Diagnosis and follow up of chronic hypersensitivity pneumonitis: utility of non-invasive measurement of airway inflammation

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DOCTORAL THESIS

Diagnosis and follow up of chronic hypersensitivity pneumonitis: utility of non-invasive measurement of airway inflammation

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LIST OF ABBREVIATIONS

APC: Antigen presenting cell

ATS: American Thoracic Society

BAL: Bronchoalveolar lavage

BCR: B-cell receptor

COPD: Chronic obstructive pulmonary disease

HRCT: High resolution computed tomography

DC: Dendritic cell

DLCO: Diffusing capacity of the lung for carbon monoxide

EBC: Exhaled breath condensate

ELISA: Enzyme-linked immunosorbent assay

ERS: European Respiratory Society

FENO: Fractional NO

FEV_1: Forced expiratory volume in one second

FVC: Forced vital capacity

IFN-: Interferon-gamma

Ig: Immunoglobulin

IL: Interleukin

IS: Induced sputum

ILD: Interstitial lung disease

IPF: Idiopathic pulmonary fibrosis

HP: Hypersensitivity pneumonitis

MHCII: Major histocompatibility complex II

MV: Minute ventilation

NSIP: Non-specific interstitial pneumonia

NO: nitric oxide

RV: Residual volumen

SIC: Specific inhalation challenge

SLB: Surgical lung biopsy

TBB: Transbronchial biopsy

TCR: T-cell receptor

Th: T-helper lymphocyte

INTRODUCTION

1 INTRODUCTION

1.1 DEFINITION

Hypersensitivity pneumonitis (HP), also known as extrinsic allergic alveolitis, is characterised by a destructuring of the pulmonary parenchyma, as a result of an immunological inflammatory response, following the repeated inhalation of an antigen, generally of an organic nature, to which the individual had previously become sensitised[1]. A progressive acknowledgment of the ubiquity of these antigens in the environment and the improvement of diagnostic tools has led to an increase in the diagnosis of HP both in the domestic and occupational settings. The pathology is typically characterised by lymphocytic alveolitis and granulomatous pneumonitis in the histological study[2].

HP continues to be a diagnostic challenge for doctors studying this pathology, due to the wide spectrum of clinical findings and the lack of a "gold standard" to perform an accurate diagnosis. Diagnosis depends on a strong initial diagnostic suspicion, an exhaustive clinical record that considers all possible exposures, and the incorporation of immunological and radiological findings and the anatomopathological study of pulmonary biopsy[2]. However, it is important to consider that these findings may be non-specific and on occasions may be similar to other pathologies[3]. HP is a treatable and preventable illness when the potentially pathogenic antigen is known, since interrupting the antigen exposure is the most effective treatment in the stages preceding the illness[4]. This reveals the importance of performing an accurate and early diagnosis. In those cases in which the cause is unknown and the patient is not receiving treatment, the natural evolution of the disease may progress towards pulmonary fibrosis followed by respiratory failure[5].

HP is part of the group of entities known as "interstitial lung diseases" (ILD) which in general terms, involve a fibrotic-inflammatory infiltration of the alveolar walls and cause a lesion in the capillary endothelium and in the alveolar epithelial cells[6]. In normal conditions, a small number of macrophages, fibroblasts and myofibroblasts live in the interstice of the pulmonary parenchyma[7]. Other components of the pulmonary interstice also include lung matrix proteins,

which consist of macromolecules related with collagen, and non-collagenous proteins such as fibronectin and laminin[8]. The fibroblastic proliferation and the excess of collagen deposits, the main identifier of DILD, may be the result of a direct insult to the endothelial surface of the alveoli, via a cellular inflammatory response, or the consequence of an anomalous regeneration and reparation process of these tissues[8,9]. ILD include many entities that are capable of damaging the pulmonary parenchyma, causing illnesses with similar clinical, radiological and physiological characteristics.

ILD were first described in 1892, when Osler[10] coined the term chronic interstitial pneumonia or cirrhosis of the lungs, which was characterised by fibrotic changes in the lungs. In 1969 Liebow and Carrington[11] performed the first classification of idiopathic interstitial pneumonias. In 2000, 2002 and 2013 the American Thoracic Society (ATS) and the European Respiratory Society (ERS) drew up three agreements in which the clinicopathological characteristics of the idiopathic interstitial pneumonias were defined and reclassified[12,13,14]. Based on the abovementioned guides, ILD were classified into three main groups[15]: a) idiopathic interstitial pneumonias b) of unknown cause or associated c) primaries or associated with other undefined illnesses. Thus, since HP is usually a disease secondary to the inhalation of avian or fungal proteins, it is included among the illnesses from the second group.

1.1.1 ETIOLOGY AND CLASSIFICATION

The list of specific HP-causing agents is extensive and permanently expanding, since new sources of exposure to the pathogenic antigens are constantly being described, and therefore, so too are new forms of HP (Table 1)[16]. The first case of this disease dates back to 1932, when Campbell[17] described “farmer’s lung” for the first time, based on the case of a farmer who had presented with an acute inflammatory pulmonary reaction related to exposure to mouldy hay. However, it was not until 1962 when Pepys et al.[18] associated this disease with the presence of specific IgG antibodies against the fungus that was colonising the hay. The other most common form of HP is that known as “bird fancier’s lung”, which corresponds to the form of the illness that is produced by being exposed to avian proteins, in those individuals who dedicate themselves professionally to caring for birds, and that Reed et al.[19] first described in 1965.

Table 1. Materials and antigens causing hypersensitivity pneumonitis. Adapted from the Pneumological Guide[16].

Disease	Antigen source	Antigen
Farmer's lung	Mouldy hay	*Saccharopolyspora rectivirgula, Thermoactinomyces vulgaris, Aspergillus flavus* and *A. fumigatus*
Bird fancier's lung	Cock pigeon, paraqueet, parrot, etc.	Serum proteins and intestinal mucin (glucoprotein), excrements, fine dust (*bloom*)
Feather duvet lung	Feather duvet and pillows	Feathers and fungus
Espartosis (estipatosis)	Esparto, ceiling plaster	*Aspergillus*, *Penicillium*
Suberosis	Mouldy cork	*Penicillium frequentans* Aspergillus sp
Air conditioner lung	Air conditioning units, humidifiers	Thermophilic actinomycetes, thermotolerant bacteria, protozoa
(Homemade ultrasonic humidifer lung)	Contaminated humidifier water	*Cephalosporium acremonium* and *Candida albicans*
Chacinero's lung (Cured meat cleaner's lung)	Mouldy cured meats	*Penicillium* and *Aspergillus*
Nacre lung	Sea shells, buttons, pearls	Proteins
Soy lung	Soy dust	Soy proteins
Machine operator's lung. *Metal working fluid*	Lubricant (cutting fluids) and refrigerating liquids	*Pseudomonas fluorescens, Aspergillus niger, Rhodococcus sp, Staphylococcus, Mycobacterium immunogenum*[7]
Spa, hot tub and shower lung	Hot water spray	*Mycobacterium avium complex and other microbacteria Cladosporium*
Hard metal lung disease (Giant-cell interstitial pneumonitis)	Tungsten carbide-cobalt in metalwork.	Cobalt + tungsten
Candida lung	Contaminated material, urine, etc.	*Candida* sp
Steam iron lung	Aerosol from the iron water	*Aspergillus fumigatus*
Mushroom worker's lung	Mushrooms	*T. vulgaris* and *Saccharopolyspora rectivirgula*
Compost lung	Compost (manure)	*T. vulgaris*, *Aspergillus*
Insecticide lung	Insecticides	Piretroides
Bagassosis	Bagazo (sugar cane)	*T. vulgaris* and *T. sacchari*
Maple bark disease	Damp maple bark	*Cryptostoma corticale*
Sequoiosis	Mouldy sawdust	*Grafium* and *Aureobasidium pullulans*
Woodworker's lung	Ramin (*Gonystylus balcanus*)	Wood
Malt worker's lung	Mouldy barley, malt	*Aspergillus clavatus* and *A. fumigatus*
Miller's lung	Contaminated wheat, etc.	*Sitophilus granarius, Sporobolomyces*

Woodworker's lung	Mouldy pulp	*Alternaria*
Cheese-washer's lung	Cheese mould	*Penicillium casei* and *Acarus siro*
Fish flour worker's lung	Fish flour factory	Fish flour
Fertilizer worker's lung	Plant waste	*Streptomyces albus*
Tobacco worker's lung	Tobacco	*Aspergillus*
Furrier's lung	Astrahkan and fox fur	Dust from the fur
Coffee worker's lung	Coffee grains	Coffee dust
Pituitary snuff-taker's lung	Hypophysis snuff	Pituitary hormone
New Guinea thatched roof disease	Thatched roof	*Streptomyces olivaceus*
Detergent worker's lung	Enzymatic detergents	Bacillus *subtilis*
Paprika splitter's lung	Paprika dust	*Mucor stolonifer*
Aerosol of contaminated water	Leak in water-cooled machinery	Six different fungi
Sauna taker's lung	Contaminated lake water	*Aureobasidium* sp
Coptic disease	Mummy wrappings	
Rodent carer's lung	Old rats	Urine proteins
Bat lung	Bat faeces	Serum proteins
Japanese summer-type alveolitis	Interior humidity	*Trichosporon cutaneum*, *Cryptococcus albidus* and *Cryptococcus neoformans*
Sericulturist's lung disease	Silk larva	Larva protein
Winegrower's lung	Vine fungus	*Botrytis cinerea*
Saxophonist's lung[10,11]	Mouth piece and case	*Ulocladium botrytis* and *Phoma* sp
Trombone player's lung[12]	Biofilm inside the instrument	*Mycobacterium chelonae and Abscesus. Fusarium* sp
Breadmaker's lung	Flour	Aspergillus fumigatus
Caused by chemical agents		
Berylliosis	Neon, TV sets, etc.	Beryllium
Isocyanate lung	Foam, adhesives, paint	Isocyanate
Epoxy resin worker's lung	Plastics, resins and epoxy	Anhydrid acids (Phthalic anhydride)
Vineyard sprayer's lung	Copper sulphate (Bordeaux mix)	Copper sulphate
Dental technician's lung	Dental prostheses	Methacrylate
Chloroethylene lung	Degreaser	Chloroethylene
Many other sporadic cases have been published.		

The different exposure forms that can lead to HP can be classified into three main groups, according to the causal agent: a) HP secondary to microbiological agents; b) HP secondary to avian proteins; c) HP secondary to chemical agents[16]. There is also a fourth group of diseases with findings compatible with HP secondary to pharmacological agents, which are generally included in the ILD secondary to drugs[20].

1.1.1.1 Microbiological agents

Microorganisms, such as bacteria and fungi, are the agents most frequently responsible for producing HP in indoor settings[21], and on many occasions, forms of this disease go unnoticed. Warm and damp environments are an ideal setting for the proliferation of microorganisms. When these are inhaled, they can cause sensitisation towards their antigens and subsequent pulmonary disease[22].

1.1.1.1.1 Bacteria

Thermophilic actinomycetes are filamentous bacteria that have some similarities with fungi and are frequently associated with the most known cause of HP, the above-mentioned farmer's lung[23]. These bacteria are found ubiquitously in the environment, especially in humid areas with temperatures of between 50 and 55 degrees Celsius, enabling them to secrete enzymes that lead to the growth of mould in diverse plants such as hay[24]. They also frequently colonise ventilation pipes or humidification systems, which provide the ideal conditions for their proliferation[25].

Atypical microbacteria are also agents that can cause this disease. HP caused by "metalworking fluids", for example, is brought about by the contamination of microbacteria in fluid circuits that are used to cool metal-milling machines on an industrial level. They are used time and time again and remain in closed circuits[26].

Other bacteria that can also occasionally cause HP include *Acinetobacter ochrobactrum, Streptomyces albus, Klebsiella oxytoca* and *Bacillus subtilis.*

1.1.1.1.2 Fungi

Many species of fungus have been associated with HP. *Aspergillus* spp. and *penicillium* spp. have been described as causal agents of several forms of this disease, such as farmer's lung[17], espartosis[27] (esparto contamination), bird fancier's lung[28] (contamination of birds' nests and

feathers) and suberosis[29] (contamination of cork) among others. *Alternaria, Cladosporium, Aureobasidium* and many other species of fungus have also been associated with this entity[30].

Figure 1. Cork worker. Suberosis.

1.1.1.2 Animal proteins

Different animal origin proteins can cause HP if they are inhaled. The most known and most frequent clinical form of this type of HP is bird fancier's lung[28]. Avian antigens are high-molecular weight proteins found in the serum, feathers and faeces of birds[31].

Figure 2. Bird fancier's lung.

1.1.1.3 Chemical agents

This is probably the least frequent cause of HP, despite some of these chemical substances being widely used in industry. For example, isocyanates, used in the production of polyurethane polymers, and subsequently used in the manufacture of adhesives, paints, glues and resins, are a well-established cause of HP[32]. Other chemical agents, such as epoxy resins may also be a potential cause of HP[33]. Literature on HP has also mentioned very exceptional cases of exposure to methyl methacrylate in dental technicians[34].

