrexate (MTX), 6-mercaptopurine (6-MP) and vinblastine (VBL). Within five years, approximately 50 percent of patients develop reactivations of their disease or are refractory to induction therapy (54). This study demonstrated significant activity of clofarabine, a second-generation nucleoside analogue, in refractory cladribine or cytarabine-resistant ICL in patients with low and high risk. Generally, cladribine and cytarabine is the most effective rescue regimen for patients with refractory high-risk LCH, although it is dangerous at high doses. In the isolated pulmonary form, honey-combing type, difficult to treat, the use of low-dose PDN (0.5-1 mg / kg / day for several months) associated with respiratory physiotherapy gives good results both in terms of outcome and quality of life. Regardless of the type and / or combination of drug used, the

response to treatment in adults is lower than that seen in children, especially in some particular forms. In multifocal forms with organ dysfunction and in those with neurological involvement, cladribine has proved effective. The phase II study demonstrated that cladribine monotherapy may be beneficial for adults with LCH SS or reactivated MS. Several studies have demonstrated the effectiveness of using cladribine and cytarabine to treat patients with central nervous system involvement, probably due to their ability to pass through the blood brain barrier. By targeting PDGFRB(platelet-derived growth factor receptor beta)-associated tyrosine kinases, Imatinib mesylate is proving to be a promising new treatment option. Recent progress concerns the use of BRAF inhibitors. The first drug used was a BRAF-V600E inhibitor, Vemurafenib. Used in 4 patients with LCH and concomitant Erdheim Chester disease, it gave a sustained response (10-16 months) even if burdened by short and long term complications, especially cutaneous ones (55).

5. Lymphangioleiomyomatosis

5.1 Definition and etiopathogenesis

Although lymphangioleiomyomatosis (LAM) has been reported in men, it is extremely uncommon in men. It predominantly affects women between the ages of 20 and 40. Angiomyolipomas, or abdominal tumors that affect the kidneys, are among its characteristic features, as are cystic lungs, lymphatic manifestations such as lymphangeomyomas, and lymphangioleiomyomas (56). As well as sporadic cases, it is also associated with a genetically dominant disorder known as tuberous sclerosis complex (TSC) (57). The TSC1 and TSC2 genes responsible for tuberous sclerosis are altered, resulting in the loss of function of their products, hamartin and tuberine (58). As an intracellular kinase that regulates cell growth and proliferation, the mechanistic target of rapamycin (mTOR) is inhibited by the amartine / tuberine heterotrimer. As a consequence of inactivating mutations of the TSC1 and TSC2 genes, mTOR is constitutively activated in both LAM associated with tuberous sclerosis and sporadic LAM (59).

Two subpopulations of LAM cells, known as spindle cells with smooth muscle proteins, such as -actin, vimentin, and desmin, are found in LAM lesions, which have the appearance of LAM cells in the lesion. Melanoma and immature melanocyte markers are expressed by epithelioid-like cells.

Consequently, LAM belongs to the family of perivascular epithelioid cell tumors, which are myogenic and melanocytic cell tumors composed of perivascular epithelioid cells. Moreover, numerous genetic and cellular findings indicate that LAM cells are neoplastic (60).

As a result of inappropriate signals activated by the mTOR cascade, TSC mutations in LAM are caused by inappropriate signals. The same patient's angiomyolipomas and lung lesions had identical TSC somatic mutations, suggesting that both types of LAM cells may have originated from the same source. Furthermore, recurrence of LAM in lung transplant patients and the presence of LAM cells in blood are evidence of metastatic behavior (61). LAM cells have been shown to express the glycoprotein CD44v6, which binds hyaluronic acid and is associated with metastatic tumors. LAM cells may also be able to adhere to the extracellular matrix more effectively with CD44v6 protein, facilitating metastatic growth (62). There is low proliferation of LAM cells without evidence of atypia; however, these cells display progressive infiltration

of the lung parenchyma, tissue destruction, angiogenesis, lymphangiogenesis, and extracellular matrix degradation. Cells that are neoplastic are similar to those that are not neoplastic.

5.2 Clinical features

As a result of the disease, a pneumothorax (spontaneous PNX) can occur acutely, resulting in chest pain and dyspnea. In addition to recurrence, pneumothorax is often bilateral and less responsive to standard treatments. The treatment for a recurrence requires pleural abrasions, talc, chemical pleurodesis, or a pneumothoraxectomy. Patients with LAM should be informed that there is an increased incidence of pneumothorax and disease progression in the event of pregnancy (in this case the patient must be followed up with close follow-up) (63). In the case of severe respiratory functional impairment the patient should be discouraged from becoming pregnant. In acute cases, the disease can also begin with hemoptysis.

Irritating cough and exertional dyspnea, on the other hand, are the most frequent symptoms of insidious onset, together with asthenia and weight loss. The disease can also manifest itself with lower back pain due to the presence of angiomyolipomas (from 1 mm to 20 cm in diameter, at risk of bleeding if> 4 cm, to be treated with embolization), which however can also be present in the asymptomatic patient. Studies have found that renal angiomyolipomas are present in 100% of TSC-LAM patients and 50% of patients with sporadic LAM.

There are two clinical phenotypes of LAM:

- with worse prognosis: onset with dyspnoea, weight loss, respiratory failure, reversible obstructive deficit, high serum levels of VEGF-D. Such patients have a shorter survival and a rapid functional decline (64);

- with better prognosis: onset with pneumothorax, age> 40 years, elevated FEV1 and Dlco values at diagnosis. Such patients have a longer survival and a slower functional decline.

5.3 Radiological diagnosis

The radiological diagnosis of LAM pulmonary involvement is carried out by means of high-resolution chest CT scan (HRCT) which detects a picture of multiple cysts, spread throughout the lung area and of various sizes (10 mm-10 cm). The typical chest-HRCT radiological picture for LAM consists in the presence of multiple (more than 10) well-defined thin-walled cysts without significant pulmonary involvement; the HRCT-chest picture compatible for LAM

consists in the presence of a few thin-walled cysts (more than two but less than 10). Histological examination confirms the diagnosis of this disease only in the presence of abnormal proliferation of smooth muscle fiber cells (LAM cells) and cystic changes. Biopsy (surgical) is indicated only in the absence of high-resolution CT findings which are not diagnostic. The 2010 ERS guidelines described three possible levels of LAM diagnosis:

- LAM defined: HRCT-chest picture characteristic for LAM with at least one other manifestation of the disease including: angiomyolipoma, chylous thoracic / abdominal effusion, lymphangioleiomyoma, lymphadenopathies (positive for LAM cells on biopsy examination), tuberous sclerosis

- Probable LAM: characteristic chest HRCT-picture for LAM with compatible clinical history or compatible chest-HRCT picture for LAM with presence of angiomyolipoma or chylous effusion

- LAM possible: only characteristic or compatible HRCT-chest picture for LAM.

In the presence of an LAM-compatible chest-HRCT picture, the abdomen must always be studied with Magnetic Resonance (MRI) or alternatively CT-abdomen with contrast medium. Vice versa, in case of finding of abdominal-pelvic lesion compatible with lymphangioleiomyoma or angiomyolipoma it is necessary to perform HRCT-thorax to exclude / confirm the possibility of LAM (65).

The follow-up of LAM patients involves the execution of a series of blood chemistry and diagnostic tests (respiratory function tests, chest HRCT, echo-abdomen, walk test, arterial blood gas analysis, echocheart) to be performed at a time of 3-6-12 months from diagnosis to mainly assess the clinical course, radiological progression of the disease and functional worsening.

5.4 Biomarkers in LAM

5.4.1 VEGF-D

In addition to forming lymphatic vessels and spreading cancer cells to lymph nodes, Vascular endothelial growth factor D (VEGF-D) is also known as a lymphangiogenic growth factor. In a decade ago, researchers determined that patients with sporadic LAM had higher serum levels of VEGF-D than healthy controls (166). Further studies showed that serum VEGF-D levels are also higher in patients who have LAM than in individuals with other cystic or chylous lung

diseases, such as PLCH or emphysema; suggesting that this parameter might be useful to identify patients with LAM (66). CT scans of women with TSC showing cystic changes found a higher level of VEGF-D than those without cystic changes. In a larger study of patients with LAM, researchers found higher levels of VEGF-D in the patients than in healthy controlsCompared to LAM patients with lower VEGF-D levels but higher lymphatic involvement, pooling patient samples based on extrapulmonary lymphatic involvement resulted in higher scores. In LAM patients with higher severity on CT scans as well as lower lung diffusion capacity, serum VEGF-D levels may be a useful indicator of lymphatic involvement. (67).

VEGF-D serum concentrations have been validated as a diagnostic test for cystic lung disease of unknown etiology in a prospective study of 48 patients. Based on the authors' findings, serum VEGF-D levels higher than 800 pg/mL are diagnostic for LAM with a sensitivity of 73 and a specificity of 100%. VEGF-D levels have been found to be diagnostic for LAM in 75 patients with cystic lung disease, with a sensitivity and specificity of 87% and 90%, respectively, placing a diagnostic threshold of 468 pg / mL as the diagnostic threshold for this condition.

As a result of this finding, VEGF-D testing is recommended by recent American Thoracic Society / Japanese Respiratory Society (ATS / JRS) 2016 guidelines for diagnosing and managing LAM when the diagnosis is radiologically suspected (65). As recommended by the European Respiratory Society (ERS) 2010 guidelines, chest HRCT is compatible when the other confirmatory characteristics are absent (e.g. TSC, angiomyolipomas, pleural effusions, ascites, chylosis). The diagnostic threshold is 800 pg/ mL.

According to the low false positive rate associated with the test, patients with typical LAM-chest HRCTs may not need a lung biopsy if VEGF-D serum concentrations exceed 800 pg/mL. A negative serum VEGF-D test does not rule out LAM, as the test has a high false negative rate. These cases may also benefit from the presence of serum VEGF-D in order to determine the severity of the disease and the effectiveness of the treatment. The safety and efficacy of Sirolimus were evaluated in this study. The Multicenter International Lymphangioleiomyomatosis Efficacy of Sirolimus (MILES) demonstrated a significant decrease in serum levels of VEGF-D in patients receiving sirolimus, but a stable level was observed in patients receiving placebo treatment (68). Sirolimus significantly improved lung function in the group with high VEGF-D values at baseline, while placebo significantly deteriorated.

In addition to confirming the diagnosis of LAM in patients who have typical chest-HRCT but do not exhibit other typical lesions, serum VEGF-D biomarker tests can also be used to evaluate the response to therapy for patients who have lymphatic manifestations (lymphangioleiomyoma, enlarged lymph nodes, chylous pleural or abdominal effusions). Although serum levels of 800 ppg / ml are not indicative of LAM, it is important to note that serum levels above 800 ppg / ml do not necessarily indicate LAM, nor can serum levels > 800 ppg / ml alone confirm the diagnosis of LAM in the absence of a characteristic HRCT-chest picture and a compatible clinical picture. As a result of combining the criteria of the ERS 2010 guidelines with serum VEFG-D levels, it is clear that invasive diagnosis with biopsy is significantly reduced.

5.4.2 Extracellular matrix metalloproteinase (MMP)

As metalloproteinases (MMPs), matrix metalloproteinases are instrumental in transforming and degrading extracellular matrix. In the lung, MMPs play a significant role in tissue remodeling and lymphangiogenesis (69). The pathogenesis of LAM has also been linked to MMPs, which are present in LAM lesions. LAM patients also have higher serum MMP-9 levels than normal individuals. Lung cysts can be caused by these substances that help cells migrate. LAM can be assessed, although nonspecifically, using serum and urinary MMP levels in patients. One patient with severe LAM was able to detect no urinary MMP-9 or MMP-2 levels during treatment with doxycycline, an MMP inhibitor.

5.5 Therapy

Everolimus and sirolimus are two inhibitors of mTORC1, which inhibit growth and proliferation of LAM cells by inhibiting the signaling pathway activated by mTOR (70).

6. Sarcoidosis

6.1 Definition and etiopathogenesis

The most common site of granulomas in sarcoidosis is the lung (20), but it is a systemic inflammatory disease characterized by granulomas in virtually any organ. herefore, sarcoidosis has a number of clinical phenotypes due to genetic variation and/or environmental influences. It is possible for the course of the disease to vary (some patients recover spontaneously, while others develop chronic inflammation and tissue fibrosis). It is highly variable and depends on gender, age and ethnicity whether sarcoidosis will affect quality of life (QOL), reduce work capacity, or increase mortality (71). Variations in incidence over time and geographically indicate the presence of unknown agents that trigger inflammation in genetically predisposed individuals, possibly microorganisms, environmental factors, or inorganic materials (72). As demonstrated by the significantly higher disease risk in first-degree relatives of patients with sarcoidosis, genetic factors clearly play a significant role in the etiology of sarcoidosis. The disease course and prognosis of disease can be predicted by HLA alleles as well as variants of other genes (73).

Typical of sarcoidosis are granulomas and non-neurotizing epithelial cells formed in the lungs from the accumulation of T-helper cells. These findings suggest that a specific antigen or antigens are responsible for triggering the immune response. Patients with identical TCRs in the lungs suggest they have been exposed to similar antigens (microorganisms or their non-degradable remains, mycobacterial antigens (ESAT6, KatG) (74) or e.g. Mycobacterium tuberculosis (75) or Cutibacterium acnes, formerly Propionibacterium acnes) by accumulating T cells in the lungs are possible triggers, although in a number of different ways. As an example, molecular mimicry (similarity between human proteins and microorganisms) can result in autoimmune reactions that can contribute to the onset of sarcoidosis. It has been demonstrated that patients with sarcoidosis have TCR receptors directed toward vimentin and antivimentin antibodies. Mesenchymal cells express vimentin, which is produced by macrophages within sarcoid granulomas as part of the cytoskeleton. Sarcoidosis patients' BALs contained anti-vimentin antibodies due to HLA-DR3 +. A diagnosis of systemic lupus erythematosus is associated with high levels of anti-vimentin antibodies, which indicate the severity of the illness.

6.2 Epidemiology

It has been noted that the incidence and prevalence of sarcoidosis as well as its clinical presentation differ significantly by geographic area, gender, ethnicity and age. Among Scandinavian countries (76), the incidence of sarcoidosis is highest (11-24 cases per 100,000 individuals per year), followed by African Americans (77), where there are 18–71 cases per 100,000 individuals per year, while Asian countries have a much lower incidence (1 case per 100,000 individuals per year). There are geographical variations in sarcoidosis distribution within countries, and some studies indicate that prevalence is higher in areas with less density of population. A typical onset age ranges between 40 and 55 years, with a peak in men (30-50 years of age) at diagnosis compared to women (50-60 years of age). A patient's characteristics determine the symptoms of sarcoidosis. A greater number of African Americans are diagnosed with advanced or multiorgan lung involvement when they are diagnosed with the disease (78). White individuals have the highest reported incidence of Löfgren's syndrome, while African Americans and Asian individuals are rarely diagnosed with the condition. Around one third of all cases of sarcoidosis are caused by Löfgren syndrome, according to Swedish statistics. There is a good prognosis for patients with this gene, with 70 to 80 percent of them going into remission.

6.3 Risk factors

• Study after study has demonstrated that genetic susceptibility plays an important role in disease risk, particularly class II HLA alleles. It is estimated that two to four times more people who have a family member with sarcoidosis are likely to develop the disease as well. This risk increases with the number of affected relatives. According to estimates, sarcoidosis is inherited at a rate of 39–70 percent.

• In the ACCESS study conducted in the United States, occupational exposures were studied retrospectively by using questionnaires. The findings indicated that patients with sarcoidosis were exposed to mold, insecticides, agricultural environments, silica dust in foundries, and firefighters as a result of exposure to various environmental agents. Inorganic materials have been shown to cause sarcoidosis in several studies. As a result of nicotine's immunomodulatory effects, smoking has consistently been associated with a lower risk of developing sarcoidosis.

Several studies have found cases of immunotherapy-induced sarcoidosis in individuals treated with immunostimulating drugs for malignant or chronic diseases (anti-IFNα, anti-IFNγ as in

psoriasis, anti-PD1 and BRAF inhibitors in melanomas). To date there is no consensual opinion on whether it is true sarcoidosis or sarcoidosis-like reactions in these subjects.

These antibodies are able to restore the proliferative capacity of CD4 + T lymphocytes, leading to the formation of granulomas. Biological anti-TNFα drugs (etanercept, infliximab, adalimubab) are rarely associated with the onset of sarcoidosis (or sarcoidosis-like reactions).

Cases of sarcoidosis have also been reported in bone marrow transplant recipients from donors with sarcoidosis.

6.4 Comorbidity and mortality

The prevalence of sarcoidosis in the general population has been found to be higher than expected by epidemiological studies because it is associated with a high disease burden and an increased mortality rate. There are 9-14 cases of sarcoidosis death per 1,000 persons-years, and 93–95 percent of patients survive five years after diagnosis. Sweden increased mortality risk by 60%, Korea by 70%, the UK by twofold, and black American women increased mortality risk by 2.4 times. According to a French study of patients with stage IV sarcoidosis, mortality is higher in subjects with more severe disease at diagnosis. Patients with sarcoidosis and immunosuppressive therapy, cerebrovascular disease, venous thromboembolism, or autoimmune diseases are more likely to contract sarcoidosis-related infections (79). Based on a systematic review and meta-analysis of 16 studies, sarcoidosis is associated with an increased risk of hematological, skin, upper digestive tract, and kidney cancers.

6.5 Pathogenesis of granuloma

The histopathological feature of sarcoidosis is the presence of non-necrotizing epithelioid cell granulomas, with varying degrees of lymphocytic inflammation (80). These granulomas are the result of exposure to unknown antigens in genetically predisposed individuals, in which there is an immune dysregulation with an exaggerated response of T-helper1 (Th1) lymphocytes. In the 1960s and 1970s, clinical studies demonstrated peripheral lymphocytopenia in patients with sarcoidosis and subsequently, with the experience of bronchoscopy with alveolar bronchial wash (BAL), an intense lymphocyte activation was detected in the affected disease sites (in the case of BAL the lung), with a prevalence of CD4 + T-helper lymphocytes. The latter differentiate into Th1 and Th17 effector cells with the production of proinflammatory cytokines (IFNγ, IL17, TGFβ) (81).

Th17 cells in sarcoidosis are poorly understood, but they appear to play a crucial role in defining its clinical symptoms (they are highly expressed in Lofgren syndrome). The immune response is crucial in the pathogenesis of sarcoidosis. Presumably there is a trigger (unknown antigen) that stimulates the response of macrophages and dendritic cells. In the presence of activation, macrophages transform into epithelioid cells that can group into giant cells with multinucleations. Serum amyloid protein A (SAA) also contributes to the aggregation of these cells into granulomas, which amplifies the immune response of Th1 lymphocytes.

The resolution of the granuloma occurs when the peptide antigen (unknown) is presented by the HLA-DR3 molecules present on dendritic cells or macrophages, with recognition by the CD4 + T cell receptor (TCR). The result is an immune response that involves a cytokine cascade that leads to the elimination of the antigen and resolution of the granuloma (type 1 immune response).

Instead, the progression of the granuloma occurs when there is no antigen recognition, perhaps due to the exposure of other molecules other than HLA-DR3. As a result, the granuloma continues to expand and the disease persists (type 2 immune response) (Figure 9).

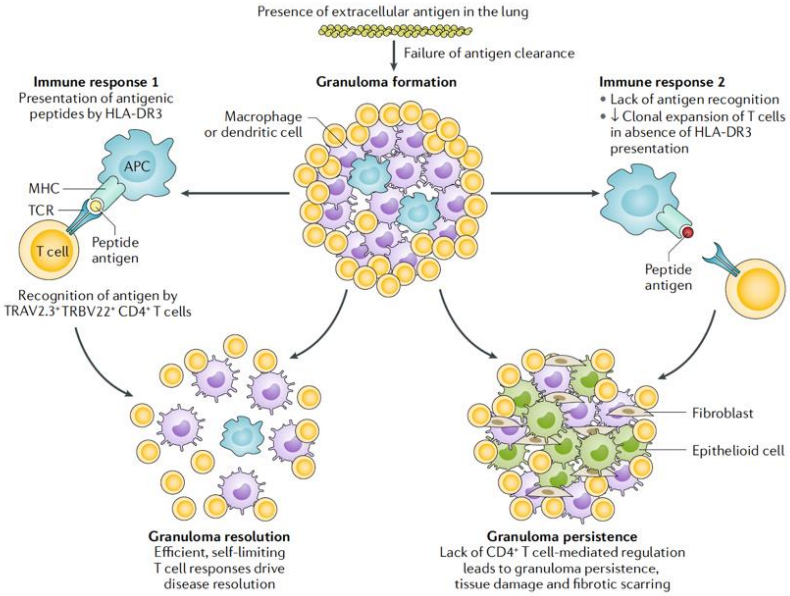


Figure 9. Pathogenesis and evolution of sarcoid granuloma

6.6 Clinical manifestations

It is possible to classify sarcoidosis clinically based on its onset, natural history and organ involvement. Sarcoidosis can begin abruptly or gradually, but it can be diagnosed incidentally during tests conducted for other reasons when asymptomatic sarcoidosis is detected. Scandinavian populations typically experience acute onset with fever, erythema nodosum, diffuse arthralgia, and subcutaneous adipose tissue inflammation. There can also be cardiac arrests or neurological manifestations associated with the pathology in some cases. A 50% remission rate is achieved after 2 years, while a 5 year remission is more uncommon (remission after 5 years). It has the most severe clinical manifestations pulmonary, cardiac and neurological (82).

The disease can also manifest itself in a syndromic manner and be diagnosed without the need for a histological examination. Lofgren's syndrome occurs in patients with a combination of genetic, immunological and environmental factors (onset mainly in the spring months). This syndrome is a very characteristic phenotype of sarcoidosis (83).

Heerfordt's Syndrome, on the other hand, is a rarer form that arises with uveitis, enlargement of the parotids, paresis of a cranial nerve (especially the VII) and is predominantly a sporadic form.

Pulmonary sarcoidosis: In the thoracic area, the organs most involved are the lungs and the mediastinal lymph nodes (80-90%) of cases. There is a radiological staging of pulmonary sarcoidosis described for the first time by John Scadding in 1950), subsequently also revealing a prognostic value. Pulmonary sarcoidosis can progress to pulmonary fibrosis progressively and with poor prognostication. Different radiological patterns were found on CT scans in the fibrotic evolution of sarcoidosis, such as traction bronchiectasis, cysts, honeycombing.

6.7 Diagnosis

The diagnosis of sarcoidosis provides a clinical consideration to the histopathological finding of non-necrotizing granulomas. Possible differential diagnoses should be excluded, and a diagnostic conclusion should be reached after discussion with a multidisciplinary group.

Unfortunately, a diagnosis of sarcoidosis is made at the first visit in only 15% of cases.

A number of laboratory tests can be performed as a first step, including calcium determination, vitamin D dosage, angiotensin converting enzyme (ACE) as well as serum interleukin-2 receptor (rIL2), but these tests have a very limited diagnostic value. More than anything else,

they are useful biomarkers for monitoring the progress of the disease and the response to therapy.

Although chest X-rays may detect the first signs suggestive of pulmonary sarcoidosis, it is high-resolution pulmonary computed tomography (HRCT) of the chest that certainly shows sarcoidosis pulmonary involvement. In patients with Lofgren's syndrome, the diagnosis is made only with a chest X-ray and by monitoring the progress of the disease. In more complex cases of pulmonary sarcoidosis, HRCT makes it possible to assess the presence of pulmonary fibrosis, or complications secondary to immunosuppressive therapy (aspergilloma) or non-infectious complications such as pulmonary hypertension. As a result of magnetic resonance imaging with gadolinium (MRI) of the heart and brain, possible pathologic involvement can be evaluated; furthermore, echocardiography is already capable of showing alterations at the level of the left ventricle (alterations in kinetics) and the right ventricle (increased diameters due to pulmonary hypertension), which indicate a poor prognosis. Nuclear radiology with positron emission tomography (PET-CT) with fluoride-deoxyglucose (18F-FDG) could also help identify occult inflammatory lesions in the lungs and heart, indicative of disease activity (84).

Bronchoscopy is currently a fundamental diagnostic test, with the possible finding of endobronchial granulomas on which to perform a biopsy examination. Alveolar bronchial lavage (BAL) is supportive in excluding alternative diagnoses, resulting in a strong diagnostic for sarcoidosis when there is significant lymphocytosis with an increase in the CD4 + / CD8 + ratio> 3.5.