1.2 PHYSIOPATHOLOGY

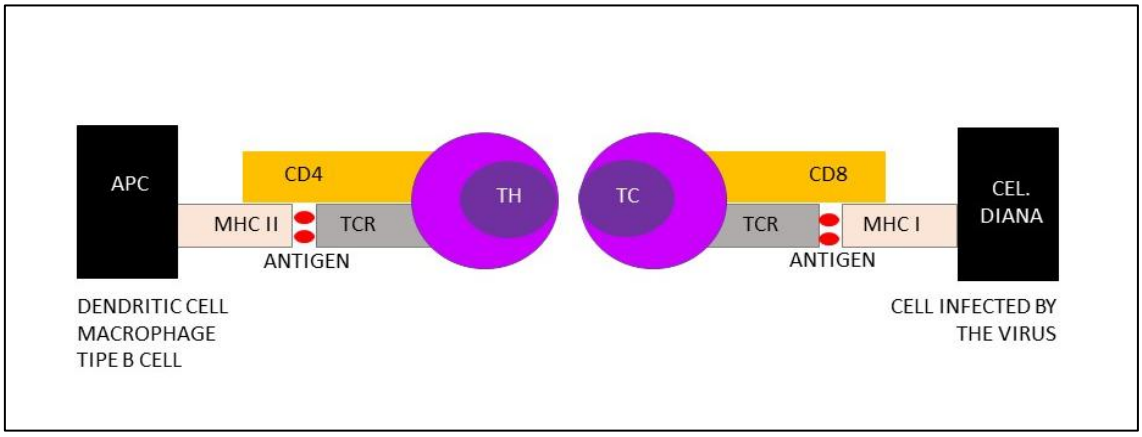
Initial findings related to the physiopathology of HP in the 1960s included as a main factor the circulating precipitins against the antigens to which the individual had previously become sensitised. This relation between the precipitins and the antigen would suggest that the disease was mediated by deposits of antigen-antibody complexes in the alveolar walls[18], which would be compatible with a Type 3 hypersensitivity reaction. However, other subsequent findings did not confirm this hypothesis: 1) some individuals had the disease, but did not have specific IgG[35]; 2) the histopathology did not reveal findings compatible with vasculitis or

neutrophilic infiltrates; 3) in animal models, the serum transmission of circulating precipitins, followed by an inhaled antigen exposure, did not reproduce the histological changes of HP[36].

Unlike other types of hypersensitivity, Type 4 hypersensitivity, which corresponds to delayed type reactivity, cannot be transferred from a sensitised individual to a non-sensitised individual with serum antibodies; lymphoid cells, in particular T lymphocytes, are needed. At the beginning of the 1970s, Moore et al,[37] observed pulmonary lesions in which there was a predominance of mononuclear cells (T cells and macrophages) in patients with HP. Likewise, diverse animal models of mice and pigs, to which the transfer of specifically sensitised cells from lymphatic nodules was performed, followed by an inhaled provocation test, managed to reproduce these same lesions, therefore reinforcing the hypothesis that HP is caused by a Type 4 hypersensitivity mechanism[38,39]. Likewise, the finding of a lymphocyte predominance in the bronchoalveolar lavage (LBA) of experimental animal models, at the end of the 1970s, helped to confirm this theory once again[40]. In the early 1980s, several authors reported that in animal models with granulomatous HP, the lesions could be inhibited using different procedures such as neonatal thymectomy or the administration of corticosteroids, thereby gathering more evidence in this line of research[41,42].

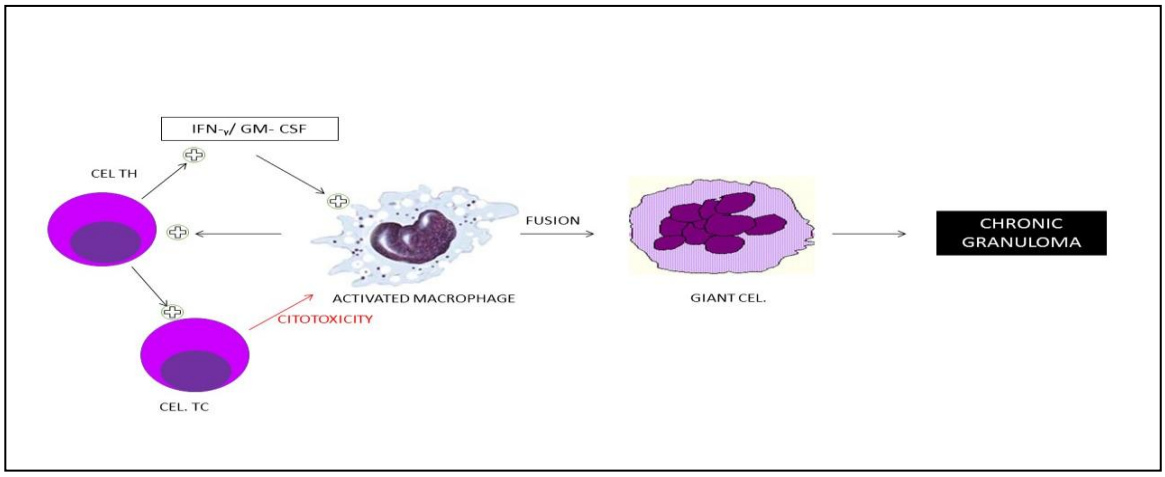
Later studies confirmed the most widespread hypothesis to date, in relation to HP generated by T-lymphocytes, mainly through an immune response generated by T-helper (Th) 1 cells, via the regulating transcription factor, T-bet[43,44,45]. After the initial programming, memory T-cells recognise the antigen by: a) a non-specific union through adhesion molecules and b) the union of the T-cell receptor (TCR) with the antigenic peptide on molecules of the major histocompatibility complex II (MHC) in an antigen presenting cell (APC)[46,47] Once the antigen has been recognised the T-cells are stimulated to turn into blasts and proliferate (figure 3).

Figure 3. The subgroups of T-helper and killer cells are restricted by the class of MHC molecules. Adapted from Roitt[48].



The stimulated T-cells release cytokines that act as generators of the hypersensitivity response that strikes, particularly because they attract and activate macrophages; they also contribute to the precursors of cytotoxic t-cells becoming killer cells, which may cause lesions in the tissues[48]. When the balance between the antigen exposure and the organism's defence is not resolved in favour of the host, the persistence of the antigen leads to a local chronic delayed hypersensitivity reaction. The continuous release of cytokines by the sensitised T-lymphocytes leads to the accumulation of a large number of macrophages, many of which give rise to collections of epithelioid cells, while others fuse together to form giant cells. Those macrophages with a bacterial antigen on their surface will become targets for the cytotoxic T-cells which will kill them. From a morphological perspective, this combination of cell types with proliferation of lymphocytes and fibroblasts associated with areas of fibrosis and necrosis has been give the name chronic granuloma and is an attempt by the organism to block or isolate an area of persistent attack[49] (figure 4).

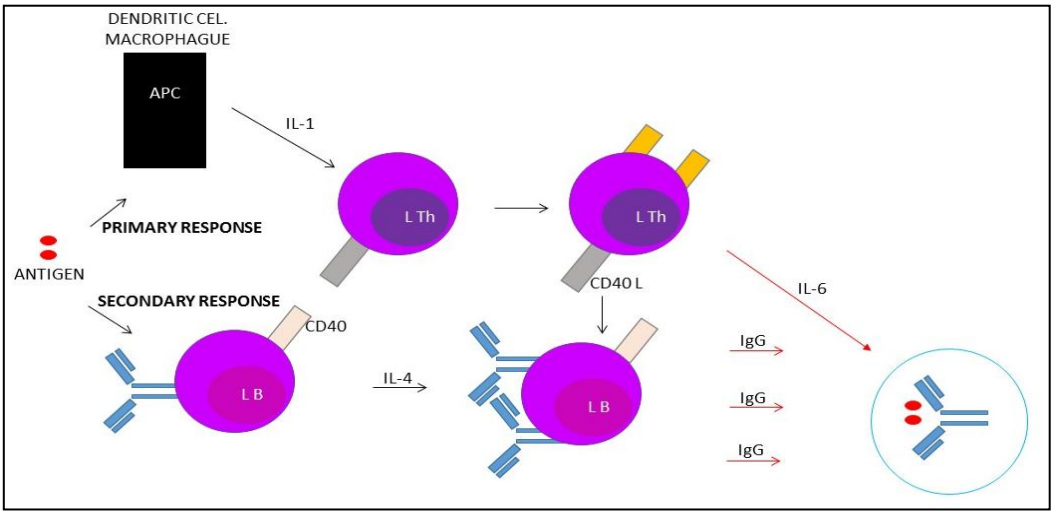
Figure 4. The cellular base of Type 4 hypersensitivity. Adapted from Roitt[48].



IL-12 is a cytokine produced by activated macrophages, which polarises the lymphocytic response towards Th1 differentiation. In turn, the Th1 lymphocytes produce IFN-y which activates more macrophages and could generate a differentiation of cytotoxic T-cells. Diverse studies of the biopsies of patients with HP have identified the activation of chemokines CXCL9 and CXC10, which in turn activate the IFN-y and the CXCR3 ligand, a Th1 chemokine receptor, which would back up the Th1 as responsible for this entity, as the main underlying mechanism[50].

In addition, this Th1 route would contribute towards activating the humoral response that takes place in HP. In this process, 2 interacting mechanisms would intervene: on the one hand, the union of the antigen with the B-cell receptor (BCR), and on the other hand, the co-stimulation of the Th lymphocyte specific for the same antigen, via the CD40 ligand. The interaction of both would enable the activation of the response mediated by a probably thymus-dependent B lymphocyte which, in turn, would encourage a secondary response generating specific IgG antibodies that are isolated in the peripheral blood of these patients (figure 5).

Figure 5. Lymphocyte T- lymphocyte B interaction.



Furthermore, $CD4^+$ T-cells can differentiate not only between subpopulations of Th1 cells, but also other subpopulations of Th2 cells, Th17 cells, follicular Th cells and regulatory T-cells. In relation to this evidence, several studies suggest that the Th1 route may not be the only route involved in the pathobiology of HP[51].

In fact, Selman et al.[50] in their analysis of microarrays of pulmonary biopsies of 12 patients with HP, in 2006, identified both activation of IL-17, which is expressed through Th17 cells, and IL-17-associated transcriptions. The same study revealed the polarisation of T $CD4^+$ cells to Th17 cells, instead of Th1 cells, via the expression of IL-17A and IL-22. Other studies on animal models with HP have revealed high levels of IL-17 in the LBA. Treatment with anti IL-17 reduced inflammation in said mice[52,53]. In this vein, a recent study by Ishizuka et al.[54] on an animal model with acute HP caused by avian protein, also observed both high levels of IL-17A mRNA in the lungs as well as levels of IL-17A protein in the blood and LBA.

In parallel, in 2008, Barrera et al.[55] researched the lymphocytes of the LBA of patients with HP in the fibrosis phase, and found an increased expression of CXCR4 —a Th2 chemokine receptor— and a reduced expression of CXCR3, as well as an increase in IL-4. Following these findings, the authors suggested a possible involvement of the Th2 cellular route. Subsequently, in 2011, Mitaka et al.[56], in an animal model with chronic HP, verified the importance of a Th2 response, by observing an increase in IL-4 and IL-13 in the LBA, compared to the control. As regards previous findings by Barrera et al. (2008) reporting the increased expression of CXCR4, for the first time in 2014, a study of 20 patients with chronic HP compared with healthy controls, revealed a significant increase in circulating fibrocytes, migrating to tissue specifically through the CXCR4/CXCL12 axis[57]. Fibrocytes are a population of circulating progenitors of the fibroblasts that stem from hematopoietic cells in the bone marrow and which have been involved in different biological processes of inflammation, repair and fibrosis in different organs[58]. In this vein, the authors suggest that fibrocytes may also be involved in the pathogenesis of chronic HP; similar findings have been observed in patients with IPF, which, in the authors' opinion, would reinforce this hypothesis and the potential role of fibrocytes in this disease[59].

It is unknown why only a small group of individuals exposed to organic proteins develop the illness, although it is speculated that this fact may be related to genetic susceptibility. Few studies have analysed the possible involvement of immune system regulation in the development of this disease, and those that have been conducted, have focused on MHC. The high number of polymorphisms and the heterogeneity of the genomic region of MHC is a considerable advantage when selecting different pathogens. However, it also presents the inconvenience of generating immune-pathological alterations. Polymorphisms of MHC- DR and

DQ, which are molecules that belong to MHC-II, have been associated with a greater risk of developing HP and could explain why only certain individuals develop the disease[60,61].

HP is less frequent in smokers than in non-smokers, in individuals subjected to the same antigenic load1. In fact, following exposure to high antigenic loads, smokers present lower levels of specific antibodies against the antigen. Although the specific mechanisms are not known, various experimental studies attribute these differences to nicotine[62]. In a study conducted by Blanchet et al.[62] on an animal model of mice with HP secondary to *S. rectivirgula,* following exposure to nicotine, a reduction was observed in the macrophage activation and in lymphocytic proliferation, as well as a dysfunctional T-cell activity.