In order to exclude other possible pathologies responsible for mediastinal lymphadenopathy, transbronchial ultrasound-guided lymph node aspiration (EBUS-TBNA) should be performed (85).

Figure 10 summarizes the diagnostic algorithm for sarcoidosis, which is based on a lung function test that does not play a diagnostic role in this disease.

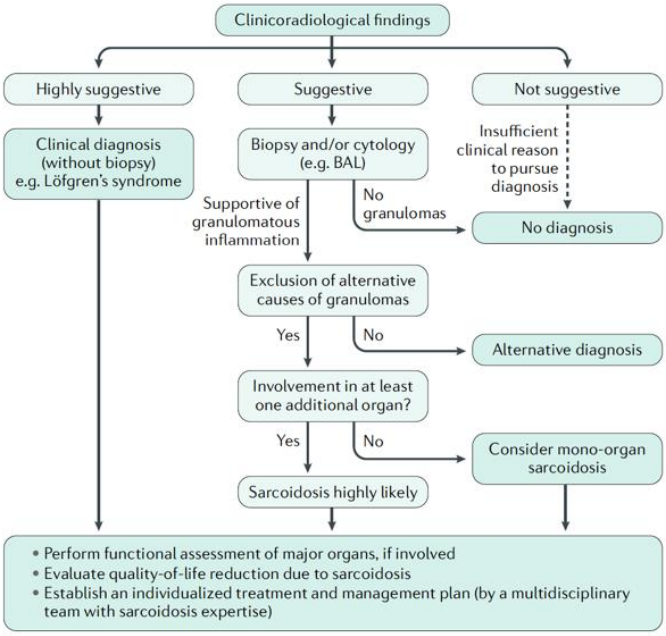


Figure 10. Diagnostic algorithm of sarcoidosis

6.8 Therapy

The clinical course of sarcoidosis is highly variable, from spontaneous resolution to severe forms with pulmonary and cardiac involvement leading to lung transplantation. The introduction of a therapy should be evaluated on the basis of the clinic, the therapeutic benefits and side effects.

There are patients who need only corticosteroids, others oxygen therapy, others implantable defibrillators due to ventricular arrhythmias, others desmopressin therapy for diabetes insipidus.

The main anti-inflammatory therapy administered in sarcoidosis is that with glucocorticoids, which must be personalized in the light of the damage it causes if taken in chronic form (arterial hypertension, iatrogenic diabetes mellitus, neuropathy, myopathy, adrenal insufficiency). There is evidence that steroid therapy improves or stabilizes the functional status of patients with evident functional deterioration. Studies show that a major lymphocytosis in BAL is associated with forms of sarcoidosis that undergo spontaneous remission, while a neutrophilia would be indicative of progressive forms that require close monitoring (86). Glucocorticoids are the first line of treatment in the symptomatic patient. Inhaled steroids cannot be used as a substitute for

oral steroid therapy. Initially, prednisolone is administered at a concentration of 0.5-0.75 mg / kg / day for four weeks, and then decreased to 10 mg / day for another four weeks, and modified accordingly. The therapy is usually continued for 12 months, and the suspension of it is evaluated according to the functional improvement and disappearance of symptoms. Relapses of the disease may occur 24 months after diagnosis in refractory forms. It has been demonstrated that patients who take low maintenance doses for six to twelve months are more likely to develop weight gain, insomnia, glaucoma, cataracts, diabetes, myopathy, personality changes, and osteoporosis. There may be relapse of the disease in some patients after discontinuing chronic cortisone therapy. When this is the case, corticosteroid therapy should be resumed at 0.5 mg / kg / day for 8-12 weeks, and followed by a maintenance dose of 5-10 mg / day in conjunction with an immunosuppressive medication. It is recommended to continue this therapy for two years at a minimum if it is well tolerated.

As a result of sarcoidosis, approximately 5-10% of patients experience hypercalcemia. Bisphosphonates can be administered to prevent this condition. If steroid therapy does not work, immunosuppressants like azathioprine, methotrexate, and mycophenolate mofetil may be administered. It may be necessary to administer cyclophosphamide in conjunction with oral steroid therapy in case of failure (87). In patients with extrapulmonary sarcoidosis that is intolerant of treatment, monoclonal antibodies that inhibit TNFalpha are used (infliximab, adalimumab).

When pulmonary sarcoidosis or hypertension reaches an advanced stage, lung transplantation may be an appropriate therapeutic intervention.

7. Rheumatologic diseases associated to ILD (RD-ILD)

There is evidence that ILDs can complicate the course of autoimmune diseases such as kidney disease, arthritic arthritis, and systemic sclerosis. Even though most patients with ILDs can have a slow progression, some are at risk of developing lethal respiratory failure as a result of extensive fibrosis (88,89).

Diagnosis of ILD is made primarily by lung function tests, HRCT and, if necessary, lung biopsy. However, these techniques have limitations that restrict their use in patient monitoring due to the high dose of radiation, the patient's poor collaboration in spirometry, invasiveness and the high procedural risks of surgical or endoscopic lung biopsy. For this reason, serum biomarkers are preferred which can identify the existence of a pulmonary interstitial disease and allow for its progression to be followed.

7.1 Rheumatoid arthritis

7.1.1 Definition

Inflammation of peripheral joints with progressive destruction and systemic symptoms is characteristic of rheumatoid arthritis, a chronic systemic autoimmune disease. The condition affects about 1% of the population, primarily women, and it can occur at any age, but it is more common in the 35-50 age group. In addition to childhood forms like juvenile idiopathic arthritis and old age, there are also childhood forms of arthritis (90).

7.1.2 Etiopathogenesis and epidemiology

The precise etiology is still unknown today, however it would seem essentially attributable to three factors: a relative lack of cortisol, essential for the proper functioning of the immune system; a deficiency of dehydro-epi-androsterone (DHEA); body infections caused by agents such as Mycoplasma, with a relatively low virulence and capable of causing inflammation and tissue destruction in the joint and periarticular areas of immunocompromised hosts (91).

A shared epitope was also identified in the HLA-DR B1 locus of class II histocompatibility antigens which would be the basis of a genetic predisposition to the disease.

It affects 1% of the population and more frequently women, with an onset that can occur at any age, although it occurs more frequently in the 35 to 50 age group. There are also forms of childhood, such as juvenile idiopathic arthritis and old age (92).

7.1.3 Extra-articular involvement

Rheumatoid arthritis can cause late subcutaneous nodules, which may be seen especially on the forearm extensor surface, as well as asymptomatic visceral nodules in severe cases. It is a type of vascular disease characterized by leg ulcers, multiple mononeuritis, pleural effusions or fibrosis of the lungs, pericarditis or myocarditis, as well as lung infiltrates or fibrosis, lymphadenopathies, scleromalacia, episcleritis, and secondary ILD are also characteristic. Sjogren's Syndrome and Felty's Syndrome can also be associated.

7.1.4 Diagnosis

More than 90% of patients can be prevented or slow down from developing irreversible joint damage by early diagnosis and treatment. In recent years, new tools have been developed for monitoring disease activity and identifying whether remission exists, thus enabling new treatment strategies to slow the progression of the disease before irreversible disability occurs (90).

The diagnosis is essentially divided into:

• Clinical evaluation

• Complete blood count (possible finding of normochromic anemia)

• Dosage of: Rheumatoid Factor, Anti-CCP (anti-citrullinated peptide antibodies), CRP (which reflects the activity of the disease)

• RX

• analysis of the synovial fluid

The Rheumatoid Factor is found in about 70% of patients with rheumatoid arthritis, but it is not specific for this pathology, being also found in other subjects affected for example by connective tissue diseases such as Systemic Lupus Erythematosus, granulomatous diseases,

chronic infections. Anti-CCP antibodies are characterized by high specificity (90%) and sensitivity (86%) and correlate with a poorer prognosis.

From a radiological point of view, initially there is only a swelling of the soft parts. The following will appear:

• Periarticular osteoporosis

• Narrowing of the joint space

• Marginal erosions (can appear at any time)

RA should be differentiated from other inflammatory arthritis (e.g., septic and crystal arthritis) by examining synovial fluid in any new-onset effusion. While active joint inflammation occurs in rheumatoid arthritis, the synovial fluid is cloudy, yellow, sterile, and generally contains 10,000 to 50,000 white blood cells per milliliter (10.0 x109 / L to 50.0 x109 / L); polymorphonuclear cells usually dominate, but lymphocytes and mononuclear cells may constitute more than 50% of the cells. There are no crystals present.

7.1.5 Therapy

The therapy uses supportive measures, such as adequate nutrition, smoking cessation, a good balance between rest and exercise, physical therapies, and the use of drugs.

7.1.6 Rheumatoid arthritis associated with ILD (RA-ILD)

Rheumatoid arthritis is associated with the most commonly occurring forms of interstitial lung disease, including Organizing Pneumonia (OP), Desquamative Interstitial Pneumonia, Interstitial Lymphocytic Pneumonia, Diffuse Alveolar Damage, and Acute Interstitial Pneumonia. In some studies, smoking, older age, high anticitrullinated peptide antibodies, high rheumatoid factor concentrations, and a family history of RA may all be risk factors for developing RA-ILD. Researchers remain uncertain of the pathophysiological basis for ILD in RA patients. Available data suggest that environmental and genetic factors may play a role. A number of variants of the human leukocyte antigen (HLA) have been linked to RA-ILD (93). In addition, cigarette smoking is associated with both RA and RA-ILD. A dysregulation of immune function within the lungs may be a contributing factor to RA development. Cigarette smokers without RA have been shown to have citrullinated proteins in their alveolar bronchial

lavage fluid, while patients at risk for RA have RA-related autoantibodies in their sputum. By investigating biomarkers for RA-ILD, we hope to identify key molecules, thereby providing additional information on the immunopathogenesis of the disease and a means of early diagnosis. Multiple citrullinated proteins and peptides have been associated with RA-ILD, including fibrinogen, vimentin, heat shock protein 90, matrix metalloproteinase 1, and interferon-gamma inducer 10. It is unclear how these proteins play a role in the manifestation of tissue-specific diseases (94).

7.2 Systemic sclerosis

7.2.1 Definition

There are three main clinical manifestations of sclerosis (systemic sclerosis, SSc): the appearance of disease-specific autoantibodies in connective tissue, organ fibrosis, and small vessel vasculopathy.

There are many different types of fibrosis in SSc, which can affect multiple tissues. The most common types are skin, lungs, gastrointestinal tract, heart, tendons, and ligaments. There may also be diffuse perivascular fibrosis present (88).

7.2.2 Epidemiology

In the United States, there are about one to two cases of SSc per 10,000 people, and the prevalence varies according to sex and age. SSc is three to four times more prevalent in women than in men. It occurs most frequently in people between the ages of 30 and 50.

7.2.3 Classification

A SSc with or without skin involvement can be divided into two main clinical subgroups based on the extent of the skin fibrosis: a SSc with limited cutaneous involvement and a SSc with diffuse cutaneous involvement. These main subgroups also differ in frequency, in the different autoantibodies associated with SSc and in cardinal clinical features.

An SSc diagnosis is made based on skin thickening between the metacarpophalangeal joints (MCP) of the fingers that extends proximally to the metacarpophalangeal joints. It is also possible to evaluate seven additional clinical features that may be variable in nature, including skin thickening, fingertip lesions (digital toe ulcers or pitting scars), telangiectasias, abnormal

capillaroscopy of nail folds, pulmonary arterial hypertension, ILD, Raynaud's syndrome, and autoantibodies that are associated with SSc (anticentromere, anti-topoisomerase I, anti-RNA polymerase III).

The classification system assigns 9 points if a patient's fingers on both hands have thickened skin proximally to the metacarpophalangeal (MCP) joints, which constitutes enough evidence to identify the patient as suffering from SSc (95) and no further application of the point system is required. The points system is based on the sum of the scores for all successful events, with a maximum score in each category. There is a maximum possible score of 19 and patients who have nine or more points are considered to have SSc.

7.2.4 Clinical manifestations

Most patients with SSc present with skin thickening and internal organ involvement, with variable clinical manifestations and prognosis. In addition to the subsets of SSc mentioned previously, it is possible to distinguish three different types: limited cutaneous SSc (lcSSc), diffuse cutaneous SSc (dcSSc), affecting the proximal limbs, abdomen and thorax, and SSc without skin involvement.

Patients with cutaneous SSc (lcSSc) usually have affected skin that extends proximally to the elbows and may also involve their trunks. In contrast, patients with diffuse cutaneous SSc (dcSSc) also have affected skin that extends proximally to the elbows and may also involve their trunks.

It is very common for limited SSc (70% of cases) to present with subcutaneous calcinosis, Raynaud's phenomenon, dysmotility in the esophagus, sclerodactyly, and telangiectasias, as well as pulmonary hypertension (96). Raynaud's phenomenon occurs simultaneously with diffuse SSc. There is a syndrome of scleroderma induced by inhalation of mineral particles known as SSc sinusoidal scleroderma, but pulmonary involvement seldom precedes scleroderma in patients with both limited scleroderma and diffuse scleroderma.

7.2.5 Diagnosis

According to some studies "the thickening of the skin of the fingers of both hands that extends proximally to the metacarpophalangeal joints" is sufficient to classify a subject as suffering from SSc; while patients with "thickening of the skin that spares the fingers" are classified as not affected by SSc. Anticentromeric antibodies are present in 70% of patients with limited SSc

while antitopoisomerase antibodies are present in 30% of patients with diffuse SSc and anticentromeric antibodies are generally absent (present in 3% of patients). Capillaroscopy is also useful for the diagnosis of SSc and is now widely used as it allows an enlargement of the nail fold which is useful in the diagnosis and management of SSc. A simple ophthalmoscope or dermatoscope can be sufficient to distinguish normal from abnormal nail fold capillaries when performing capillaroscopy.

7.2.6 Systemic sclerosis associated with ILD (SSc-ILD)

Along with vascular damage and autoimmunity, SSc leads to progressive tissue and organ fibrosis due to excessive extracellular matrix (ECM) deposition. SSc affects not only the skin, but also various internal organs, such as the lungs, heart, kidneys, gastrointestinal tract, and musculoskeletal system, leading to skin fibrosis as well as various systemic effects. A common systemic manifestation of SSc is ILD, which affects the lungs (97). The prevalence of clinically significant ILD in patients with SSc is approximately 50% within the first five years after diagnosis. ILD accounts for 33% of all SSc-related deaths, which is the leading cause of death in SSc patientsThe forced vital capacity (FVC) and lung diffusion capacity for carbon monoxide (DLco) of these patients should be measured frequently in order to detect ILD timely. As of today, there are no treatments available to reverse pulmonary fibrosis, which occurs as a consequence of progressive inflammation and fibrosis of the lung tissue. In order to manage this disease effectively, it is imperative to intervene early on so that it does not progress. No matter how severe the disease is, SSc patients with ILD should initiate treatment when their lung function deteriorates or their radiographic condition worsens. Generally, ILD caused by Ssc is treated with immunosuppressive therapy using CYC and mycophenolate mofetil (MMF). (98). CYC is preferred because it has a lower side effect profile and is more tolerable.

8. Overview of Lung Mucins

The respiratory tract is protected and lubricated by a mucus layer. Mucus glycoproteins (mucins) and antimicrobial proteins comprise it, along with water, ions, lung secretions, serum proteins, and ions (ions) (99,100). Mucins are high-molecular-weight glycoproteins produced by goblet cells on the surface epithelium, submucosal glands, and serous cells. Several amino acids tandem repeats (TRs) are present in these proteins, especially serine, threonine, and proline. These proteins contain an extensive glycosylated protein structure.

In mucins, the secretion mode (secreted mucins) or the tethering mode (transmembrane mucins) are classified. It is possible to further separate secreted mucins into mucins that form gels and mucins that do not form gels. Currently, 21 human MUC genes have been identified, with 16 identified in the lung. These genes encode 10 TM mucin types, 4 gel-forming mucin types, and 2 nongel-forming mucin types (101,102). Around 90 percent of mucin in sputum comes from MUC5AC and MUC5B combined: MUC1, MUC4, and MUC16.

8.2 Secreted Mucins

Among the glycoproteins found in mucus, mucins play the most important role (103). Furthermore, they both have a similar gene structure consisting of a large central exon containing the entire TR domain, nonrepetitive domains, as well as flanking 5′ and 3′ regions that contribute to polymerization via disulfide bonds. Mucin TR domains differ from one another in their sequences and length, and they can contain polymorphisms of the variable number tandem repeat type (VNTR). Among their major roles is providing scaffolding for O-linked carbohydrates.

It is noted that secreted gel-forming mucins have high molecular weights (5 to 40 MDa), large size (600 to 900 nm), high glycosylation percentages (50%–80%), and the ability to oligomerise and form viscoelastic gel, forming a matrix that traps bacteria and serves as a barrier to the respiratory system. In addition, they are able to capture, retain, and release molecules which have biological activity, such as cytokines, growth factors, and trefoil factors. Due to their association and dissociation properties, mucins have the ability to influence inflammation and immune responses as well as post-injury epithelial repair. The mucus is also lubricated by the gel-forming mucins. As a result, secreted non-gel-forming mucins are observed as monomers instead of oligomerizable gels.

The two main and best-described mucins that form gels are MUC5AC and MUC5B. MUC5AC and MUC5B levels are elevated in asthma patients and chronic obstructive pulmonary disease (COPD) patients, respectively (104). The function of MUC2 and MUC19 in the lung is not known, as is the function of MUC7 and MUC8. However, COPD patients have been observed to have decreased expression of MUC2, and despite not being investigated in airway mucus, saliva has been well characterized for secreted non-gel-forming mucin MUC7. According to reports, MUC7 has an antifungal activity and interacts with bacteria thanks to a histatin-like domain in its N terminus. This suggests that MUC7 plays an essential role in innate defense. Several studies have associated genetic variants of the toll-interacting protein (TOLLIP), which regulates innate immune responses, with sporadic IPF. In an effort to understand the origin of lung fibrogenesis, innate immune mechanisms have been implicated, as well as toll-interacting proteins and their functions (105). MUC7 appears to be an essential component of IPF disease, as indicated by these findings. African-Americans and Northern Europeans with MUC7*5 polymorphisms, which have nine fewer potentially O-glycosylated sites, are at a significantly reduced asthma risk. These results indicate that this association is caused by allelic differences in bacterial interactions because of the glycosylated domain at the N terminus.

8.3 Transmembrane Mucins

An epithelial cell's apical surface is covered with transmembrane mucins, which are large glycoproteins. Cell surface mucins are characterized by non-covalent sodium dodecyl sulfate-labile bonds holding dimerizations of two dissimilar subunits together (α and β chains). It is highly glycosylated and entirely extracellular. Smaller subunits (β chains) are composed of a single-pass TM domain, a cytoplasmic tail, and an extracellular part (serine protein, enterokinase, or agrin domains) (Figure 11). (106).

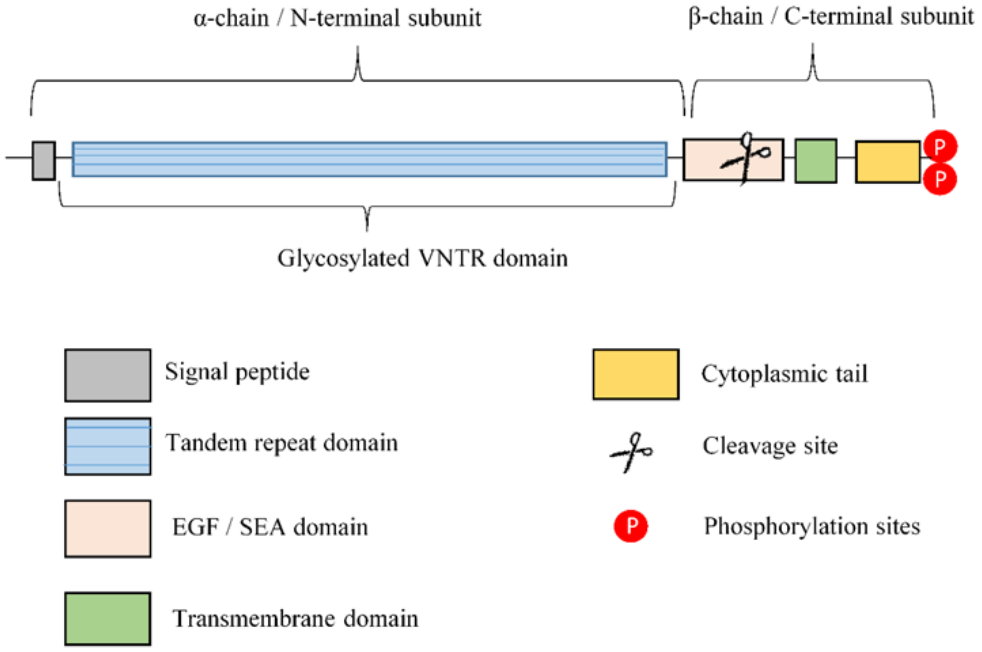


Figure 11. J. Clin. Med. 2019, 8(9), 1447; https://doi.org/10.3390/jcm8091447

It functions as a classical innate immune receptor, sensing an external environment and activating intracellular signaling pathways. As such, it is pivotal to the maintenance of mucous membranes and repairing damage. According to some theories, mucins are released from extracellular sites in response to mechanical forces, microbe interactions, pH changes, ionic concentration changes, or hydration changes, as well as inflammatory stimuli, such as tumor necrosis factor-α (TNF-α) and neutrophil elastase. High glycosylation levels in this extracellular domain contribute to barrier formation in addition to protecting the protein backbone from proteolytic attacks by bacteria and hosts. In addition to the putative phosphorylation sites present in all TM mucins intracellular tails, these tails differ in sequence and length and do not contain conserved domains. It appears that TM mucins have a high degree of functional divergence and, most likely, differ in their signaling specificities (107). It is nevertheless believed that TM mucins contribute significantly to cellular proliferation, apoptosis, and epithelial to mesenchymal transition (EMT) processes, in accordance with IPF observations. According to the above information, the lung is predominantly composed of MUC1, MUC4, and MUC16 TM mucins.

9. Krebs von den Lungen-6 (MUC1)

In humans, the MUC1 gene encodes KL-6, a mucin that is located on chromosome 1. This protein has long been associated with cancer, as it is overexpressed and abnormally glycosylated in many types of cancer (including ovarian, colon, lung, pancreatic, and gastrointestinal cancers) (108). It has been found that patients with IPF who have overexpression of the MUC1 gene have pulmonary fibrosis. The serum prognostic marker most commonly used in breast cancer is the soluble form of MUC1, also known as CA15-3.

An epithelial transmembrane domain anchors the glycoprotein to the apical epithelium. Extracellular N-terminus subunits, also known as KL-6, are composed of a 20 amino acid domain repeated in tandem with a 25-125 repeat VNTR from SEA domains and transmembrane domains, as well as a 72 amino acid cytoplasmic tail. In addition to 18 phosphorylation sites, this subunit interacts with various effectors involved in cell proliferation, apoptosis, transformation and gene expression (109). In the vicinity of the plasma membrane, metalloproteinases such as MT1-MMP and MMP14 can release the extracellular domain into the lumen following proteolytic cleavage of the SEA domain. In patients with idiopathic pulmonary fibrosis, the presence of MUC1-N / KL-6 in biological fluids is thought to be attributable to the elevated levels of MT1-MMP present in pulmonary fibrosis (Figure 12).