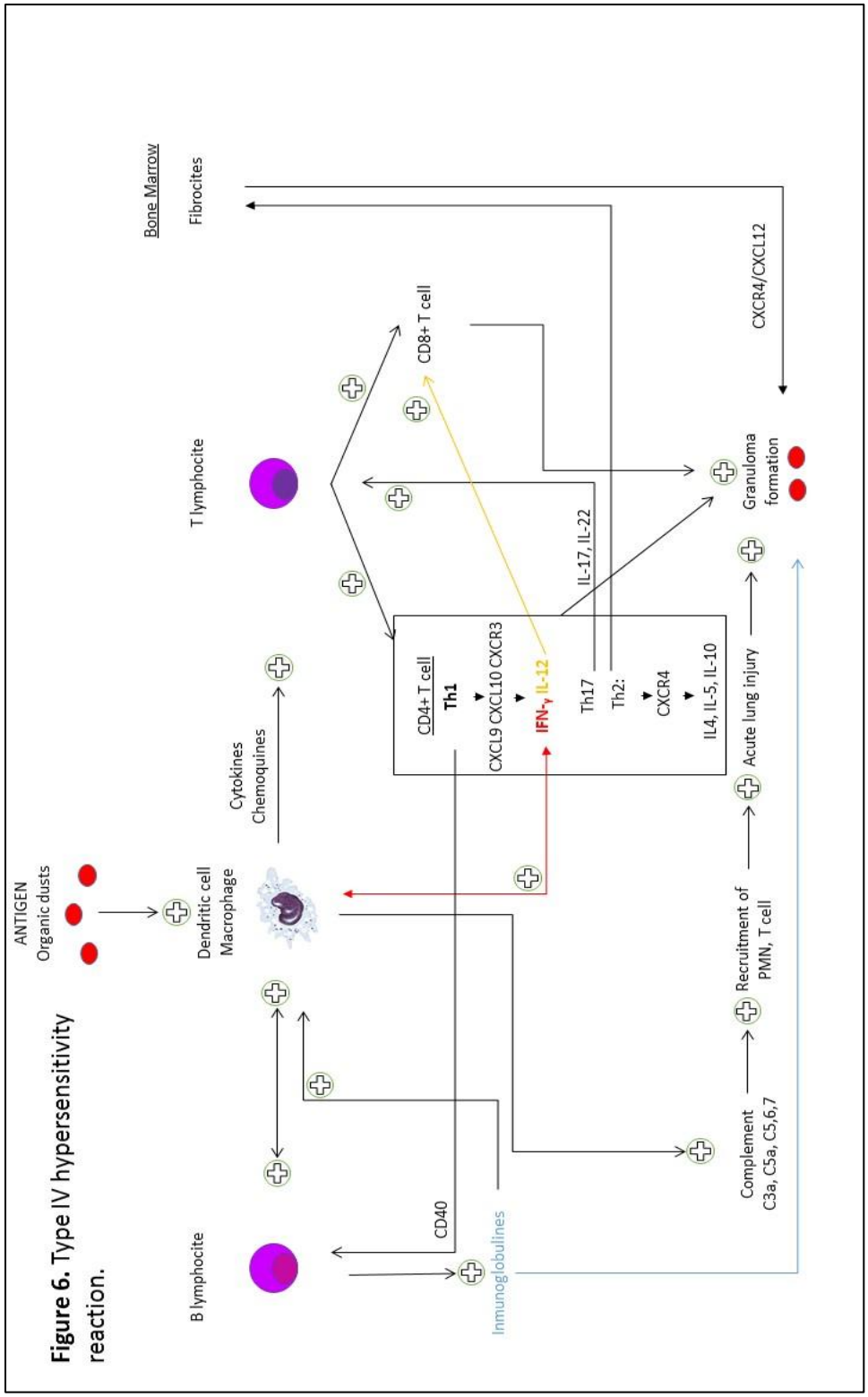


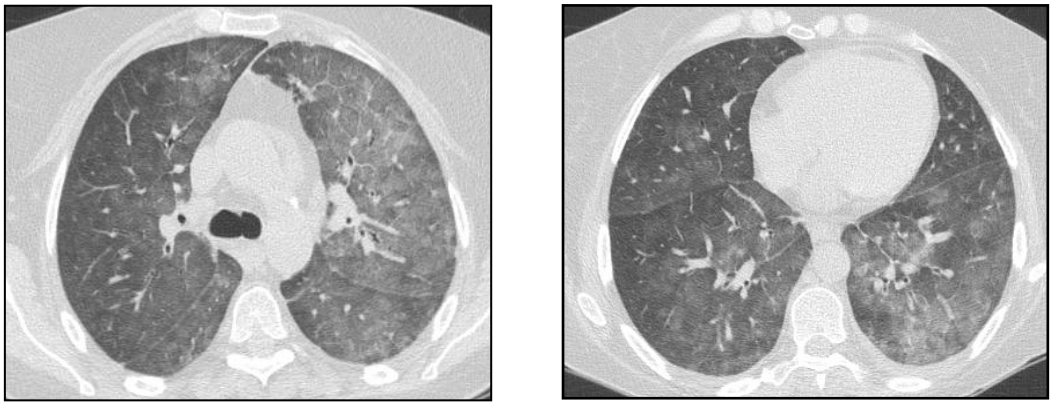
Figure 6. Type IV hypersensitivity reaction.

1.3 CLINICAL FORMS

1.3.1 ACUTE FORM

The acute form of HP is the most characteristic and specific presentation. It is related with intermittent and high intensity exposure to the antigen causing the disease. “Farmer’s lung”, which occurs in people who work in fields and are in contact with damp hay, is one of the most typical examples of this type of presentation[17]. “Bird fancier’s lung” is another frequent form that occurs when these clean bird cages and are exposed to avian antigens[19]. The beginning of the cycle is abrupt, and patients present clinical symptoms that are compatible with a viral upper respiratory tract infection with a temperature, myalgia, severe headache and a dry cough, dyspnea, tachypnea and thoracic oppression. Physical examination reveals bibasal crackles. Nail clubbing is very unusual. The signs and symptoms last between 6 and 24 hours and normally stop several days after exposure has ended1.

Figure 7. Thoracic CT: acute phase of hypersensitivity pneumonitis.



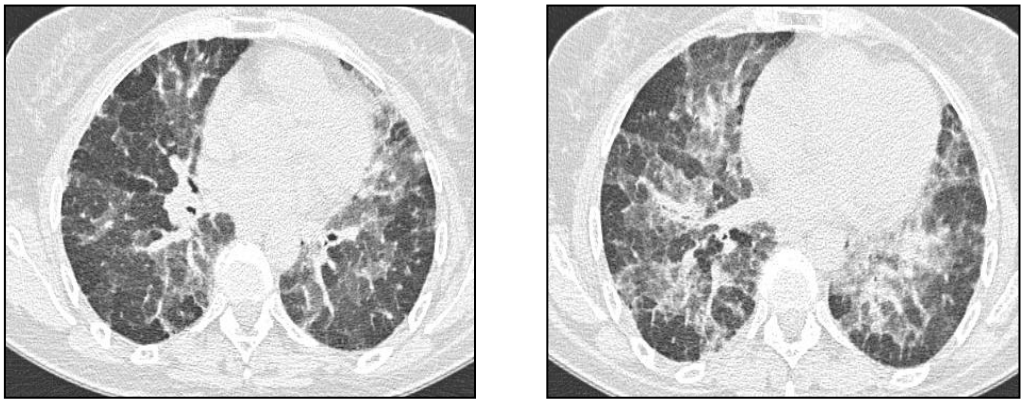
1.3.2 SUBACUTE FORM

Occasionally more latent symptoms are observed, with slight fever and exertion dyspnea after more prolonged antigen exposures at lower intensities than those previously described. After repeated episodes the disease may evolve to its chronic form[63].

1.3.3 CHRONIC FORM

The chronic form tends to result from continued exposure to low intensities of potentially pathogenic antigens. The onset of clinical manifestations tends to be insidious with slowly progressive exertion dypsnea, dry cough and weight loss. Usually, patients do not tend to associate these symptoms with antigen exposure and the slow evolution and non-specific clinical symptoms hinder diagnosis. Physical examination tends to reveal dry bibasal crackling. Nail clubbing is observed in 20% to 50% of patients1.

Figure 8. Thoracic CT: chronic phase of hypersensitivity pneumonitis.



However, currently, some authors maintain that, since there are no clearly established criteria to differentiate between the three forms, HP could be classified into two clusters. One of the clusters would encompass those patients with more recurring systemic symptoms and a normal thorax radiography and would have a more favourable progression; the other would refer to those patients with nail clubbing, hypoxemia, restrictive ventilatory defects and fibrosis in the high resolution CT of the thorax, and would have a more unfavourable progression[64].

1.4 DIAGNOSIS

1.4.1 DIAGNOSTIC CRITERIA

Different algorithms have been put forward to establish the diagnosis of HP, but the majority of them have not been validated. Moreover, diagnosis is especially difficult in those cases of chronic HP, in which symptoms can be very non-specific and are not clearly related to a causal agent. At the end of the 1990s, Schuyler and Cormier[65] listed the most accepted criteria, which probably are the most useful, to perform a correct HP diagnosis (table 2). Later, in 2004, Selman et al.1 described criteria for the diagnosis of HP, with the specific provision that they would establish diagnostic premises for each form of the disease (acute, subacute and chronic) (table 3).

Table 2. Major and minor criteria for the diagnosis of hypersensitivity pneumonitis. Patients had to present at least 4 of the major criteria and 2 of the minor criteria. Other conditions with similar characteristics were ruled out. Adapted from Shuyler et al.[65].

Major criteria
Symptoms consistent with HP
Evidence of appropriate antigen exposure in medical history and/or detection of specific precipitins in serum and/or BAL findings consistent with HP on chest plain films or chest CT
Lymphocytosis in BAL (when performed)
Histological changes consistent with HP
Positive SIC (reproduction of symptoms and laboratory abnormalities following exposure to the suspected antigen)
Minor criteria
Bilateral basal crackles
Decrease in DLCO
Arterial hypoxemia, at least following exercise

Table 3. Diagnostic criteria of HP. Adapted from Selman et al.[1]

Criteria for acute HP
Evidence of exposure, documented by history and specific antibodies
Flu-like syndrome
Increased BAL lymphocytes and neutrophils
Partial but significant improvement after removing the patient from the suspected environment and worsening after re-exposure
Criteria for subacute HP
Evidence of exposure with cause-effect relationship and precipitins against the offending antigen
BAL lymphocytes (usually more than 50%)
Diffuse micronodular pattern with air trapping and ground-glass attenuation on HRCT
Chronic HP (in addition to the criteria mentioned for subacute HP)
Environmental or laboratory-controlled challenge test with the suspected antigen
Lung biopsy

1.4.2 DIAGNOSTIC TOOLS

1.4.2.1 Medical history

The primordial facet in diagnosis is anamnesis. At times, the examination may easily discover the cause of the clinical picture if the patient looks after birds or works in fields with damp hay. However, on many occasions, the antigen is not as easily identifiable and especially in chronic cases, the doctor must have good knowledge of the different exposures potentially causing the disease, in order to be able to question the patient. In this regard, the doctor must know that rather than questioning and thinking about exposure to a rare or unknown substance, it is much more likely that the hidden cause is due to an unusual exposure to an already known causal agent. In this regard, cases have been reported of patients with chronic HP after inhaling the excrements of starlings populating a park beside their house[66] or chronic HP following exposure to fungi colonising cured meats in the operators handling them, in the form known as "Chacinero's lung"[67].

1.4.2.2 Physical examination

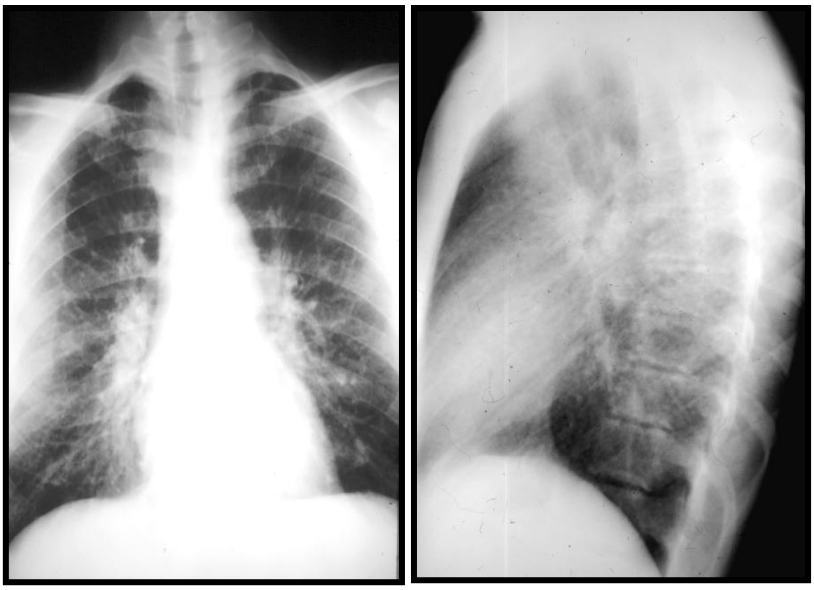
Auscultation may be normal or "inspiratory rales" may be heard. In more advanced cases Velcro-type inspiratory sounds may be heard. A characteristic but infrequent sound is the tele-inspiratory whistle known as "chirping rales" which Laënac had described as "*le cri d'un petit oisseau*"[68].

Patients presenting chronic forms of the disease may present nail clubbing.

1.4.2.3 Thoracic radiography

A radiography of the thorax is a tool of limited use to support the HP diagnosis. In the acute form, findings compatible with a diffuse pattern, with abundant ground-glass attenuation and micronodules, can be observed. The sub-acute forms can show a micronodular pattern and reticular opacities. The chronic form mainly reveals a reticular pattern and a honeycomb pattern[28]. Unlike IPF, the findings are more diffuse and are mainly in the middle and upper zones[69]. Pleural affectation is infrequent[70,71]. The thorax radiography can be normal in 30% of patients, and even in some patients with advanced lung disease.

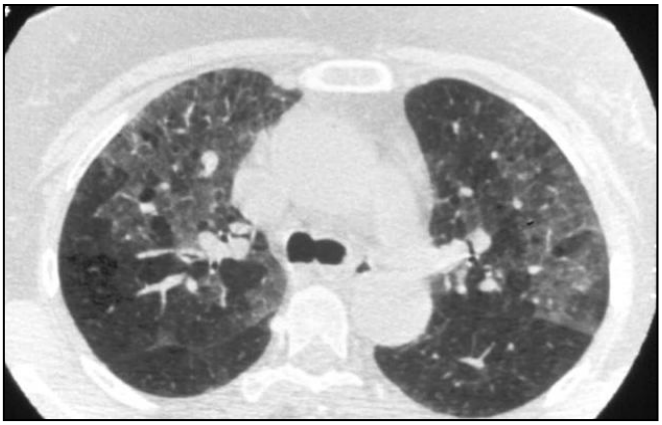
Figure 9. Thoracic radiography: pattern of interstitial lung disease compatible with hypersensitivity pneumonitis.



1.4.2.4 High-resolution thoracic CT

In the acute and subacute forms, the most characteristic findings consist of a patchy ground-glass pattern. Centrilobular nodules are often found, as well as a mosaic pattern associated with bronchiolitis, which in a suitable clinical context are highly suggestive of HP[72,73]. The chronic phase of HP is characterised by the presence of fibrosis, although generally overlapping signs of active disease can be observed, such as loss in volume, nodular reticular opacities and honeycombing. The distribution may dominate upper or lower zones, but it is generally different to the typical predominant basal honeycomb pattern of IPF[12]. Findings compatible with emphysema may be found in 20% of non-smoking patients with chronic HP[74].

Figure 10. Thoracic CT compatible with chronic hypersensitivity pneumonitis.