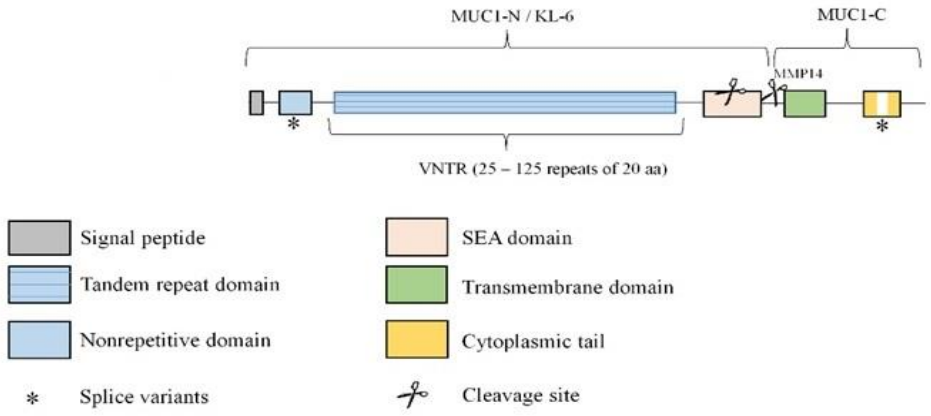


Figure 12. (J. Clin. Med. 2019, 8(9), 1447; https://doi.org/10.3390/jcm8091447)

A key transmembrane mucin in the lungs, KL-6 is mainly involved in cell proliferation, growth, and apoptosis, and is expressed at higher levels in injured or regenerating pneumocytes. Type

2 pneumocytes produce epithelial mucins that have not been fully clarified in terms of their pathophysiologic role. Repairing injured alveolar epithelium relies on Type 2 pneumocytes, which cover the space left by damaged Type 1 pneumocytes desquamating from the basement membrane (108). The increased production of TGF-beta by activated Type 2 pneumocytes in patients suffering from idiopathic pulmonary fibrosis has been demonstrated, and TGF-beta is known to act as a mitogen for fibroblasts, suggesting that Type 2 pneumocytes may play a role in fibrotic formation (51).

By immunizing a mouse with human lung adenocarcinoma cells, Kohno et al (Japan) developed a monoclonal antibody designated KL-6 that can recognize sialylated sugar chains (110). The serum biomarker KL-6 was initially suggested as a diagnostic tool for lung, breast, and pancreatic cancer, however it has proved less accurate than other tumor markers in terms of diagnostic accuracy. KL-6 was identified as a serum biomarker of lung disease in 1992 by the Diagnostic Division of Eidia Co. Ltd. (Tokyo, Japan), which resulted in the development of a new ELISA to measure KL-6 levels (108). A diagnostic marker of ILD, KL-6, was approved by the Japanese Health Insurance Program in 1999. In ordinary Japanese clinical settings, the Chemiluminescenze Enzyme ImmunoAssay (CLEIA) system has been available for detecting serum KL-6 levels, but not in Western European countries, including Italy. By using Lumipulse fully automated instrument, the CLEIA method can be performed in just one hour with a first result available in 35 minutes.

To distinguish between fibrotic ILD and healthy subjects, researchers suggested a serum cut-off value of 465 U/mL in 2002 (111).

A total of 17 patients were immunohistochemically evaluated using KL-6 antibody on lung tissue staining with typical and nonspecific interstitial pneumonia, hypersensitivity pneumonitis, collagen vascular disease-associated interstitial pneumonia, viral pneumonia, and bronchobronchioectasis, according to Ohtsuki. A spontaneous pneumothorax led to the resection of ten cases of presumably normal pulmonary tissue (112).

There were continuous linear patterns of antibodies to KL-6 surrounding damaged areas in most interstitial pneumonias as well as some normal areas, suggesting that type 2 pneumocytes overexpress KL-6. Patients with ILD have been found to have higher levels of KL-6 in their sera, although it has been unclear whether this is solely caused by their lower respiratory tracts.

A number of polymorphisms have been identified that affect serum levels of KL-6 as well as ethnicity. ILD patients with MUC1 rs4072037 A/A genotypes have a poor prognosis as well as severe pulmonary dysfunction and a high rate of disease progression. Horimasu et al. demonstrated that rs4072037 genotype distribution differed from control groups in German and Japanese cohorts. In the same study, the authors found that the German cohort's KL-6 cut-off values were significantly higher than the Japanese cohort's (113).

According to a recent study, KL-6 is a reliable prognostic biomarker that provides insight into the response of IPF patients to nintedanib treatment. The FVC and KL-6 levels of IPF patients treated with nintedanib for 12 months remain stable. In spite of this, there is no complete understanding of how KL-6 works (114). Separated KL-6 also promotes migration, proliferation, and differentiation of alveolar cells in addition to lung fibroblasts. The bleomycin-induced lung fibrosis was reduced by antibodies against KL-6, whereas knock-out mice of MUC1 exhibited improved lung function, survival, and fibrotic lung tissue remodeling (113).

Many authors have found that patients with sarcoidosis have higher levels of KL-6 in their serum than controls. As Janssen et al. proposed, KL-6 has the best discriminative capability when it comes to assessing sarcoidosis severity and distinguishing patients from controls. Interestingly, Bergantini et al found that serum KL-6 concentrations were proportionally higher in patients with fibrotic phases of sarcoidosis than in those with other radiological stages (115). The BAL KL-6 levels in another population have been shown to correlate with BAL KL-6 levels reported by d'Alessandro et al. At a cut-off of 221 U/mL, sarcoidosis was distinguished from other ILDs using this correlation with CD4/CD8 ratio (116).

Currently, there are very few data available on KL-6 in patients with hypersensitivity pneumonitis (HP). The only data available on KL-6 in HP patients is pertaining to the fibrotic variants, since this biomarker was detected to better study interstitial lung involvement (117). A study by Okamoto et al. determined that serum KL-6 concentrations could be used to diagnose and manage chronic HP in patients with high levels of KL-6 in comparison to IPF and sarcoidosis, with a cutoff level of 1500 U/ml used to distinguish between IPF and sarcoidosis.

Only two studies investigated the role of KL-6 in the diagnosis of miscellaneous cystic ILD: LAM and PLCH. The authors demonstrated higher KL-6 concentrations in PLCH (118) and a rare interstitial lung disease, LAM causes greater alveolar damage than controls. Moreover, they showed a prognostic role of KL-6 for monitoring LAM patients (119).

KL-6 has shown the strongest sensibility and accuracy for ILD diagnosis in rheumatic autoimmune diseases (ARD). More than 10 studies showed the utility to perform KL-6 assay in RA patients to identify the interstitial lung involvement as well as to monitor disease activity (120–122). Recently, it has been reported the efficacy of baricitinib to reduce the serum KL-6 concentrations in RA patients, including a subgroup with interstitial lung impairment.

ILD is a life-threatening complication among ARD patients. No definite diagnostic work-up has been to date validated for these patients, nor robust evidence exists about the optimal management, as this condition is usually poorly responsive to conventional immunosuppressants, which have proved controversial efficacy. A study by Nakajima et al. examined serum KL-6 in ARD patients with and without interstitial lung disease. The authors proposed that KL-6 be used as a marker of the severity of the disease in ARD patients (123). There has been considerable research done in SSc with good demonstration of KL-6's ability to discriminate between disease severity and pulmonary function tests with moderate to high correlations with quantitative HRCT scores of lung involvement (124).

10. Aim of the study

Among idiopathic interstitial pneumonias, the most common is IPF, characterized by a progressive clinical course, sometimes interrupted by high mortality events, defined as “acute exacerbation”. In the last decades, it has been reported that also patients with non-IPF ILDs, such as fibrotic HP or connective tissue diseases associated with ILD (CTD-ILD: RA-ILD, SSc-ILD), can show a progressive worsening of the disease regardless of treatments. Moreover, many recent studies have suggested the presence of common pathogenetic pathways between IPF and non-IPF ILDs, like shortening of telomeres, epithelial cell dysfunction and immune dysregulation. For these reasons, a new progressive fibrotic phenotype has been recently proposed to include all ILD patients (both idiopathic and non-idiopathic) that show an inexorably worsening of disease, based on clinical and functional progression of the disease. Therefore, identification of prognostic biomarkers for ILDs is more compelling than ever. In IPF, many biomarkers have shown to have prognostic significance, but their utility in the clinical practice to predict outcome and answer to therapy is limited and no marker has been proven in non-IPF disease. Anyway, up to now, there is no single validated bioindicator that can be routinely used in the clinical management of ILD patients: many studies support the prognostic value of serum KL-6.

The aim of the present study was to assess the role of KL-6 as diagnostic marker of fibrotic ILD and predictor of the fibrotic progression. At the moment of diagnosis and before any pharmacological treatment, the primary aim was to compare KL-6 concentrations in IPF, fibrotic HP, LAM, PLCH, sarcoidosis, RA, and SSc with and without ILD. The secondary aim was to characterize and compare the progressive phenotype of IPF, RA- and SSc-ILD patients assessing the functional progression in accordance with serial serum KL-6 concentrations.

11. Material and methods

11.1 Patients

The study involved 107 patients (median age *IQR*, 65 (54-71) years) who were being monitored at the Rheumatology Unit at Siena University Hospital and at the Sarcoidosis and Interstitial Lung Disease Referral Centre at Siena.

Thirty-five had diagnoses of IPF (median age *IQR*, 69 (63-76) years; 26 males), 18 sarcoidosis (median age *IQR*, 53 (48-63) years; 2 males), 10 PLCH (median age *IQR*, 55 (51-68) years; 3 males), 5 LAM (median age *IQR*, 43 (41-45) years; one male), 24 fibrotic HP (median age *IQR*, 70 (65-74) years; 13 males). International criteria were used for multidisciplinary discussions, and international ATS/ERS guidelines were followed for diagnosing (6,80). Exclusion criteria were patients with a follow-up inferior to 24 months, fibrotic HP patients without any histological confirmation diagnosis, patients with active malignant diseases and acute lung infections, PLCH patients without similar predominantly nodular radiological HRCT patterns and sarcoidosis patients with Lofgren syndrome, acute disease onset or spontaneous resolution of disease.

Among rheumatologic diseases, thirteen patients had diagnoses of RA (median age *IQR*, 66 (59-68) years; 2 males) based on American College of Rheumatology criteria and 4 out of 13 had ILD involvement (96,125). Twenty-two had diagnoses of SSc (median age *IQR*, 64 (57-68) years; 4 males) according to international criteria. Eighteen out of 22 had ILD involvement and 10 out of 18 revealed radiological diagnosis of PPFE. Diagnosis of ILD was made according to ATS/ERS guidelines.

A chest high resolution computed tomography (HRCT) and lung function tests were administered to all patients as part of the diagnostic work-up for ILD. For each patient, peripheral blood sampling was collected for immunological analysis at the moment of diagnosis.

Serial serum samples were collected before therapy started (t0) and 24 months later (t1) from patients with IPF, SSc- and RA-ILD. LFT parameters were repeated according to our center's follow-up protocol and performed in accordance with ATS/ERS guidelines (126).

Medical records were reviewed for demographic and clinical information, including comorbidities, family history, lung function parameters, and radiological findings. This data was entered into an electronic database for statistical analysis.

We also enrolled 22 healthy controls (median age (IQR) 54 years (42-60); 6 males). All of them had normal lung function test parameters and no history of concomitant pathologies.

According to the Declaration of Helsinki, the study has been approved by the regional ethical review board of Siena, Italy (C.E.A.V.S.E. Markerlung 17431 and RHELABUS 22271).

11.2 Lung function test

An ATS/ERS standard plethysmograph with corrections for temperature and barometric pressure was used to measure lung function as per ATS / ERS standards. The forced expiratory volume in the first second (FEV1) and the lung diffusing capacity for carbon monoxide (DLCO) were measured.

11.3 Definition of progressive fibrosing phenotype

Progressive phenotype of ILD (PF-ILD) is defined as developing any of the following within 2 years of diagnosis despite treatment: a decline in FVC by 10%; a decline in FVC by 5%, with a 15% decline in DLCO, or with worsened symptoms or radiological appearances, or a decrease in FVC of 5%, with an increase in the extent of fibrosis with worsening symptoms.

11.4 KL-6 assay

According to previous studies, serum samples were collected from all patients, and Krebs von den Lungen-6 (KL-6) concentrations were determined by KL-6 reagent assays (Fujirebio Europe, Belgium) based on serum samples from all patients (115). By measuring the change in absorbance, KL-6 concentrations, expressed in Units per mL, were determined by agglutination of sialylated carbohydrate antigen by antigen-antibody reaction in samples.

11.5 Statistical analysis

A median is the value between the five quartiles (IQR) or the mean minus the standard deviation. We performed a one-way ANOVA (Kruskal-Wallis test) and Dunn test for multiple comparisons using nonparametric methods. The comparison between two groups was

performed through non-parametric Mann-Whitney U-test analysis. The Chi-squared test was used for categorical variables, as appropriate.

Patients were stratified according to diagnosis: IPF, fibrotic HP, LAM, PLCH, sarcoidosis, RA, RA-ILD, SSc, SSc-ILD at the time of diagnosis (T0).

In order to determine the best diagnostic cut-off values for KL-6, a regression decision tree was constructed to determine the best clustering variables based on the Gini criterion for ILDs (IPF, fibrotic HP, RA-ILD, SSc-ILD) as compared to non-ILDs (HC, LAM, PLCH, sarcoidosis, RA, SSc). In order to assess binary classifier accuracy, we created a series of test/training partitions by utilizing a confusion matrix. In order to determine the best thresholds for each binary classifier based on specificity and sensitivity, Youden's J method was used.

A multinomial logistic regression analysis was performed to model the probability of patients' diagnosis versus HC group given the values of a set of quantitative (age and KL-6) and/or qualitative (gender smoking habit) descriptive variables.

During the research, we formed a group of patients with non-fibrotic involvement (LAM, PLCH, sarcoidosis, RA, SSc) with the aim of investigating potential binary classifiers for the diagnosis of fibrotic patients (IPF, fibrotic HP, SSc-ILD, RA-ILD). In order to determine the best clustering variables based on the Gini criterion for fibrotic and nonfibrotic lung involvement, we constructed a regression decision tree to determine the optimal clustering variables. A confusion matrix was used to evaluate the accuracy of potential binary classifiers by creating a series of test/training partitions.

Progressive (IPF, SSc-ILD) and non-progressive (RA-ILD) patients were identified according to fibrotic progression at HRCT and LFT parameters performed during follow-up period.

In an exploratory analysis, supervised Principal Component Analysis (PCA) was used to determine trends in immunological properties (KL-6) and clinical features (FEV1, FVC, and DLco) using a two dimensional representation of the multidimensional data set.

In order to determine the best clustering variables for progressive versus non-progressive patients in order to improve KL-6's predictive prognostic role, a regression decision tree based on the Gini criteria was constructed to determine the best clustering variables. To assess the accuracy of potential binary classifiers using a confusion matrix, a series of test/training

partitions were constructed. Youden's J method was used to select the best thresholds for each binary classifier based on its specificity and sensitivity.

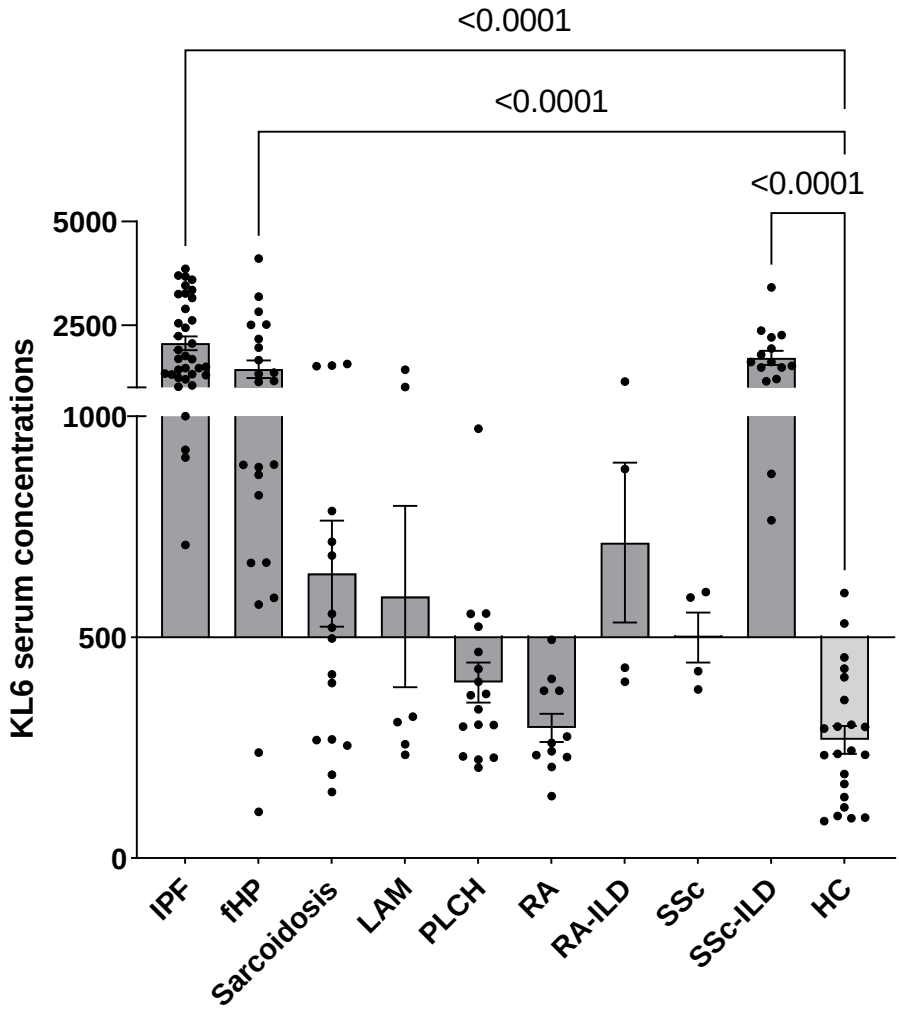
An analysis of correlations was carried out using the Spearman test. It was considered statistically significant if the p value was less than 0.05. GraphPad Prism 9.3 and XLSTAT 2021 were used for statistical analysis.

12. Results

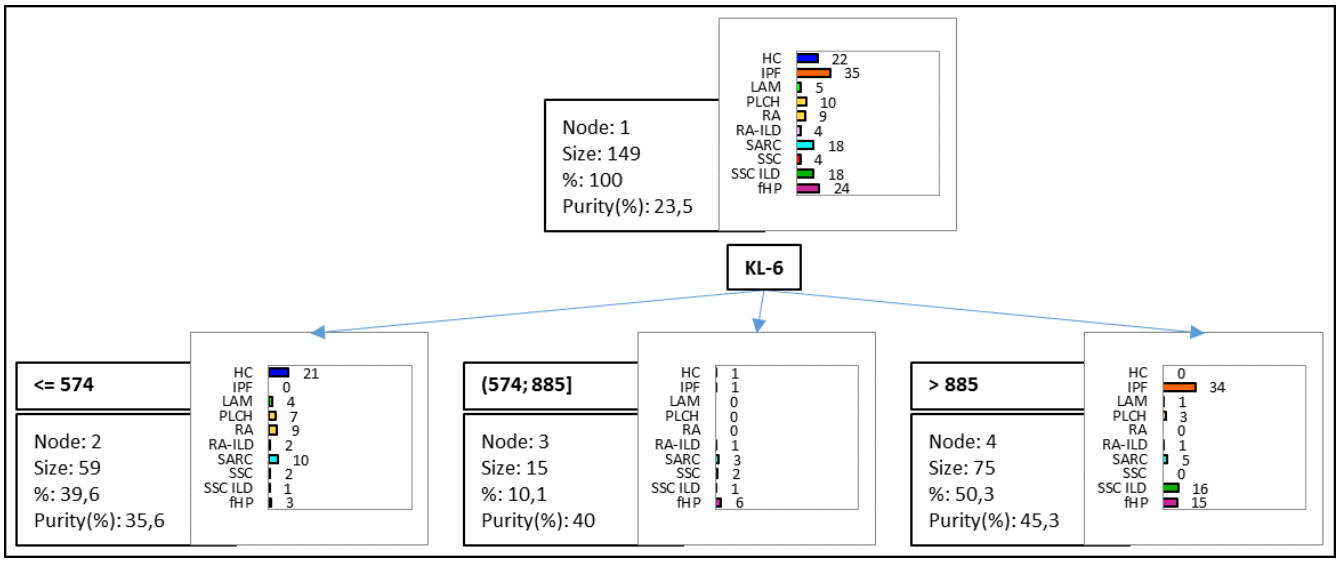
The following demographic information, smoking habits, and lung function test parameters are provided in Table 1. Our study showed that in both IPF and fibrotic HP populations, males were more prevalent, while females were more prevalent in sarcoidosis, LAM, PLCH, RA, and SSc patients. Former smokers were more prevalent in IPF and PLCH patients. Functional parameters revealed mild restrictive deficits among IPF, PLCH, and fibrotic HP patients, as well as a moderate reduction in DLco. Most patients were between 50 and 70 years of age and were younger in sarcoidosis than fibrotic HP and IPF ($p=0.0162$ and $p=0.0045$, respectively) as well as in LAM than IPF patients ($p=0.0405$).

12.1 Demographic and immunological analysis between patients and controls

Comparative analysis (figure 13) showed KL-6 concentrations above 500U/mL in patients than controls. In particular, higher KL-6 concentrations were reported in IPF, fibrotic HP and SSc-ILD patients than HC ($p<0.0001$).

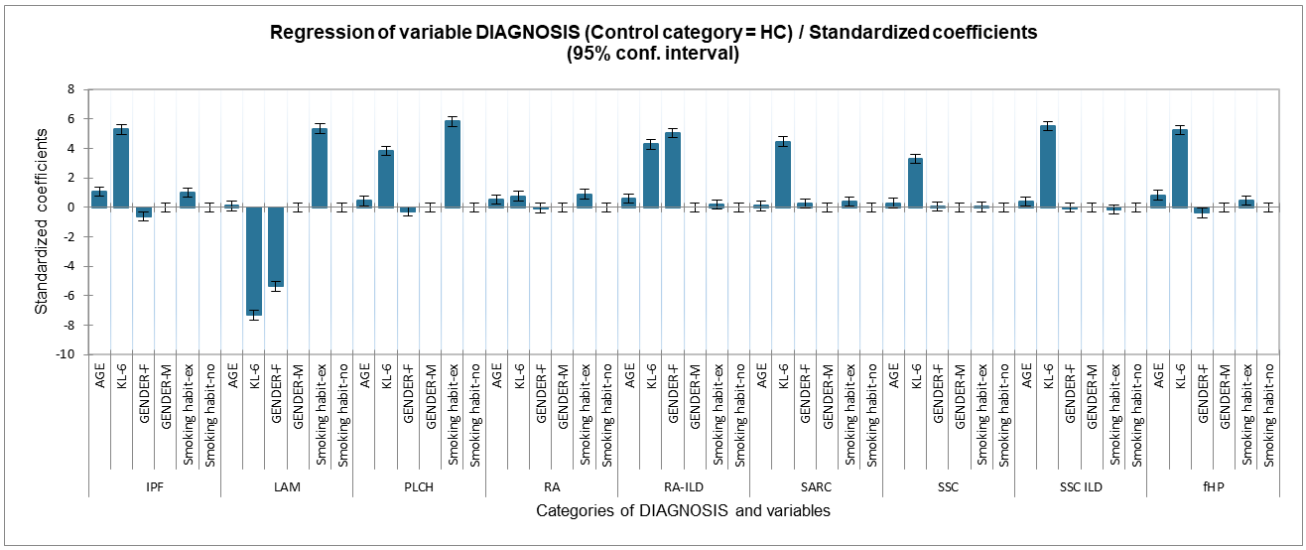


In order to better classify patients than controls according to cut-off values of KL-6 concentrations, a regression tree analysis was performed. The model (figure 14) showed KL-6 >885U/mL for 97% of IPF patients, 89% of SSc-ILD patients and 62% of fibrotic HP patients. KL-6 concentrations ≤ 574 classified 100% of RA, 95% of controls, 80% of LAM, 70% of PLCH, 55% of sarcoidosis, 50% of SSc and RA-ILD patients.