1.4.2.5 Pulmonary function testing

The most frequent findings consist of restrictive ventilatory defects and an alteration in gas exchange consisting of a reduction in the CO transfer test and hypoxemia during exercise[75]. These alterations are compatible with all ILD in general, and are not specific to HP. In the acute form, and in the final phase of chronic HP, hypoxemia may be observed when at rest[28]. Some patients tend to exceptionally present an obstructive lung disease[28].

1.4.2.6 Immediate and delayed hypersensitivity skin testing

Skin testing against causal antigen extracts as a diagnostic method are either not mentioned in the different reviews of skin diseases[76,77] or are not usually considered to be determining[78] or helpful[79] factors. However, if these are correctly performed with a suitable antigen extract, some studies have demonstrated the diagnostic usefulness of immediate skin testing (15 minutes), both in the diagnosis of farmer's lung (83% sensitivity and 72% specificity using hay extract)[80], and bird fancier's lung (90% sensitivity and 85% specificity)[81].

In delayed hypersensitivity skin testing in the case of HP the response is usually reduced, as occurs in sarcoidosis[82,83].

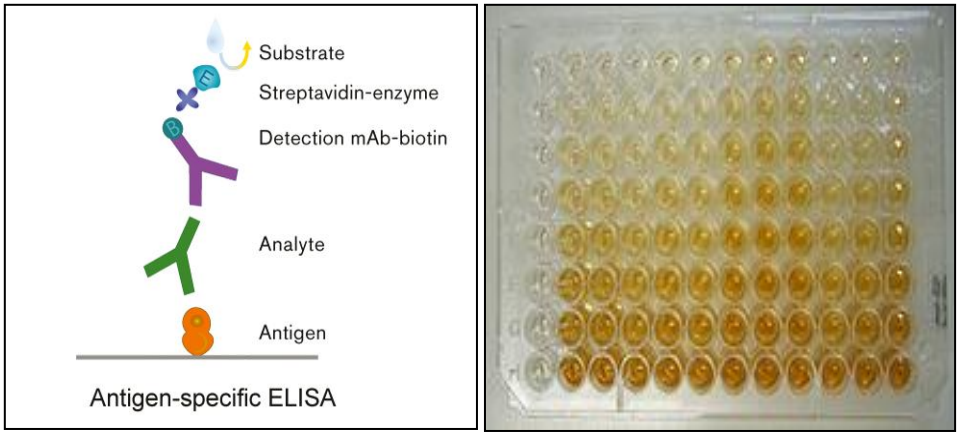
1.4.2.7 Determination of specific IgG antibodies

The determination of these markers is useful in the diagnostic approach and may occasionally suggest a possible exposure that might have gone unnoticed. The immune response to the inhaled antigens can be detected by the production of specific IgG in serum[65,84]. Different qualitative (precipitins, immunoelectrophoresis) and quantitative (ELISA, ImmunoCAP, Immulite) methods to determine IgG antibodies are available, but the results vary considerably[85]. The selection of the antigens to be studied tends to need to be performed according to the main antigens causing the disease in a particular environment.

A high specific IgG antibody titer associated with consistent clinical characteristics is highly suggestive of HP. Lacasse et al.[86] demonstrated that positive antibodies in serum are a positive predictor of HP (odds ratio: 5.3; 95% CI:2.7-10.4). However, the presence of specific IgG antibodies against the trigger antigen does not necessarily demonstrate the disease, but sensitisation to the antigen with which contact has been had. In this regard, according to Costabel et al.[87] between 30% and 60% of healthy farmers produce specific IgG antibodies against the antigens to which they are exposed, although it is important to highlight that a positive specific IgG antibody titer in a specific clinical context calls for HP to be assessed as a possible diagnostic alterative.

A possible limitation of this technique lies in the fact that between 10% and 15% of patients with HP do not produce specific IgG antibodies, confirming that the absence of these findings does not exclude the pathology[88,89].

Figure 11. Determination of specific immunoglobulin G using the ELISA technique.



1.4.2.8 Bronchoalveolar lavage (BAL)

In 1974 Reynolds and Newball described the bronchoalveolar lavage technique for the first time[90]. Initially, this technique was developed as a procedure to analyse inflammatory and immunological cells in the lower respiratory tract, both in healthy lungs and in various types of interstitial diseases. In this first study, BAL was performed on 32 individuals who were participating as a control group, and 26 patients with ILD, which were mainly IPF and HP. These clinical characteristics of BAL meant that at the end of the 1980s BAL was mainly used for the study of ILD[91,92]. However, over time, the use of this technique has not become widespread, and although in the main European centres it is a routine technique, in the United States its use remains controversial. In this regard, in recent IPF diagnosis guides, its systematic practice is not recommended[14,93,94].

The presence of a lymphocytic pattern in the BAL is highly suggestive of granulomatous disease: the observation of a lymphocytosis higher than 25% is characteristic of HP and of sarcoidosis; although it has also been described in entities such as berylliosis, drug toxicity (patients undergoing treatment with amiodarone and methotrexate, among others), non-specific interstitial pneumonia, organising pneumonia, lymphoid pneumonia and lymphoma,

radiation pneumonitis, Wegener's granulomatosis, Crohn's disease, and primary biliary cirrhosis[95]. The presence of a differential cell count with 50% or more lymphocytes is highly suggestive of HP or cellular non-specific interstitial pneumonia; the first option being more likely if accompanied by a T CD4/CD8 lymphocyte ratio of less than 2[96]. As regards HP, it is important to know that as the disease evolves towards fibrosis, the lymphocyte figure decreases in the BAL[97,98], although in 80% of chronic HP a lymphocyte percentage of more than 20% is maintained[99].

The finding of neutrophilia (>3%) in the BAL in the absence of infection, and accompanied or not by the presence of high figures of eosinophils, is present in 70-90% of cases of IPF, and has been related, in some studies, with the degree to which the fibrosis has spread[93,94]. The presence of mastocytes in the BAL has also been related with the spread of the fibrotic process in some ILD, such as HP, among others[100].

The presence of an eosinophil in the BAL with eosinophil figures greater than 25% is highly suggestive of eosinophil pneumonia[101], although eosinophil figures higher than 1% have also been described in HP and other ILD[93].

1.4.2.9 Specific inhalation challenge (SIC)

The majority of guides or review articles maintain that the diagnosis of HP must be performed according to clinical, radiological and inflammatory criteria[102,103]. In this context, it is rather surprising that in a disease with a clearly immunological foundation, greater diagnostic value is not afforded to immunological tests. In this context it would be logical to assume that bronchial challenge tests would be an essential element for the diagnosis of this entity[104]. However, various authors believe that as a result of a lack of standardised tests, both in terms of defining the inhalation protocols and establishing positivity protocols, and due to a risk of possible serious reactions, these tests should only be performed in selected patients and in specialised centres with qualified staff[105,106]. Despite the heterogeneity of the different studies analysing the usefulness of bronchial challenge tests for the diagnosis of HP, the sensitivities and specificities of same are adequate (Table 3), confirming their usefulness for the diagnosis of this disease[107,108]. In this same vein, a recent study by Muñoz and al.[109], performed on 113 patients with suspected HP demonstrated a high diagnostic usefulness of bronchial challenge

tests with a sensitivity and specificity of 73% and 84% respectively, if all the tests are analysed, regardless of the causal agent, or of 85% and 86% if only exposures to avian or fungal protein antigens are assessed. In general the exposure method consisted of nebulisation of an extract of the antigen suspected of being the cause of the disease. Patients were asked to inhale 2 mL of the suspected antigen in a dilution of 1/100 (0.01 mg(mL)[110]. FVC, FEV, DLCO and basal body temperature measurements were taken prior to inhalation, 20 minutes after exposure and subsequently every hour, for the following 8 hours and 24 hours later. A hemogram and thoracic radiography were performed and oximetry measurements were taken prior to exposure and 8 hours later. In all cases, a bronchial challenge test was performed with placebo (saline serum) the day before the test with the suspected antigen. By employing this technique, only 9 patients (8%) presented reactions to the bronchial challenge test. All the reactions were temporary and only 3 patients required the administration of oral corticosteroids.

The test was considered positive when one of the following criteria was met: 1) a decrease in the FVC >15% or a decrease in the DLCO >20% compared to basal values; 2) a 10% to 15% decrease in the FVC, as well as one of the following criteria related to the basal parameters: a) 20% increase in leucocytes; b) 3% decrease in the oximetry; c) appearance of patchy infiltrates in the thoracic radiography; d) more than 0.5ºC increase in body temperature; e) significant clinical symptoms (cough, dyspnea…); 3) a decrease in the FVC >10% accompanied by at least 3 of the abovementioned criteria. If the test result was negative, the same procedure was repeated with the antigen at a dilution of 1/10 (0.1 mg (mL).

This study probably demonstrates that the bronchial challenge test should not be considered as a test to be exclusively performed in particular cases in centres interested in the topic, but rather, that it should be widely spread, since the complexity of its performance is minimal if the adequate antigens are available and if it is performed with a correct protocol; the usefulness, as we have observed, is good with few adverse effects. The bronchial challenge test also enables false diagnoses of IPF to be ruled out, as was recently demonstrated in a series of 46 patients who met the IPF criteria and in whom eventually, with the help of SIC it was demonstrated that 43% of them were actually patients affected by HP[111]. This fact is transcendent on account of the different treatment to be applied in both entities[112].

However, the SIC has some limitations. As mentioned above, there is no standardisation either in terms of an inhalation protocol or the positivity criteria of same[105] (table 4). Furthermore, previous studies have shown that the sensitivity and specificity of the SIC can oscillate between 73-92% and 82-100% respectively, and therefore, a negative result would not rule out a HP diagnosis[109]. In this regard, our group has demonstrated in previous studies that changes in the inflammatory profile in induced sputum after the SIC can be useful to define the positivity of this test, in a similar way to that which occurs in occupational asthma[113]. However, sputum induction and processing techniques are time-consuming and are not available in the majority of centres. As will be incorporated later in this PhD thesis, inflammatory parameters can be easily quantified in exhaled air. Nevertheless, these non-invasive methods of measuring respiratory tract inflammation have not been tested to date for the diagnosis of HP[114]. Therefore, one of the objectives of this thesis is to perform an in-depth study of inflammation markers in exhaled air, particularly changes in the pH of the AEC (Chapter 1) and in the FeNO (Chapter 2) following the antigen exposure using the SIC, with a view to improving the diagnostic usefulness of this test.

Figure 12. Temperature, FVC and DLCO curves in a positive bronchial challenge test in patient exposed to avian antigens.

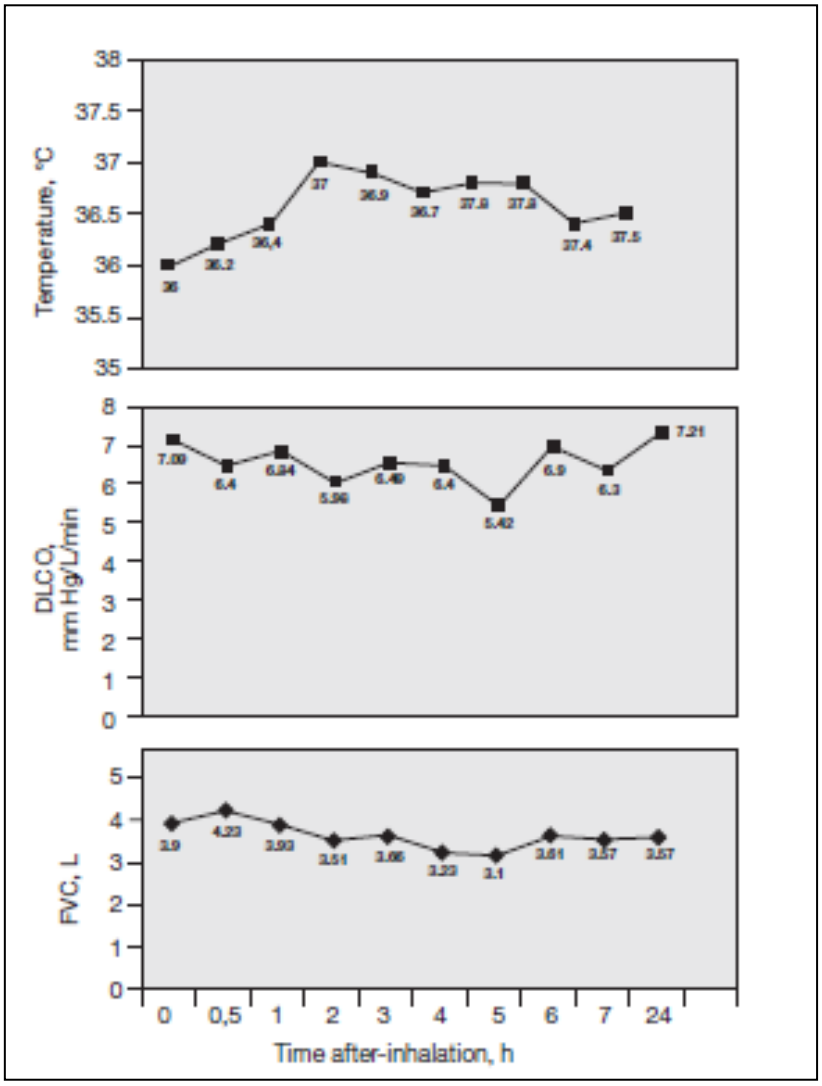


Table 4. Criteria to interpret bronchial challenge tests. Adapted from Quirce et al.[115]