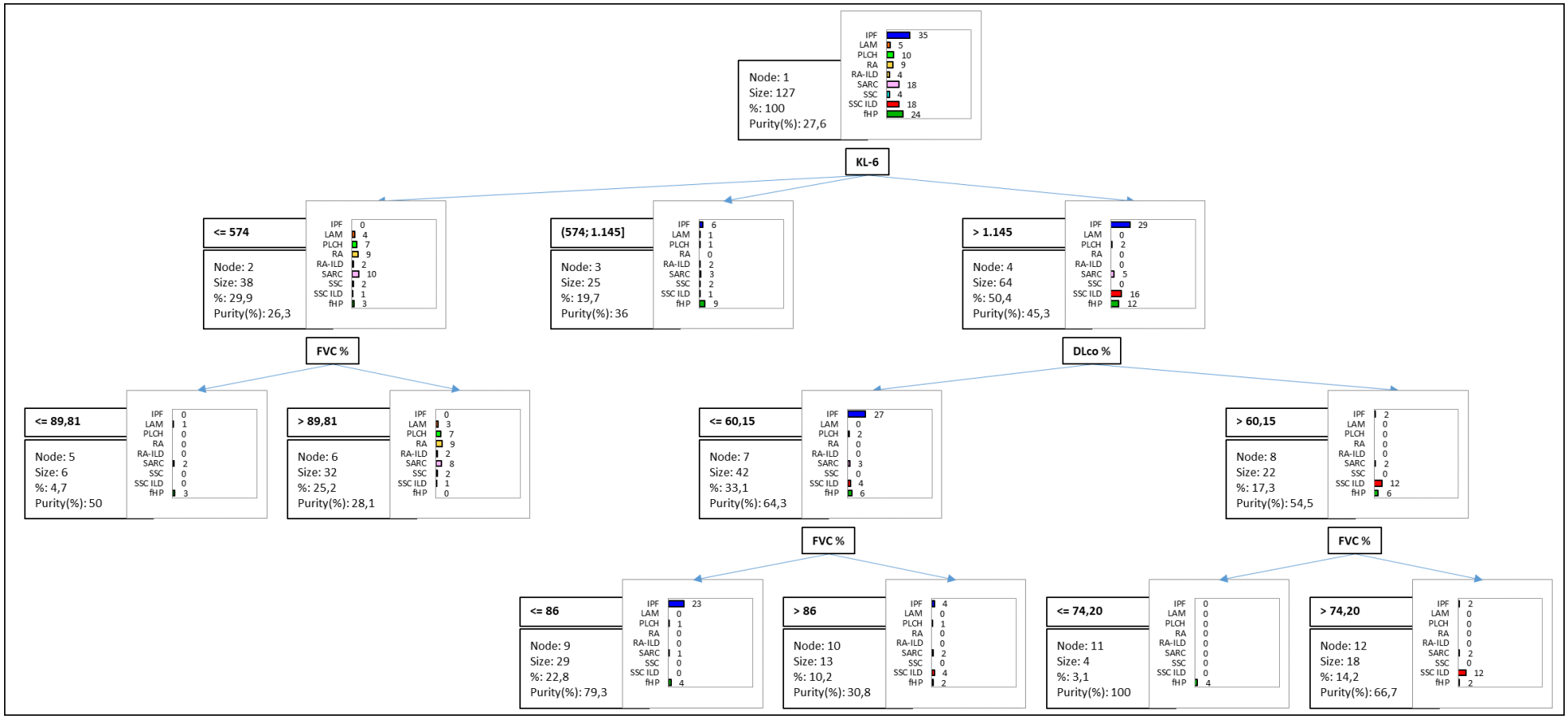
A multinomial logistic regression analysis was performed to model the probability of patients' diagnosis versus HC group given the values of a set of quantitative (age and KL-6) and/or qualitative (gender smoking habit) descriptive variables.

Chi2 was associated with the Log ratio (L.R.) in the goodness-of-fit statistics <0.0001 (figure 15). The Type II analysis indicated that KL-6 concentrations (p=0.004) and smoking habit (p=0.005) were the most important variables that affected the diagnosis of ILD based on the probability associated with the Chi-square tests.



Using a decision tree model (cross-validated by confusion matrix) was used to identify the best clustering variables for ILD diagnoses. The model obtained (figure 16) using functional parameters and KL-6 concentrations cut-off values showed a classification for 70% of IPF

patients with KL-6 >1145U/mL, DLco ≤60.15% and FVC ≤86%. While 67% of SSc-ILD patients with KL-6 >1145U/mL, DLco >60.15% and FVC >74.2%.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Size: 127
%: 100
Purity(%): 27,6
IPF 35
LAM 5
PLCH 10
RA 9
RA-ILD 4
SARC 18
SSC 4
SSC ILD 18
fHP 24
KL-6
<= 574
Node: 2
Size: 38
%: 29,9
Purity(%): 26,3
IPF 0
LAM 4
PLCH 7
RA 9
RA-ILD 2
SARC 10
SSC 2
SSC ILD 1
fHP 3
(574; 1.145]
Node: 3
Size: 25
%: 19,7
Purity(%): 36
IPF 6
LAM 1
PLCH 1
RA 0
RA-ILD 2
SARC 3
SSC 2
SSC ILD 1
fHP 9
> 1.145
Node: 4
Size: 64
%: 50,4
Purity(%): 45,3
IPF 29
LAM 0
PLCH 2
RA 0
RA-ILD 0
SARC 5
SSC 0
SSC ILD 16
fHP 12
FVC %
DLco %
<= 89,81
Node: 5
Size: 6
%: 4,7
Purity(%): 50
IPF 0
LAM 1
PLCH 0
RA 0
RA-ILD 0
SARC 2
SSC 0
SSC ILD 0
fHP 3
> 89,81
Node: 6
Size: 32
%: 25,2
Purity(%): 28,1
IPF 0
LAM 3
PLCH 7
RA 9
RA-ILD 2
SARC 8
SSC 2
SSC ILD 1
fHP 0
<= 60,15
Node: 7
Size: 42
%: 33,1
Purity(%): 64,3
IPF 27
LAM 0
PLCH 2
RA 0
RA-ILD 0
SARC 3
SSC 0
SSC ILD 4
fHP 6
> 60,15
Node: 8
Size: 22
%: 17,3
Purity(%): 54,5
IPF 2
LAM 0
PLCH 0
RA 0
RA-ILD 0
SARC 2
SSC 0
SSC ILD 12
fHP 6
FVC %
FVC %
<= 86
Node: 9
Size: 29
%: 22,8
Purity(%): 79,3
IPF 23
LAM 0
PLCH 1
RA 0
RA-ILD 0
SARC 1
SSC 0
SSC ILD 0
fHP 4
> 86
Node: 10
Size: 13
%: 10,2
Purity(%): 30,8
IPF 4
LAM 0
PLCH 1
RA 0
RA-ILD 0
SARC 2
SSC 0
SSC ILD 4
fHP 2
<= 74,20
Node: 11
Size: 4
%: 3,1
Purity(%): 100
IPF 0
LAM 0
PLCH 0
RA 0
RA-ILD 0
SARC 0
SSC 0
SSC ILD 0
fHP 4
> 74,20
Node: 12
Size: 18
%: 14,2
Purity(%): 66,7
IPF 2
LAM 0
PLCH 0
RA 0
RA-ILD 0
SARC 2
SSC 0
SSC ILD 12
fHP 2

12.2 Clinical and immunological analysis of progressive fibrotic phenotype

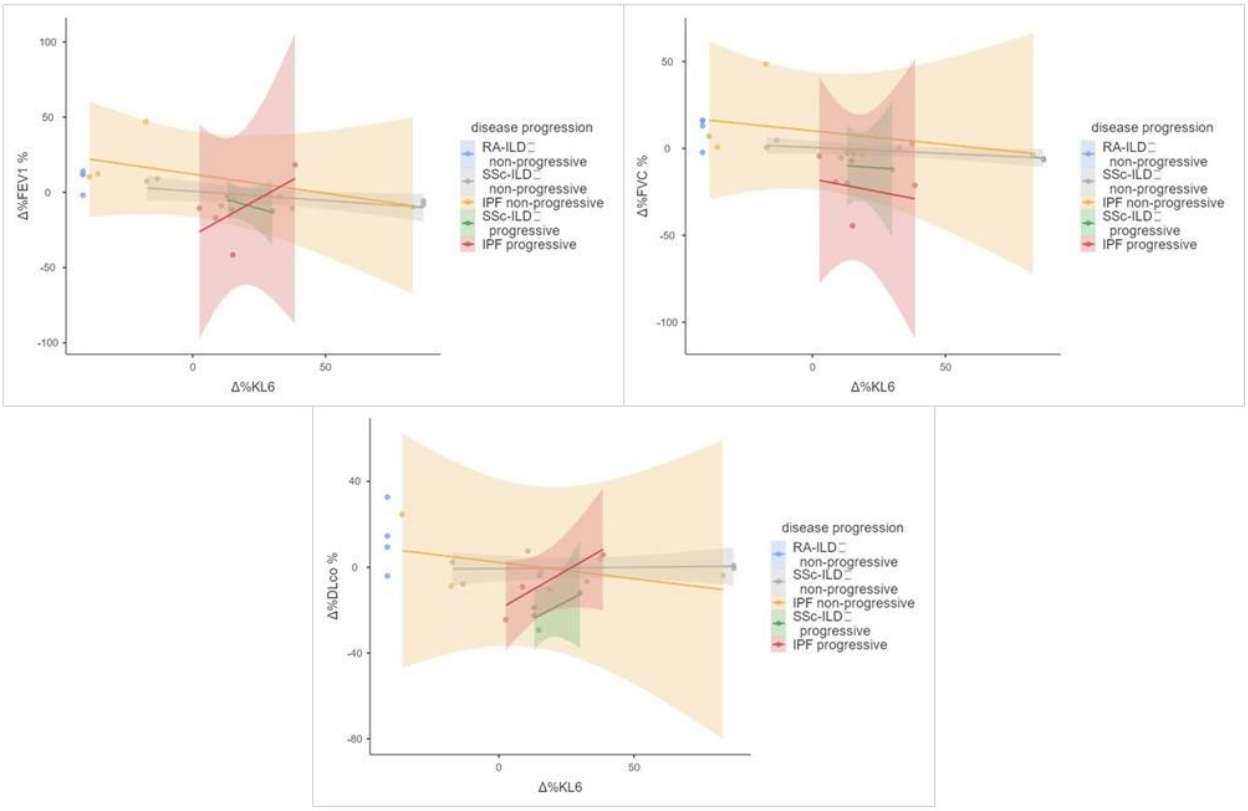
To improve the prognostic role of KL-6, ILD patients were stratified according to progressive fibrotic phenotype. IPF, RA-ILD and SSc-ILD patients with a progressive phenotype had higher T0 KL-6 concentrations than non-progressive patients. However, KL-6 values were greater over the time.

Inverse correlations between T0 KL-6 serum concentrations and T1 FVC and T1 DLco percentages were found (r=-0.314, p=0.046 and r=-0.327, p=0.038, respectively).

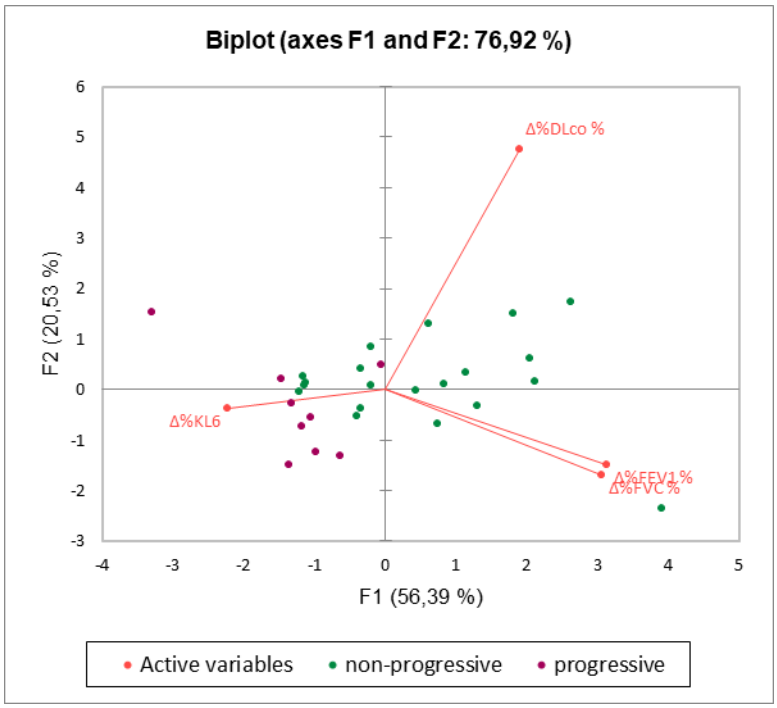
Assessing the rate of variation (T1-T0) of FEV1, FVC and DLco percentages, patients who showed progressive phenotype reported pulmonary functional decline of such parameters over the time (table 2).

	KL6T0	**KL6 T1**	**Δ%KL6**	**Δ%FEV1 %**	**Δ%FVC %**	**Δ%DLco %**
non-progressive	1297 ± 754	1539 ± 1423	4.95 ± 4.68	4.17 ± 14.2	4.69 ± 13.5	3.24 ± 12.1
progressive	2349 ± 1490	2787 ± 2046	17.0 ± 11.6	-9.94 ± 16.8	-16.4 ± 13.5	-14.2 ± 11.7

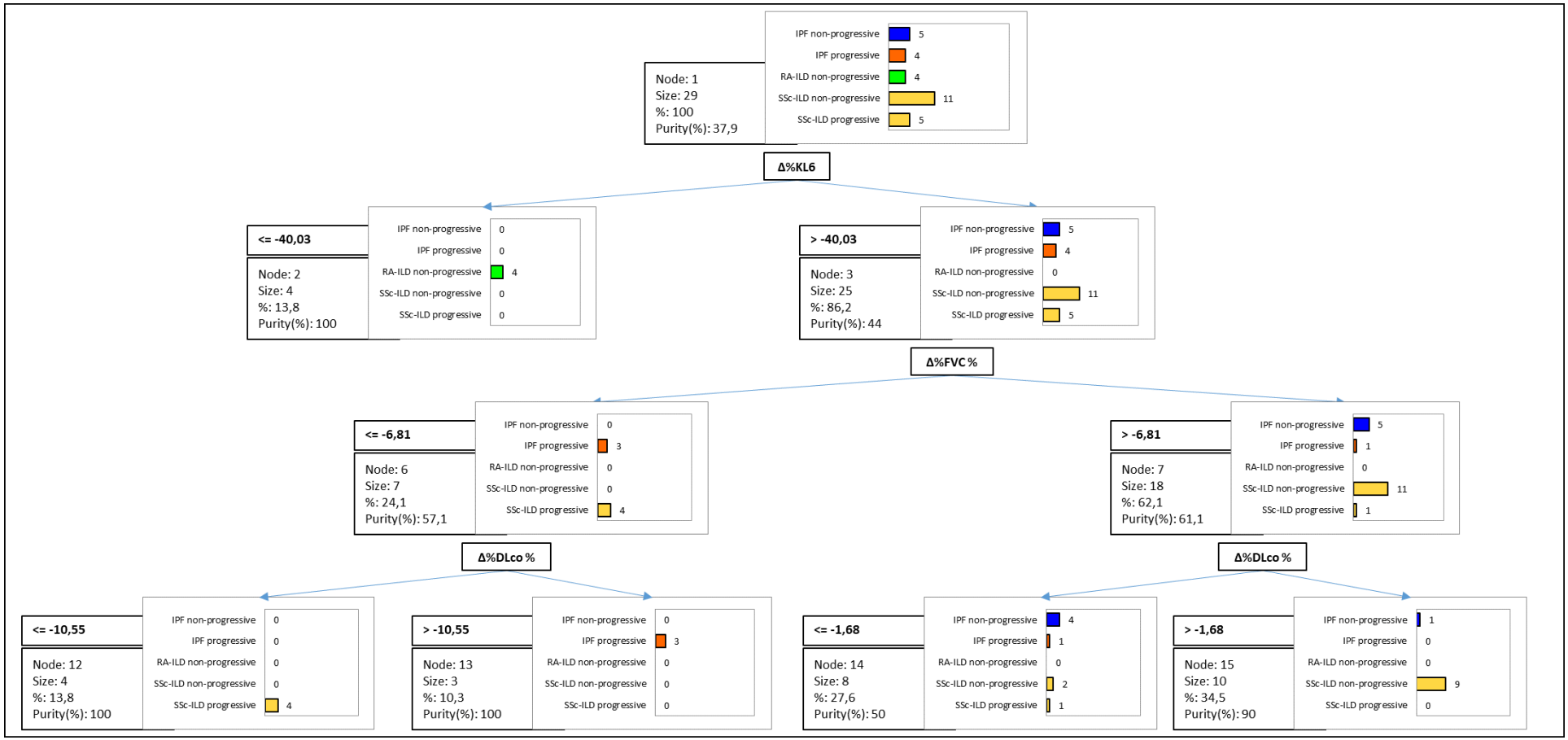
Progressive patients (IPF and SSc-ILD) had pulmonary functional decline and greater KL-6 concentrations over the time in respect with non-progressive patients (figure 17).



The PCA plot (Figure 18) distinguished the progressive disease clusters: progressive and non-progressive patients showed that they separated on the basis of ΔKL-6, ΔFEV1, ΔFVC, ΔDCco. The first and second components explained 56.39% and 20.53% of the total variance.



To identify the best cut-off values to cluster progressive and non-progressive patients, a regression tree analysis was performed. The model showed ΔKL-6 ≤ -40.03 for 100% of RA-ILD non-progressive patients. ΔKL-6 > -40.03, ΔFVC > -6.81 and ΔDLco > -1.68 cluster for 82% of SSc-ILD non-progressive patients and ΔDLco ≤ -1.68 for 80% of IPF non-progressive patients. On the other hand, ΔKL-6 > -40.03, ΔFVC ≤ -6.81 and ΔDLco > -10.55 cluster for 75% of IPF progressive patients and ΔDLco ≤ -10.55 for 80% of SSc-ILD progressive patients.


Node: 1
Size: 29
%: 100
Purity(%): 37,9
IPF non-progressive 5
IPF progressive 4
RA-ILD non-progressive 4
SSc-ILD non-progressive 11
SSc-ILD progressive 5
Δ%KL6
<= -40,03
Node: 2
Size: 4
%: 13,8
Purity(%): 100
IPF non-progressive 0
IPF progressive 0
RA-ILD non-progressive 4
SSc-ILD non-progressive 0
SSc-ILD progressive 0
> -40,03
Node: 3
Size: 25
%: 86,2
Purity(%): 44
IPF non-progressive 5
IPF progressive 4
RA-ILD non-progressive 0
SSc-ILD non-progressive 11
SSc-ILD progressive 5
Δ%FVC %
<= -6,81
Node: 6
Size: 7
%: 24,1
Purity(%): 57,1
IPF non-progressive 0
IPF progressive 3
RA-ILD non-progressive 0
SSc-ILD non-progressive 0
SSc-ILD progressive 4
> -6,81
Node: 7
Size: 18
%: 62,1
Purity(%): 61,1
IPF non-progressive 5
IPF progressive 1
RA-ILD non-progressive 0
SSc-ILD non-progressive 11
SSc-ILD progressive 1
Δ%DLco %
Δ%DLco %
<= -10,55
Node: 12
Size: 4
%: 13,8
Purity(%): 100
IPF non-progressive 0
IPF progressive 0
RA-ILD non-progressive 0
SSc-ILD non-progressive 0
SSc-ILD progressive 4
> -10,55
Node: 13
Size: 3
%: 10,3
Purity(%): 100
IPF non-progressive 0
IPF progressive 3
RA-ILD non-progressive 0
SSc-ILD non-progressive 0
SSc-ILD progressive 0
<= -1,68
Node: 14
Size: 8
%: 27,6
Purity(%): 50
IPF non-progressive 4
IPF progressive 1
RA-ILD non-progressive 0
SSc-ILD non-progressive 2
SSc-ILD progressive 1
> -1,68
Node: 15
Size: 10
%: 34,5
Purity(%): 90
IPF non-progressive 1
IPF progressive 0
RA-ILD non-progressive 0
SSc-ILD non-progressive 9
SSc-ILD progressive 0

13. Discussion

In the present study, serum KL-6 concentrations were evaluated in IPF, fibrotic HP, LAM, PLCH, sarcoidosis, RA and SSc with and without ILD and compared with healthy volunteers.

The highest KL-6 concentrations were reported in IPF, fibrotic HP and SSc-ILD patients than HC. The best KL-6 cut-off value to identify fibrotic ILD patients was 885U/mL, while KL-6 concentrations below 574U/mL classified 100% of RA, 95% of controls, 80% of LAM, 70% of PLCH, 55% of sarcoidosis, 50% of SSc and RA-ILD patients. From logistic regression analysis, the variables that most influences the ILD diagnosis was the KL-6 concentrations and smoking habit.

The decision tree model classify 86% of IPF patients with KL-6 >1145U/mL, DLco ≤60.15% and FVC ≤86%. The fibrotic progression was evaluated in our cohort through radiological features and LFT parameters. Pulmonary functional decline and greater KL-6 concentrations over the time were identified in progressive than non-progressive patients.

There was an inverse correlation between serum KL-6 concentrations at T0 and the percentages of FVC and DLco at T1 in the progressive group compared to the non-progressive group.

Studies of immunohistochemistry have demonstrated that KL-6 is highly expressed on Type 2 pneumocytes, although it is not yet clear whether the elevated concentrations of KL-6 in sera from patients with ILD represent exclusively lower respiratory origins (112).

As the most common fibrotic ILD, IPF, more than 400 publications have been published concerning KL-6's role in the treatment of ILD patients. Although no specific differential diagnostic cut-off value of KL-6 was proposed to differentiate several types of fibrotic ILD, KL-6 was demonstrated as useful marker for predictive prognosis and response to therapy in IPF patients (127).

In this study, it has been observed that serum KL-6 concentrations identified fibrotic patients and in line with Ishii et al, who demonstrated high baseline KL-6 values related to significant worse survival, our cohort showed increased T0 and T1 KL-6 serum concentrations in progressive than non-progressive patients. Moreover, higher KL-6 values were correlated with FVC and DLco decline in progressive patients pointing out the potential of KL-6 in serum for predicting functional disease progression.

As a result of acute exacerbations of IPF, serum levels of KL-6 have been reported to increase. Moreover, a recent study showed that increasing serum KL-6 levels is associated with a rapid decrease in predicted FVC, as well as lower survival rates for patients with IPF who have higher levels of KL-6 (128). As a result, secreted KL-6 is considered a useful biomarker for predicting IPF outcome and evaluating disease activity.

There has been a good correlation between KL-6 and pulmonary function tests and quantitative HRCT scores of lung involvement in SSc studies, which found that it had a good ability to stage disease severity. The serum KL-6 level in SSc patients was investigated as a marker of disease activity and was correlated with the presence of pleuroparenchymal fibroelastosis, which was a worse prognostic indicator (129).

We can speculate that high values of KL-6 in sera from fibrotic and progressive patients (IPF and SSc-ILD) are derived from activated regenerating Type 2 pneumocytes and the presence of alveolar epithelial damage suggesting the potential of KL-6 values along with LFT parameters to identify a new and similar progressive phenotype in IPF ILD and non-IPF ILD patients.

In the last decades, it has been reported that also patients with non-IPF ILD (such as SSc-ILD patients) can show a progressive worsening of the disease regardless of treatments. Moreover, many recent studies have suggested the presence of common pathogenetic pathways between IPF and non-IPF ILD, like shortening of telomeres, epithelial cell dysfunction and immune dysregulation (130).

For these reasons, a new progressive fibrotic phenotype has been recently proposed to include all ILD patients (both idiopathic and non-idiopathic) that show an inexorably worsening of disease, based on clinical and functional progression of the disease. Our study shines a spotlight on the potential of KL-6 to identify such phenotype along with pulmonary functional parameters.

Type 2 pneumocyte epithelial mucins have a pathophysiologic role that has not been completely clarified. In order to repair injured alveolar epithelium, Type 2 pneumocytes cover the spaces where Type 1 pneumocytes have desquamated from the basement membrane (107). Activated Type 2 pneumocytes produce significant amounts of TGF-beta in IPF patients, and TGF-beta is a mitogenic factor for fibroblasts. Therefore, Type 2 pneumocytes may be involved in the cellular and molecular pathways that lead to fibrosis. (131).

Our findings not only confirmed the prognostic role of peripheral KL-6 values from fibrotic ILD patients but also demonstrated the clinical usefulness to add their measurements along with pulmonary functional parameters at the time of diagnosis for predicting progressive patients.

The results of our study provide insights into the diagnostic as well as prognostic role of KL-6 in patients with fibrotic ILD, and multicentric prospective studies would be useful for validating these findings in other patients with fibrotic ILD.

14. Conclusion

The development of numerous anti-fibrotic therapies has been a result of continuous advancements in our understanding of the pathophysiology of IPF. Currently, only two treatments (pirfenidone and nintedanib) have been approved, and both have limited efficacy. KL-6 proved to be a reliable marker for diagnosis and prognosis of fibrotic ILD patients with predictive value in progressive fibrotic patients and a useful marker to identify the new and similar progressive phenotype of IPF and SSc-ILD patients assessing the functional progression in accordance with serial serum KL-6 measurement.

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