References	Patients	Antigen	Criteria for positive response	Validity of the criteria
Hendrick	144 ICT in 29 suspected HP (18 proved HP); 2 controls	Avian antigens	1 Rise in body T° >37.2°C 2 Increase in blood neutrophils >2500/mm3 3 Decrease in FVC >15% 4 Decrease DLco >15%	Equivocal result: 15/144 • Sensitivity: 78% (criteria 1); 68% (criteria 2); 48% (criteria 3); 17% (criteria 4) • Specificity: >95% for all criteria
Ramirez-Venegas	17 chronic HP; 17 other ILD; 5 controls	Avian antigens	*At least one of the following criteria:* 1 Decrease in FVC >16% 2 Decrease in PaO2 >3 mmHg 3 Decrease in SaO2 >3% 4 Rise in body temperature >0.5°C	• Equivocal result: 3/39 • Sensitivity: 76-100% • Specificity: 82-86%
Ohtani	17 chronic HP; 17 other ILD; 5 controls	Avian antigens	*Three or more of the following criteria:* 1 Increased radiologic abnormalities 2 Increase in P(A-a) O2 >10 mm Hg and/or a decrease of DLco >20% 3 Decrease in FVC >15% 4 Increase in blood leukocyte count >30% 5 Increase in C-Reactive Protein >1.0 mg/dl 6 Increase in body T° >1.0°C and/or systemic symptoms 7 Respiratory symptoms	• Equivocal result: 3/11 • Sensitivity: 73% • Specificity: 100%
Morell	17 chronic HP; 17 other ILD; 5 controls	Avian antigens	1 FVC decrease >15% or DLco decrease >20% 2 10–15% FVC decrease plus at least one	• Equivocal result: 5/59 • Sensitivity: 92% • Specificity: 100%

			of the following criteria: a) White blood cell increase _20% b) Decrease in SaO2 >3% c) Significant radiologic changes d) Rise in body temperature >0.5_ C e) Clinical symptoms (e.g. cough, dyspnea) 3 FVC decrease <10% plus _3 above mentioned criteria	
Ishizuka	28 HP; 19 other ILD;	Avian antigens	Two or more of the following criteria: 1 Increased radiologic abnormalities 2 Increase in the alveolar–arterial oxygen pressure difference (P[A-a]O2) by more than 10 mm Hg and/or a decrease of DLCO by more than 20% 3 Decreased VC by more than 15% 4 Increase in the peripheral WBC count by more than 30% 5 Increased CRP by more than 1.0 mg/dl 6 Increased body temperature by more than 1.08C and/or the development of systemic manifestations, including chills and general fatigue 7 Development of respiratory symptoms (cough and dyspnea)	• Equivocal result: 7/47 • Sensitivity: 79%† • Specificity: 95%

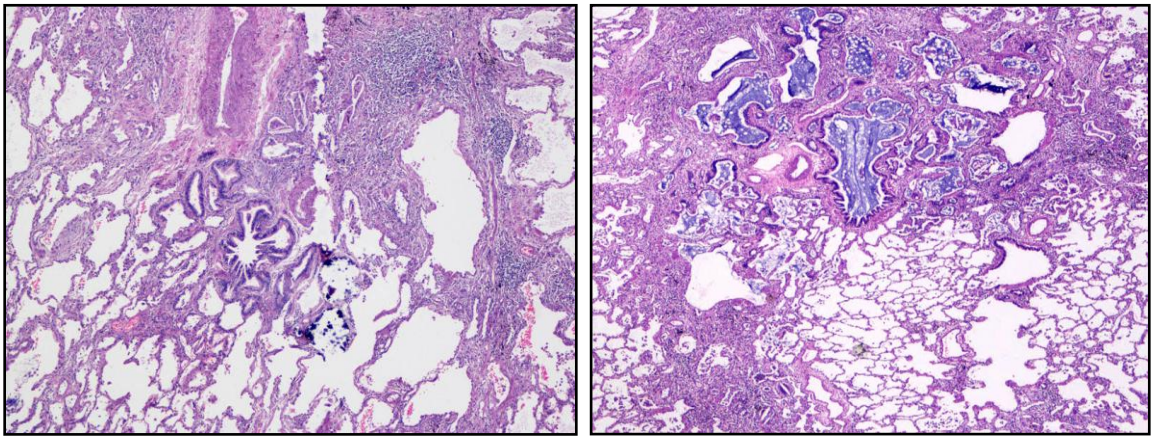
1.4.2.10 Transbronchial biopsy. Cryobiopsy

Taking advantage of the practice of fibrobronchoscopy to obtain the BAL, in many centres a transbronchial biopsy (TBB) is performed simultaneously, although its diagnostic usefulness in

ILD is questionable[116]. It can be useful in the case of granulomatous diseases, such as HP and sarcoidosis, and in lymphangitic carcinomatosis, diffuse alveolar damage, alveolar proteinosis and eosinophilic pneumonia[117]. Its usefulness in the study of interstitial disease is low as a result of pulmonary affectation generally being patchy or heterogeneous and because the samples obtained are small. In these samples, as a result of the use of forceps, the structural preservation of the pulmonary tissue tends to be deficient[118]. According to some studies, the usefulness of the technique in the diagnosis of DILD is 30%[119].

During recent years, the technique of pulmonary biopsy using cryoprobes has been introduced, and become established as a reliable and highly useful diagnostic technique in interstitial disease. In 2009, Babia et al.[120] published, for the first time, the potential usefulness of cryobiopsy in the study of interstitial diseases, revealing that in a significant number of cases the information obtained from the cryobiopsy was useful in the establishment of a definitive diagnosis, findings which were subsequently reproduced in other studies[121]. Hernández-Gonzalez et al. described a series of 33 patients with interstitial disease in which pulmonary cryobiopsy obtained a diagnostic usefulness of 76%[122].

Figure 13. Pulmonary biopsy revealing non-necrotizing granulomas, fibrosis, dilation of the airway and bronchiolar metaplasia.



1.4.2.11 Surgical pulmonary biopsy (SLB)

A surgical pulmonary biopsy should only be indicated as a last resort when the medical history, physical examination and other abovementioned additional examinations cannot establish a

certain diagnosis[2]. Surgical pulmonary biopsy is an invasive procedure and presents an average mortality of 3.6% (CI 95%, 2.1-5.5) according to a meta-analysis that included 2,148 patients with interstitial pulmonary disease[123]. Patients with more deteriorated pulmonary function (FVC < 55% and DLCO < 30%), mechanical ventilation or undergoing immunosuppressive treatment were at higher risk of suffering complications. The diagnostic usefulness did not vary according to the subtype of interstitial pulmonary disease being studied.

1.4.2.12 Study of the HP inflammatory profile, using non-invasive methods

Considering the inflammatory origin of the majority of respiratory diseases, non-invasive methods of studying pulmonary function are increasingly being used in the diagnosis or monitoring of these diseases[124]. Thus, the study of cellularity in induced sputum or of biomarkers in exhaled air or in exhaled breath condensate has proved to be useful in the evaluation of certain respiratory diseases[125]. However, there is little experience of the use of these techniques in the assessment of patients with HP.

1.4.2.12.1 Sputum induction (SI)

The usefulness of SI is well-known in diseases such as bronchial asthma or COPD[126]. However, its role in DILD is not as evident[127,128]. Very few studies analyse SI in HP and all of these confirm the increase in the total cell count and in the lymphocyte count, compared to healthy controls[129,130,131]. In the most recently published study concerning SI in HP, Villar et al.[113] analyse the inflammatory profile in two groups of patients with HP secondary to different antigens. The study reveals a similar basal inflammatory profile in patients with HP caused by fungi and by birds. However, after the bronchial challenge test, those patients with HP from fungi presented an increase in neutrophils and proinflammatory cytokines, while those patients with HP from birds, presented an increase in eosinophils and Th2 cytokines. These results led the authors to speculate that different antigens could generate different inflammatory mechanisms.

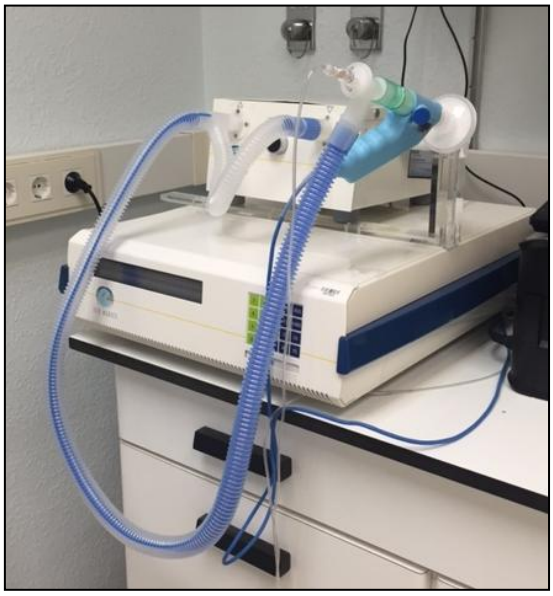
1.4.2.12.2 Fractional exhaled nitric oxide (FeNO)

The FeNO is a non-invasive marker that enables eosinophilic inflammation in the airways to be analysed swiftly[132]. FeNO levels depend on different factors such as the demographic characteristics of patients, smoking habits and physiological and biochemical factors[133]. Different studies maintain that their values are reduced in diseases such as COPD[134,135], bronchiectasis and primary ciliary dyskinesia, and increased in patients with asthma[136].

In patients with atopic asthma in particular, activation of the inducible nitric oxide synthases (iNOS) has been reported in the epithelium of the airway, at the expense of the STAT-6 route, through the action of proinflammatory Th2 cytokines such as IL-4 and -13, leading to the release of high levels of NO in the exhaled air[137,138]. Some studies suggest that FeNo could be a direct marker of a Th2 inflammatory mechanism[139]. Some authors have suggested that in patients with DILD, evolution towards fibrosis could be mediated by this Th2 inflammatory mechanism[140]. However, there is very little evidence of the usefulness of FeNO in these diseases.

Since some authors have suggested that the Th2 inflammatory route could be involved in those patients with HP who evolve to fibrosis, it seems logic to think that a Th2 inflammation biomarker such as FeNO, could be useful in chronic HP[140]. In this vein, recently Guilleminault et al.[141] analysed a cohort of HP patients in whom the NO levels were higher than in other ILD. As a result, they proposed that FeNO could be a useful marker for the diagnosis of HP. Due to the existence of these antecedents, in the second chapter of this thesis we consider analysing possible changes in FeNO values after antigen exposure using SIC (Chapter 2).

Figure 14. Equipment for the measurement of fractional exhaled nitric oxide.



1.4.2.12.3 Exhaled breath condensate (EBC)

Exhaled air contains valuable information about the physiological and bio-pathological processes affecting the airways, the pulmonary parenchyma and other organic system processes separate to the lungs[142].

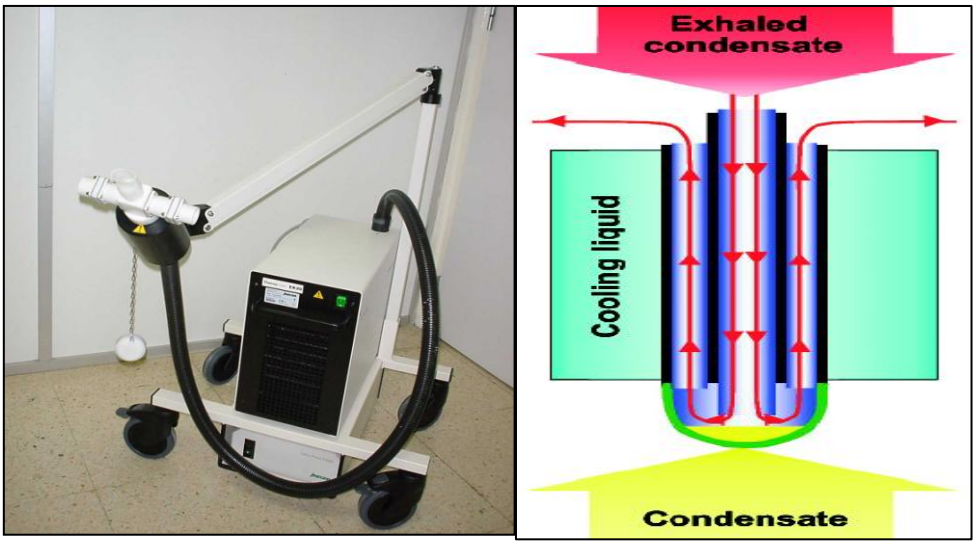
The exhaled air is made up of a gas phase and an aqueous phase. In the gas phase physiological gases and other endogenous volatile compounds are found, such as nitric oxide, carbon monoxide and hydrocarbons, among many others. The aqueous phase is made up of a suspension of tiny drops stemming from the aerosolization of the epithelial lining fluid. It is considered that the fraction of epithelial lining fluid contained in the CE is representative of the extracellular pulmonary microenvironment with its different biological mediators[143].

When the exhaled air, saturated with water, goes through an environment with low temperatures, the water vapour condenses, causing the small drops in suspension, stemming from the aerosolization of the epithelial lining fluid found in the exhaled air, to increase their volume and precipitate over the surface of a specially designed condenser system[143].

The pH of the AEC is a recently implemented tool that could be useful in the study of various respiratory diseases[144]. Several studies have revealed a drop in the pH value in uncontrolled

bronchial asthma[145], in the context of respiratory infections in patients with bronchiectasis, COPD or cystic fibrosis[146]. However, there is much less experience in DILD: higher levels have been reported in patients with pulmonary fibrosis[147] and lower levels have been reported in patients with asbestosis[148]. To date, no study analysing the role of AEC pH in HP has been performed. As discussed previously, recently our group has demonstrated the presence of bronchial inflammation in induced sputum in patients with HP, with an increase in total cellularity and lymphocytes in these patients compared to the healthy population[113]. Therefore, another of the objectives of this thesis was to analyse the usefulness of the pH of AEC to study the degree of inflammation in chronic HP and quantify its changes following antigen exposure using the SIC (Chapter 1).

Figure 15. Equipment to measure the exhaled breath condensate.



1.5 DIFFERENTIAL DIAGNOSIS

The differential diagnosis encompasses the wide spectrum of all ILD. Chronic HP in the fibrosis phase often requires great effort by the doctor to be able to differentiate it from NSIP and IPF. Our specific experience in this regard, as mentioned above, consists of the study of a series of 51 patients with initial criteria for IPF according to the 2011 international standards[14], who we studied exhaustively from an etiological point of view with directed anamnesis, collection of material from the antigenic source, study of the antibodies against birds and fungi as well as

other antigens in the patients' serum, BAL and TBB, bronchial challenge test against the suspected antigen and, where appropriate, surgical pulmonary biopsy. In this study of patients diagnosed between 2004 and 2009 and monitored until 2012, it was concluded that 43% of these IPF were actually chronic HP[111]. As reported by Wuist et al.[149] the diagnosis of chronic HP requires demonstrated exposure to a causal antigen or the suspicion of a potential exposure, related to the onset of symptoms; sensitisation to the suspected antigen and compatible findings in the CT of the thorax. However, serum IgG against a variety of common antigens have low sensitivity and specificity, and are useful when they are positive, but not negative. Chronic HP may be a diagnostic dilemma in those cases in which a temporal relation cannot be established between exposure and disease. Thus, it seems particularly relevant to avail of new diagnostic tools that enable us to establish a precise diagnosis of chronic HP, in such a way that patients can benefit from the indicated treatment in each case.

1.6 NATURAL HISTORY AND PROGNOSIS

The prognosis for HP varies considerably according to the clinical form of the disease (acute, subacute or chronic)[150]. Some patients present progression of the disease, despite avoiding the causal antigen and following a pharmacological treatment, although it is not known why this progression takes place in one subgroup of patients and not in others 2.

In general terms, the prognosis for acute HP is favourable and by avoiding the causal antigen and following an initial treatment with corticosteroids, the disease tends to go into remission. A study conducted in patients with bird fancier's lung showed that these had an excellent prognosis if the duration of the exposure was less than 6 months. Normalisation of the pulmonary function was observed in practically all the patients, after exposure had ceased[151]. However, when the disease is in the chronic phase, despite avoiding the antigen and following the corticosteroid treatment, the disease tends to progress towards respiratory insufficiency and death, if a lung transplant is not performed[2,111]. Various studies highlight the role of the spread of fibrosis in the thoracic CT[152,153] or histological findings in the pulmonary biopsy[154], as HP survival predictor factors.

HYPOTHESIS

2 HYPOTHESES

Bearing in mind the limitations of the specific inhalation change mentioned in the introduction of this thesis, the hypotheses considered in this PhD thesis are the following:

1. The pH of the EBC and/or the FeNO can be useful tools to improve the diagnostic usefulness of SIC.
2. The pH of the EBC can be useful to analyse the degree of inflammation in different types of chronic HP.

OBJECTIVES

3 OBJECTIVES

1.-To quantify the possible changes in the pH values of the EBC and FeNO in patients with HP following the SIC, and assess its potential usefulness as a marker of this test.

2.-To analyse the usefulness of the pH of AEC to study the degree of inflammation in chronic HP caused by birds and fungi.

CHAPTER 1

4 CHAPTER 1: CHANGES IN PH IN EXHALED BREATH CONDENSATE AFTER SPECIFIC BRONCHIAL CHALLENGE TEST IN PATIENTS WITH CHRONIC HYPERSENSITIVITY PNEUMONITIS: A PROSPECTIVE STUDY.

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Key words: Inflammation, pulmonary fibrosis, environmental exposure.

4.1 ABSTRACT

Introduction: The aim of this study was to investigate the influence of the specific inhalation challenge (SIC) on changes of pH values in exhaled breath condensate (EBC) in patients with hypersensitivity pneumonitis (HP).

Material and methods: A prospective study of 85 patients with suspected HP, of whom 63 were diagnosed with HP due to exposure to avian or fungal antigens. In all cases, EBC samples were collected before and after completion of the SIC and pH values were determined.

Results: Taken as a whole, patients with HP did not present changes in EBC pH after SIC. However, considering only patients with exposure to molds, those diagnosed with HP had a significantly more acid pH post-SIC than those with another diagnosis ($p = 0.011$). This fact is not observed in patients exposed to bird's antigens. A ROC curve showed that a reduction in EBC pH of 0.3 units or more after SIC in patients diagnosed with HP due to exposure to molds had a sensitivity of 30% (CI: 12.8 to 54.3%) and a specificity of 100% (CI: 65.5 to 100%).

Conclusion: EBC pH may be useful in interpreting SIC results in patients with HP, especially in those patients exposed to molds. Further studies are now required to test the validity of these proposals.

4.2 INTRODUCTION

Hypersensitivity pneumonitis (HP) is a complex syndrome of variable intensity and clinical history, caused by an immune-mediated inflammation of the lung parenchyma due to the continued inhalation of a wide range of antigens[1]. The increased recognition of exposure to environmental antigens and improvements in the diagnostic tools available have allowed the identification of a variety of environmental and occupational settings that can cause this disease.

HP is difficult to diagnose because of the wide spectrum of clinical variants and the absence of a "gold standard" diagnostic test. In general it is diagnosed on the basis of clinical criteria, among which Schuyler and Cormier's criteria are the most frequently used[2]. As HP is an

immunologically mediated disease, some authors have suggested that the specific inhalation challenge (SIC) could be a useful diagnostic tool[3]. Although the SIC can demonstrate a direct link between exposure to an antigen and impaired lung function in the patient[4,5], its use in the diagnosis of the HP is limited by the lack of standardization of both the inhalation protocols and the criteria required to define a positive response[6,7]. Thus, while some authors prioritize the symptoms, others prioritize falls in forced expiratory volume (FVC) and diffusing capacity of the lung for carbon monoxide (DLCO) to establish positivity[3].

In view of the inflammatory nature of most respiratory diseases, the use of non-invasive methods to study lung function for diagnosing or monitoring these pathologies is becoming increasingly common[8]. The study of induced sputum cellularity or of biomarkers in exhaled breath or exhaled breath condensate has proven useful in the evaluation of certain respiratory pathologies[9,10]. However, the experience with these techniques in patients with HP is limited. Some studies using induced sputum have reported an increase in the total cell count and the lymphocyte count in patients with HP compared to the healthy population[11,12,13]. Also using induced sputum, our group recently demonstrated that bronchial inflammation is present in patients with HP, and is mainly evidenced by increases in neutrophils that worsen following exposure to the offending antigen during SIC[14]. However, other non-invasive methods for studying pulmonary inflammation have not been analyzed to date in patients with this disease.

The aim of this study was to investigate the influence of SIC on changes of pH values in exhaled breath condensate (EBC) in patients with hypersensitivity pneumonitis (HP).

4.3 MATERIAL AND METHODS

Study population

This prospective, cross-sectional study included all patients older than 18 years, referred to our hospital with suspected HP between 2005 and 2013 and who underwent a SIC (Figure 1). The study was approved by the local Ethics Committee and all subjects gave informed consent prior to participation (Hospital Vall d'Hebron Ethics Committee approval PS09/01486).

Diagnostic protocol

Prior to the administration of the SIC, a thorough clinical history was performed along with additional tests: blood count, immunoglobulin G specific for birds and fungi[4,15], blood calcium levels, angiotensin converting enzyme, lactate dehydrogenase, chest X-ray, high resolution computed tomography, pulmonary function tests including spirometry, static lung volumes and carbon monoxide transfer test, immediate and delayed hypersensitivity skin testing, bronchoscopy with bronchoalveolar lavage and/or transbronchial biopsy and/or criobiopsy. In all cases the diagnosis was made using Schuyler and Cormier's criteria[2]. None of the patients were taking steroid therapy.

Lung function studies

All patients underwent forced spirometry, static lung volume study by plethysmography, and determination of the diffusing capacity of the lung for carbon monoxide (DLCO) using the single breath-hold method. These studies were performed on a MasterLab system (MasterLab, Jaegger, Germany) following the joint recommendations of the European Respiratory Society and American Thoracic Society[16] and the Spanish Respiratory Society[17].

Determination of specific IgG antibodies

Specific IgG to avian sera (pigeon, parrot and parakeet), *Aspergillus fumigatus, Penicillium frequentans* and *Mucor mucedo* were measured by an ELISA technique based on the method previously described[4,18]. Wells of high binding microtiter plates (Costar, Cambridge, MA, USA) were coated with 2 μg protein/well in 0.1 M Na_2CO_3/$NaHCO_3$ buffer (pH 9.6) at 4ºC overnight. The wells were then washed three times with washing buffer (0.1M phosphate buffered saline, pH 7.5 containing 0.005% Tween 20) and blocked with 1% bovine serum albumin in phosphate buffered saline for 1 hour at 37ºC. The specific IgG assays were performed in duplicate by incubating the serum samples at an appropriate dilution for 2 hours at 37ºC, and wells were washed four times between each step. A solution of horseradish peroxidase-labeled anti-human IgG monoclonal antibody (clone MH16-1ME, 0.5 mg/mL) diluted at 1:1000 was added to each well and plates were incubated for 2 hours at 37ºC. The reaction was developed with 3,3',5,5'-tetramethylbenzidine (Sigma Chemicals), 3% H2O2 for 20 minutes at room temperature in the dark and stopped with 2M H2SO4. Optical density at 450 nm was measured with a microplate reader (Titertek Multiskan Plus MKII). Results were expressed as

absorbance units at 450 nm ($A_{450\ nm}$). Values above the mean plus two standard deviations of the results obtained in a control population of 30 healthy individuals previously studied in our laboratory were deemed positive.

Bronchoscopy techniques: bronchoalveolar lavage (BAL) and transbronchial biopsy (TBB)

Bronchoalveolar lavage was performed according to the recommendations of the European Respiratory Society[19]. The TBB procedure used has been described by other authors[20].

Antigen extract preparation for specific inhalation challenge

Commercialized extracts (Bial-Aristegui, Bilbao, Spain) from *Penicillium frequentans*, *Aspergillus fumigatus* and *Mucor mucedo* were used to study fungi[18]. The avian sera and pigeon bloom extracts were prepared in our laboratory, as previously described[3,18].

Specific inhalation challenge

Informed consent was obtained from all patients prior to performance of SIC. A de Vilbiss 646 nebulizer and Mefar MB3 dosimeter were used, which release the antigenic solution during the first second of each inhalation. The technique consisted of inhaling 2 mL of solution at a 1/100 (0.01 mg/mL) dilution. The patients' FVC, forced expiratory volume in one second (FEV1), DLCO and temperature were assessed at baseline, at 20 minutes following exposure, and every hour thereafter for the next eight hours. The SIC was considered positive according to previously published criteria[3,18]. In patients testing negative on SIC, exposure was repeated 24 hours later, at an antigen dilution of 1/10 (0.1 mg/mL). In all cases a baseline test was performed with placebo solution the day before inhalation of the putative causal agent.

EBC collection

EBC was collected during tidal breathing with a commercially available condenser (EcoScreen; Jaeger, Würzburg, Germany), as described elsewhere[9]. Smokers were advised not to smoke during the 48 hours prior to the completion of SIC. Each EBC sample was divided into 500- µL aliquots in two to four plastic tubes. Other aliquots were used to measure the pH before and after deaeration. Baseline EBC pH was recorded 24 hours before the SIC and post-SIC EBC pH was recorded 24 hours after the last antigenic exposure.

Measurement of pH in EBC

pH was measured in one of the aliquots immediately after EBC collection and after deaeration with helium (350 mL/min for 10 min), using a calibrated pH meter (Model GLP 21; Crison Instruments SA; Barcelona, Spain) with an accuracy of 0.01 pH, and a probe for small volumes (Crison 50 28; Crison Instruments SA). The probe was calibrated daily with standard pH 7.02 and 4.00 buffers[21].

Statistical analysis

The Mann-Whitney test and chi-square test were applied to compare continuous and nominal variables, respectively, with a two-sided significance level of 0.05. The consistency of EBC was estimated by evaluating the sensitivity (SE) and specificity (SP)[22] of the method, the positive (PPV) and negative (NPV) predictive values, and the likelihood ratio of a positive (LR+) and negative (LR-) value with 95% confidence intervals (95% CI) using the Wilson method[23]. Receiver-operating characteristic (ROC) curves were constructed to determine the cut-off values that best differentiated between having the disease or not[24]. All analyses were done with SPSS, version 17 (Chicago, IL, USA) and, SAS version 9.2 (SAS Institute Inc., Cary, NC, USA).

4.4 RESULTS

A total of 99 patients were studied. Fourteen patients were excluded because the agents suspected of producing HP were neither birds nor molds (Figure 1). Of the 85 patients studied, 63 were diagnosed with chronic HP, 42 of whom had been exposed to avian proteins and 21 to fungal agents. Of these 63 patients, 52 had a positive SIC. Out of the 52 patients with a positive SIC, 33 were exposed to bird antigens and 19 to molds[3,18]. After SIC, 20 patients presented a decrease of DLCO > 20%, 15 a decrease of FVC between 10-15% plus an increase > 0,5ºC in body temperature, 11 a decrease > 15% in FVC plus a decrease > 20% in DLCO, and finally 6 patients presented a decrease of FVC% > 15%.

Twenty-two patients received diagnoses other than HP, and in all of them the SIC was negative: five were diagnosed with nonspecific interstitial pneumonia, five with sarcoidosis,

eight with bronchiectasis, two with non-classifiable pulmonary fibrosis and two with idiopathic pulmonary fibrosis. Of these 22 patients, 12 were exposed to bird's antigens and ten to fungi.

The clinical characteristics of the study population are presented in Table 1. No significant differences in baseline characteristics were observed according to diagnosis (HP or non-HP) or exposure to bird's antigens or molds.

EBC pH values before and after the SIC are displayed in Figure 2. The mean reduction in EBC pH after SIC in patients with HP was 0.02 in those exposed to bird's antigens and 0.15 in those exposed to molds; it was not statistically significant in either case (p values 0.903 and 0.634 respectively, Table 2). However, considering only patients with exposure to molds, those diagnosed with HP had a significantly more acid pH post-SIC than those with another diagnosis ($p = 0.011$) (Figure 2). In fact, in general, post SIC pH was significantly lower in patients diagnosed with HP than in patients with a different diagnosis ($p = 0.010$).

The sensitivity, specificity and positive and negative predictive values of EBC pH according to the established diagnosis of HP are shown in Table 3. An ROC curve showed that a reduction of EBC pH of 0.3 units or more after SIC had a sensitivity of 25% (CI: 15.9 to 38.7%) and a specificity of 81.8% (CI: 58.9 to 94%) for the diagnosis of HP in the total study population. Analysing only patients diagnosed with HP due to exposure to fungal antigens, a fall in EBC pH of 0.3 or greater showed a sensitivity of 30% (CI: 12.8 to 54.3%) and a specificity of 100% (CI: 65.5 to 100%) (Figure 3). In the group of patients diagnosed with HP due to exposure to avian proteins, a fall in EBC pH of 0.3 or higher showed a sensitivity of 23.8% (CI: 12.6 to 39.8%) and a specificity of 66.6% (35.4 to 88.7%).

No correlation was observed between the EBC pH before SIC and baseline FVC, DLCO or BAL lymphocytes. Additionally, no correlation was observed between the acidification of pH after SIC and fall in FVC, DLCO or temperature increase.

Considering the false negatives of the SIC, in the group of patients diagnosed of HP due to molds ($n = 2$) one patient experienced a drop in EBC pH > 0.3. In the group of patients diagnosed of HP due to avian proteins ($n = 9$), two patients experienced a drop in EBC pH > 0.3.

4.5 DISCUSSION

The present study demonstrates that a drop in EBC pH of 0.3 or higher after SIC has a sensitivity of 30% (CI: 12.8 to 54.3%) and a specificity of 100% (CI: 65 5-100%) for diagnosis of HP due to exposure to molds. In the case of HP due to bird proteins, a fall in EBC pH of 0.3 or more after SIC had a sensitivity of 23.8% (CI: 12.6 to 39.8%) and a specificity of 66.6% (35.4 to 88.7%). This study is the first to analyse the diagnostic performance of the EBC pH in the context of antigen exposure through SIC, in patients with HP due to exposure to bird's antigens or to molds.

These results may be helpful for establishing a firm diagnosis of HP. In acute forms of the condition the clinical criteria currently in use are sufficient [2], but establishing a firm diagnosis in chronic or subacute forms is much more difficult. It has recently been shown that up to 50% of cases of idiopathic pulmonary fibrosis may be evolving forms of HP that have not been properly diagnosed[25] and it is in this context where the SIC may be most useful[26].

The SIC has been used to diagnose since the 1960s, but there is still no consensus regarding the variables on which the test's interpretation should be based. This situation may influence its sensitivity and specificity. In a study with 29 patients basically using clinical criteria such as the onset of symptoms and signs mimicking influenza, Hendrick et al.[5] reported a sensitivity of up to 85% and a specificity of 95%. Also basing positivity on clinical symptoms, Ohtani et al.[7] found no false results in a study of 11 patients with bird fancier's lung. However, other authors using criteria based on falls in the values obtained in lung function tests reported sensitivities ranging from 82% to 92% and specificities between 76% and 100%[4,6].

Establishing a positive diagnosis from the appearance of symptoms during the test necessitates a high level of exposure to the antigen, a requirement which may of course have a negative effect on the test's safety. The use of criteria based on lung function studies to determine diagnosis may allow lower levels of antigen exposure. For example, maximum exposure in Ohtani et al.'s[7] study was 680 µg of avian protein, whereas Morell et al.[4] used a maximum dose of 200 µg of protein. However, this lower antigen exposure during SIC may yield false negatives: evaluating 113 patients, of whom 88 were diagnosed with HP due to different agents, Muñoz et al[26] recently found a sensitivity of 73% and a specificity of 84%, and 24 patients finally diagnosed with HP had negative SIC. The results of this study allow us to

hypothesize that the measurement of EBC pH during SIC may reduce the number of false negatives and thus improve the test's diagnostic accuracy. In this regard, three patients in the present study diagnosed with HP but with a negative SIC, experienced a decline of EBC pH greater than 0.3 units after the SIC.

EBC pH measurement is a recently introduced tool which may be useful in assessing various respiratory diseases[27]. Several studies have shown that pH values may fall in non-controlled asthma[28] or in the context of respiratory infections in patients with bronchiectasis, COPD or cystic fibrosis[29]. However there is less experience in the context of interstitial diseases: higher levels have been found in individuals with pulmonary fibrosis[30], and lower levels in patients with asbestosis[31]. EBC pH is the result of a balance between various buffer systems and the production and release of acids and bases in the airways[32]. In healthy individuals, EBC is determined to a significant extent by the NH_4, HCO_3 and CO_2 produced during breathing[33], with the most acidic pH being found in the alveolar lumen in the proximal airway. Inflammatory processes trigger a range of mechanisms which produce acidification of these more proximal airways as a possible innate defense mechanism[34]. These mechanisms are basically the production and excretion of superoxide ions and protons by the respiratory epithelial cells, the inhibition of glutaminase activity in epithelial cells, and finally the recruitment of macrophages and neutrophils whose lysis in the context of inflammation raises the acidity level of the environment[35-36]. In fact the acidification produced by neutrophil recruitment may well explain some of the findings of the present study. In this sense, previous studies have shown that EBC pH values are decreased during asthma exacerbations but they are not related with spirometric values[37]. Furthermore Kostikas et al[38] demonstrated that EBC pH levels are negatively correlated with the number of eosinophils in sputum and positive correlated with neutrophilic airway inflammation.

Although the presence of lymphocytic inflammation in the alveoli is characteristic in HP, in the bronchi it has been shown that there may be neutrophilic inflammation, especially in cases in which molds are the causative agent [12,14]. Our group has also recently confirmed that individuals with HP present significantly increased levels of neutrophils in induced sputum following the challenge with fungal agents[14]; this may explain the decrease in pH found in the present study after the challenge test in patients with HP caused by molds. Taken together, these findings also allow us to hypothesize that the mechanism of action of the HP may differ depending on the causative agent.

This study has a number of limitations, some of them deriving from factors that may have influenced the pH values recorded. The most important of these factors is smoking: lower levels of pH in EBC have been reported in healthy individuals exposed to tobacco smoke than in non-exposed subjects[39-40]. In our study, however, this is unlikely to have been a determinant, as we did not find significant differences in smoking habits in our four study groups. Another limitation is the small number of patients ultimately included. Future studies are needed, with larger numbers of patients, to confirm the sensitivity and specificity of EBC pH for the diagnosis of HP. Moreover, the environmental exposure to specific antigens before SIC is difficult to demonstrate, especially in patients exposed to molds. If experimental exposure to antigen can change pH of EBC, natural exposition can also influence it, so we can not rule out that this environmental exposure might influence the results. Finally, another possible limitation is the fact that EBC was recorded 24 hours after the antigen challenge, which may have affected pH levels observed. As yet, the variability of EBC pH after SIC has not been assessed either in occupational asthma or in HP[41]. In any case, the decision to record EBC after 24 hours was based on the protocol established by different groups in the context of occupational asthma, in which markers of inflammation are analysed using noninvasive methods in order to help establish the positivity of SIC[42].

In conclusion, the results of this study suggest that the use of EBC pH may be helpful in the interpretation of SIC in patients with HP. Furthermore, its clinical assessment in the context of this test in relatively straightforward, since it is non-invasive, easy to perform, reproducible and cost-effective. The results of this study also suggest that the use of EBC pH after SIC can reduce the test's false negative rates. Further studies are now required, with larger numbers of patients, to test the validity of these proposals.

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Authors contributions: Dr Munoz had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Dr Ojanguren:

contributed to data collection, analysis and interpretation of data, drafting the manuscript for important intellectual content, and reading and approving the final manuscript. Drs Munoz, Morell, Cruz and Villar: contributed to study conception and design, analysis and interpretation of data, drafting the manuscript for important intellectual content, and reading and approving the final manuscript. Dr Cruz and Sanchez-Ortiz: contributed to laboratory analysis and interpretation of data and reading and approving the final manuscript.

All authors approved the final version of the manuscript.

LIST OF ABBREVIATIONS

CI - Confidence intervals

DLCO - Diffusing capacity of the lung for carbon monoxide

EBC - Exhaled breath condensate

FEV1 - Forced expiratory volume in one second

FVC - Forced vital capacity

HP - Hypersensitivity pneumonitis

LR- - Likelihood ratio of a negative value

LR+ - Likelihood ratio of a positive value

NPV - Negative

PPV - Positive

ROC - Receiver-operating characteristic

SE - Sensitivity

SIC - Specific inhalation challenge

SP - Specificity

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Table 1. Demographic data and clinical characteristics of the study subjects.

	HP n = 63			**Non-HP n = 22**		
	Birds n = 42	**Molds n = 21**	**P**	**Birds n = 12**	**Molds n = 10**	**p**
Age, mean (SD), years	57 (11.9)	59 (17.91)	0.789	59 (9,42)	58.33 (14.6)	0.892
Sex, M/F	13 / 29	8 / 13	0.8	6/6	5 / 4	0.449
Smoking (%)						
Smoker	12.8	10.5		11.1	7	
Non-smoker	61.5	52.6		55.6	57	
Ex-smoker	25.6	36.8		33.3	36	
Crackles, n (%)	22/35 (62,9)	9/14 (64,3)	0,37	6/8(75)	6/8(75)	0,7
Clubbing, n (%)	5/35 (14,3)	0/14 (0)	0,3	2/8(25)	1/8 (12,5)	0,5

Table 2. Immunological, functional pulmonary data, and results from BAL and EBC of the study subjects.

	HP n = 63			**Non-HP n = 22**		
	Birds n = 42	**Molds n = 21**	**P**	**Birds n = 12**	**Molds n = 10**	**p**
Avian IgG, + / -	22 / 15	-----	-	5/3	-----	-
Fungal IgG, + / -	-----	11/7	-	-----	2/ 6	-
FVC %, mean (SD)	74.6 (14.3)	75.53 (12.46)	0.881	75.6 (17.0)	77.33 (12.17)	0.776
DLCO %, mean (SD)	59.5 (17.6)	63.47 (20.33)	0.465	60.3 (15.0)	64.44 (15.89)	0.351
Bronchoscopy (n)	35	17		9	9	
Transbronchial biopsy (n)*	25	13	-	3	7	-
Criobiopsy (n)*	4	0		2	1	
Bronquial biopsy (n)*	0	0		1	0	
Surgical biopsy(n)*	5	0	-	0	0	-
BAL (n)	35	17	-	9	9	-
BAL lymphocytes* %	19.8 (21)	22.41 (17.91)	0.663	20.33 (20.43)	16.1 (11.95)	0.685
SIC, +/-	33 / 9	19 / 2	-	0 /12	0 / 10	-
EBC pH before SIC	7.9(0.86)**	7.78(1.03) ***	0.624	8.14(0.52)	7.92(0.67)	0.245
EBC pH after SIC	7.88(0.74)**	7.63(1.02) ***	0.270	8.07(0.56)	8.31 (0.19)	0.273

IgG, immunoglobulin G; FVC, forced vital capacity; DLCO: diffusing capacity of the lung for carbon monoxide; BAL, bronchoalveolar lavage; * Number of patients in whom the test result was consistent with HP; **p = 0.90; *** p = 0.634

Table 3. Exhaled breath condensate diagnostic yield

	All n=85	**Birds n=54**	**Molds n=31**
Sensitivity % (CI)	25.8 (15.9-38.7)	23.8 (12.6-39.8)	30 (12.8-54.3)
Specificity % (CI)	81.8 (58.9-94.0)	66.6 (35.4-88.7)	100 (65.5-100)
PPV	80 (55.7-93.3)	71.4 (42.0-90.4)	100 (51.7-100)
NPV	20 (6.6-44.2)	0.2 (0.09-36.1)	41.6 (22.8-63.0)
PLR	1.42 (0.53-3.79)	0.71 (0.27-1.87)	---
NLR	0.9 (0.77-1.07)	1.14 (0.89-1.46)	0.7 (0.52-0.93)

Data are expressed as % (95% CI); PPV, positive predictive value; NPV, negative predictive value; PLR, positive likelihood ratio; NLR, negative likelihood ratio.

FIGURES

Figure 1. Study sample and agents tested.

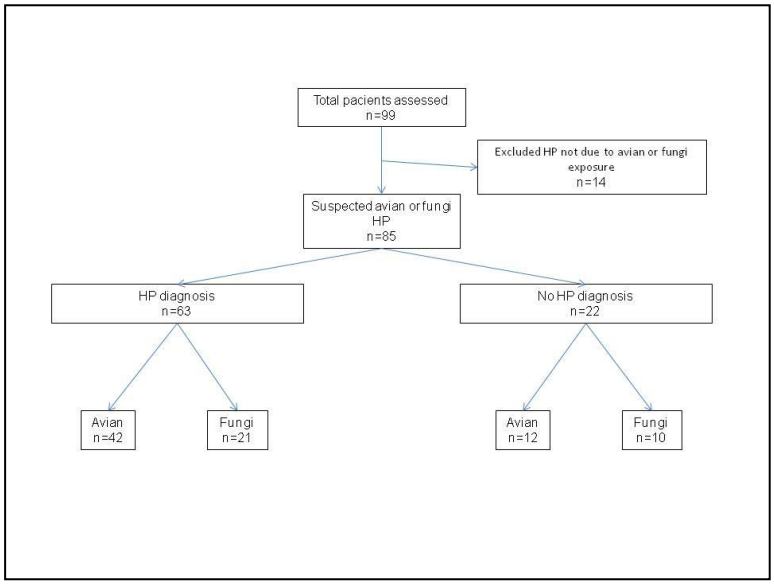


Figure 2. EBC pH before and after SIC in patients diagnosed with HP and in those with diagnoses other than HP.

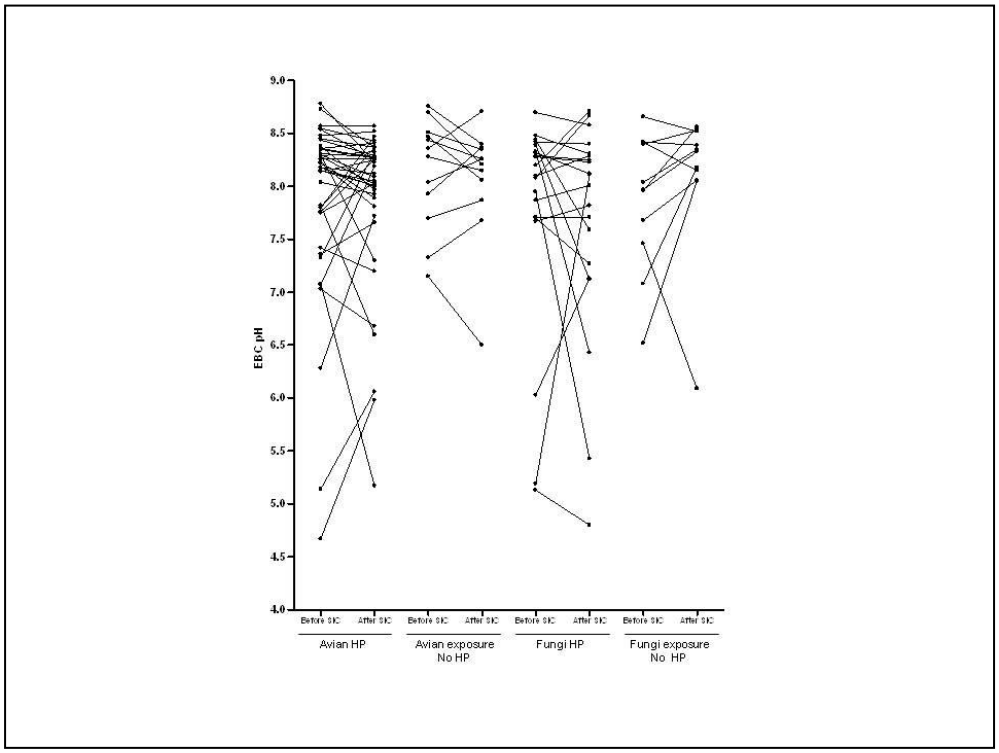
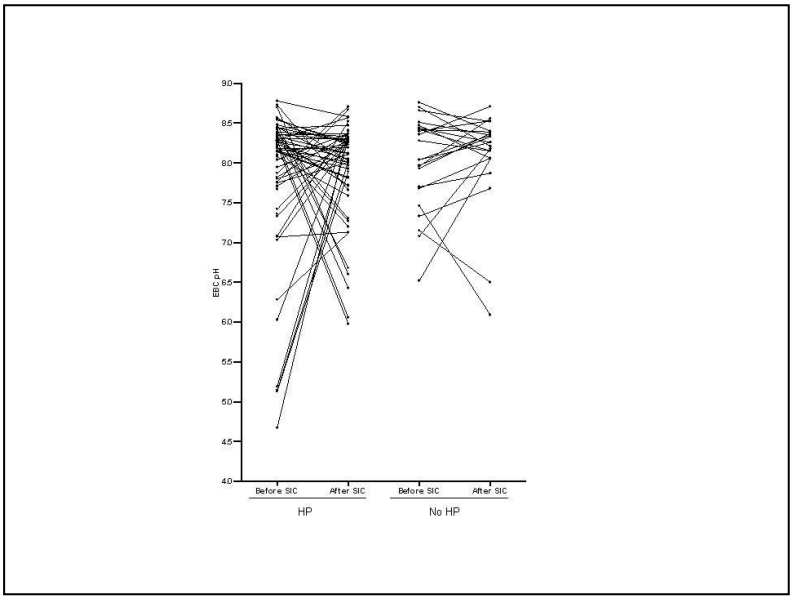


Figure 3. ROC curves: assessment of the most relevant difference in EBC pH after SIC in patients with suspected HP. A: Birds, B: Molds.



CHAPTER 2

5 CHAPTER 2: UTILITY OF EXHALED NITRIC OXIDE FRACTION FOR THE DIAGNOSIS OF HYPERSENSITIVITY PNEUMONITIS.

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Key words: Inflammation, interstitial lung disease, pulmonary fibrosis, specific inhalation challenge.

BACKGROUND: There is very little evidence of the utility of the exhaled fraction of NO (FeNO) for the diagnosis of interstitial lung disease (ILD) and nearly all of it is related with connective tissue disease. Some authors have suggested that in patients with hypersensitivity pneumonitis (HP), evolution to pulmonary fibrosis may be mediated by a Th2 mechanism, which could redound in a potential utility of FeNO. The aim of this study was to investigate the values of FeNO before and after antigenic exposure with the specific inhalation challenge (SIC) and to analyze its potential utility for the diagnosis of HP.

METHODS: It was a prospective, cross-sectional study of all patients older than 18 years referred to our center for suspected chronic HP between May 2012 and May 2014 and who underwent a SIC. FeNO was collected before and after SIC.

RESULTS: The study sample comprised 25 patients. Eleven were diagnosed with chronic HP; six had been exposed to avian proteins and five to fungal agents. Of these 11 patients, seven had positive SICs. In the 14 patients with diagnoses other than HP, all the SICs were negative.

No significant differences in baseline characteristics were observed according to HP diagnosis, except in the BAL lymphocyte count. No differences were found after the test in patients diagnosed with HP; nor were there differences in baseline FeNO in patients diagnosed with HP and those who received alternative diagnoses.

CONCLUSIONS: The results suggest that FeNO measurement is not useful for the diagnosis of chronic HP.

DISCUSSION

6 DISCUSSION

The results obtained confirm several of the hypotheses set out. A drop in the AEC pH equal to or greater than 0.3 after the SIC revealed a sensitivity of 30% and a specificity of 100% for the diagnosis of HP as a result of exposure to fungi. In the case of HP caused by avian proteins, a drop equal to or greater than 0.3 in the AEC pH after the SIC revealed a sensitivity of 23.8% and a specificity of 66.6%. However, the determination of FeNO did not prove to be useful for the diagnosis of chronic HP. Patients with chronic HP did not present differences compared to patients with the diagnosis of other ILD as regards FeNO values before and after the SIC. These results gave rise to doubts about the usefulness of the FeNO measurement and it has been ruled out as a diagnostic indicator of this disease.

Although the SIC has been used for the diagnosis of HP for decades, to date, no consensus has been reached about which variables should be taken into account for the interpretation of the test, a fact that may condition the sensitivity and specificity of same. Establishing a positive diagnosis on the basis of the appearance of symptoms during the test entails greater antigen exposure which may condition a decrease in the security of same. However, establishing the diagnosis according to criteria based on the study of pulmonary function may mean less antigen exposure could be used. With this approach, the maximum exposure described in the studies by Othani et al.[155] was 680 µg of avian protein, while Morell et al.[28] use a maximum of 200 µg. In fact, in a recent study Muñoz et al.[109] evaluated 113 patients, 88 of whom were diagnosed with HP to different agents. They encountered a sensitivity of 73% and a specificity of 84%, with the number of patients finally diagnosed with HP with a negative SIC being 24.

The SIC enables a causal association to be established with an antigen suspected of causing the disease when the result of same is positive. The SIC seeks to reproduce, in the laboratory, under medical supervision, the antigen exposure that has taken place in real life under controlled conditions and maximum safety for the patient. As a result, and as demonstrated by the recently mentioned results, obtaining a positive result on the basis of criteria established through signs, symptoms and pulmonary function values, enables us not only to diagnose the pathology with security, but also establishes the main treatment, which consists of avoiding

the antigen. The present works allow us to establish on the one hand, the theory that the pH measurement of the AEC during the SIC can reduce the number of false negatives, and therefore improve the diagnostic usefulness of this test. On the other hand, they rule out the usefulness of the FeNO measurement for the diagnosis of HP and its role in improving the diagnostic usefulness of the SIC. ,

The AEC pH is a recent tool that may be useful in the evaluation of different respiratory diseases[144]. The AEC pH is the result of a balance between several buffer systems, and the production and release of acids and bases in the airways[156]. In healthy individuals the AEC is largely determined by the NH4, HCO3 and CO2 produced during respiration[157] with the pH in the alveolar lumen being more acidic than in the more proximal airway. Those diseases that condition inflammatory processes trigger different mechanisms which bring about an acidification of these more proximal airways as a possible innate defence mechanism. These acidification mechanisms in the airway are essentially the production and excretion of superoxide ions and protons by the respiratory epithelial cells, inhibition of the glutaminase activity in the epithelial cells, and lastly, the recruitment of macrophages and neutrophils whose lysis in the context of inflammation leads to increased acidification in the environment[158,159]. It is precisely the acidification produced by this neutrophilic recruitment which may explain some of the findings of the present study.

In HP, although at an alveolar level the presence of a lymphocytic inflammation is characteristic, at a bronchial level it has been demonstrated that a neutrophilic inflammation may exist, and is more notable in those cases in which the causal agent is fungus[130,]. Likewise, recently our group demonstrated that following the challenge test with fungal agents in individuals with HP, a significant increase occurs in the neutrophils in the induced sputum. This could explain the finding in the present study of a reduced pH following the challenge test in those individuals with HP caused by fungus. The combination of these findings also points to the hypothesis that the action mechanism of HP may differ according to the causal agent.

This study has a series of limitations, some of which are related to factors that could condition the pH value found. Firstly, a factor that could condition the pH measurement is the patient's tobacco habit. Lower levels of pH in the AEC have been reported in healthy individuals exposed to tobacco smoke, compared to non-exposed individuals[160,161]. However, this fact should not be considered a determining factor since we have not found significant differences as regards

the tobacco habit in the four study groups. Lastly, another possible limitation would be in relation to the fact that the AEC is taken 24 hours after the antigen exposure, which could affect the pH levels observed. There are no studies determining the variability of the AEC pH in relation to the SIC whether in the context of occupational asthma or HP[162]. The decision to take the AEC after 24 hours is in accordance with the protocol established by different groups in the context of occupational asthma to analyse non-invasive inflammation markers as an aid towards establishing the positivity of the SIC[163].

The second study in this thesis analyses the usefulness of FeNO for the diagnosis of HP. FeNO is a marker of eosinophilic inflammation of the airway, which can be measured in a non-invasive manner[132]. Its usefulness for the diagnosis and monitoring of eosinophilic bronchial asthma appears in the main international guides, as well as its applicability in a subgroup of patients with COPD[135,136]. However, there is very little experience in relation to its usefulness in interstitial pulmonary diseases[164]. The association of the Th2 route and high levels of FeNO could suggest that the measurement of this marker could be helpful in the diagnosis of HP. Several studies have demonstrated that HP is mainly mediated by a Th1 inflammatory mechanism[165,166]. In fact, the main cytokines intervening in this route are IL-12, IL-18 and TNFα and they probably play a more important role in the formation of the granuloma[167]. Mroz et al.[51] described a series of 12 patients with HP who presented higher levels of IL-12, IL-18 and TNFα in the BAL, compared to 13 healthy controls. Ye et al. [168] reproduced these results in a series of 16 patients with HP compared to 11 healthy controls. However, other authors have reported interesting findings in relation to the role of the response mediated by the Th2 cells in the progression of the disease towards pulmonary fibrosis[51]. Barrera et al.[55] described an increase in the expression of CXCR4, a Th2 cell receptor, as well as a reduction in CXCR3, a Th1 cell receptor, lymphocytes in the BAL and higher levels of IL-4. In light of these results, the authors suggested that the Th2 inflammatory route could be involved in these patients. Mitaka et al.[169] also found higher levels of IL-4 and IL-13 in the BAL of mice with chronic HP, compared to the controls, which once again reflects the possible involvement of the Th2 route.

The only study to date that has related FeNO to chronic HP has been that published by Guilleminault et al.[141] This study describes a series of 13 patients with chronic HP who present significantly higher levels of FeNO than 3 other groups of DILD including IPF (n = 18), drug-induced pneumonia (8n = 8) and ILD associated with connectivopathies (N = 22). The authors concluded that FeNO is a useful tool for detecting patients with pulmonary fibrosis secondary

to chronic HP. However, the results found in the second study of this thesis do not support this theory. In a similar sample of patients, no correlation was found between the FeNO values and the different parameters characterising this disease. Moreover, the fact that there were no variations in its values before and after the SIC, during which immunological mechanisms are activated, raises doubts about the recommendation to use this molecule as a biological marker of the disease.

Nevertheless, it is interesting to observe that there are some differences between the two populations, which may explain some of the discrepancies in the results. Firstly, the gender of the patients: 11 of the 13 patients with chronic HP described by Guilleminault et al.[141] were men. A recent study with more than 500 participants demonstrated higher levels of FeNO in men than in women[170]. Secondly, the tobacco-free period: the 13 patients with chronic HP from the series by Guilleminault et al.[141] presented a longer tobacco-free period than the patients from the other 3 groups, and tobacco is a factor that has been associated with 40-60% lower FeNO levels[171]. Thirdly, poly-medication: the fact that the patients were poly-medicated at the time at which the FeNO measurement was taken, may also have influenced the results. Other factors to be considered would be the clear association between eosinophilic inflammation and FeNO levels; as such, the study of the eosinophil values in the peripheral blood in the BAL should be highly relevant. In this regard, neither the study by Guilleminault et al.[141], nor our study could present differences in the number of eosinophils in the peripheral blood or in the BAL. Lastly, it is also important to highlight that neither of the two studies reported the atopic status or the IgE levels of the assessed population, a fact that is associated with FeNO increases of up to 60%[172].

A limitation to be considered in this study lies in the low number of patients included. This is partly due to the relative rarity of the disease; HP represents between 5-15% of all ILD[69]. Considering this limitation, the results suggest that FeNO is not a valid tool for the diagnosis of chronic HP.

CONCLUSIONS

7 CONCLUSIONS

The following conclusions can be drawn from the works already published:

Chapter I: Changes in pH in exhaled breath condensate after specific bronchial challenge test in patients with chronic hypersensitivity pneumonitis: a prospective study.

- The pH of the AEC could be useful for the diagnosis of chronic HP.
- A 0.4 drop in the pH of the AEC following the SIC has a 100% specificity for the diagnosis of chronic HP caused by fungi.
- The use of the pH of the AEC after the bronchial challenge test could reduce the number of false negatives.
- The degree of pulmonary inflammation may differ according to the causal agent.

Chapter II: Usefulness of fractional exhaled nitric oxide for the diagnosis of hypersensitivity pneumonitis.

- The FeNO measurement is not useful for the diagnosis of chronic HP.

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