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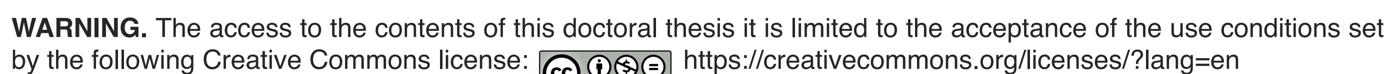
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Universitat Autònoma
de Barcelona

Doctoral Program in Medicine – Department of Medicine

DOCTORAL THESIS

PATHOPHYSIOLOGICAL MECHANISMS INVOLVED IN ACUTE AND CHRONIC HYPERSENSITIVITY PNEUMONITIS

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Barcelona, 2022

LIST OF ABBREVIATIONS

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ANOVA	Analysis of variance
APC	Antigen-presenting cell
AUC	Area under the curve
BAL	Bronchoalveolar lavage
BCA	Bicinchoninic acid
BRHP	Bird-related hypersensitivity pneumonitis
BSA	Bovine serum albumin
CD	Cluster of differentiation
CI	Interval of confidence
COP	Cryptogenic organizing pneumonia
CPFE	Combined pulmonary fibrosis and emphysema
CV	Coefficient of variation
CVD-IP	Collagen vascular disease-associated interstitial pneumonia
CXCL	C-X-C motif chemokine ligand
DC	Dendritic cell
DLCO	Diffusing capacity of the lungs for carbon monoxide
DTT	Dithiothreitol
ECM	Extracellular matrix
ELISA	Enzyme-linked immunoassay
FEV_x	Forced expiratory volume in x seconds
Fc	Constant region of immunoglobulins
FOXP3	Forkhead box P3
FVC	Forced vital capacity
HP	Hypersensitivity pneumonitis
HRCT	High resolution computed tomography

HRP	Horseradish peroxidase
HV	Healthy volunteers
ICT	Immunochromatographic test
IEF	Isoelectric focusing
IFN	Interferon
Ig	Immunoglobulin
IGLL-1	Immunoglobulin lambda-like polypeptide-1
IL	Interleukin
ILD	Interstitial lung disease
IPF	Idiopathic pulmonary fibrosis
IPG	Immobilized pH gradient
KL-6	Krebs von den Lungen-6
LOD	Lower limit of detection
LOQ	Lower limit of quantification
MHC	Major histocompatibility complex
MMP	Metalloproteinase
MW	Molecular weight
NK	Natural killer
NOD	Nucleotide-binding oligomerization domain–like receptor
NSIP	Nonspecific interstitial pneumonia
OD	Optical density
PBS	Phosphate buffered saline
ProE	Proproteinase E
PRR	Pattern recognition receptor
PVDF	Polyvinylidene difluoride
ROC	Receiver operating characteristic

r_s	Spearman's rank correlation coefficient
RT	Room temperature
SD	Standard deviation
SIC	Specific inhalation challenge
SP-D	Surfactant protein D
T	Tween-20
TGX	Tris-glycine extended
Th	Helper T cell
TLC	Total lung capacity
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T cell
UIP	Usual interstitial pneumonia
VA	Alveolar volume
YKL-40	Chitinase 3-like-1

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SUMMARIES

SUMMARY

Hypersensitivity pneumonitis (HP) is an interstitial lung disease (ILD) characterized by a bronchoalveolar inflammation usually caused by the inhalation of avian and fungal proteins. The immunopathological pathways involved in the disease after antigen exposure are still unknown. In addition, the diagnosis of HP remains challenging because of the absence of a gold standard. In fact, HP patients are diagnosed on the basis of a combination of clinical, imaging and laboratory findings. In this context, YKL-40 (chitinase 3-like-1) and Krebs von den Lungen-6 (KL-6) are two promising biomarkers that may play an important role in the management of HP diagnosis. Moreover, to establish correct HP diagnosis, identification of the triggering antigen is an essential step. To achieve this, specific IgG antibody determination is a useful technique. In addition, the environmental detection of the causative antigen will help to control complete antigen avoidance, which is crucial to preventing disease progression and to improving respiratory symptoms in patients with HP. However, in clinical practice, no methods are currently available for directly measuring antigen exposure in the environment.

This PhD thesis aims to assess the immunopathological pathways that are activated in HP, and to improve diagnostic accuracy and prognosis in patients. The first study focuses on the cellular immune response and the cytokine pattern involved in a murine model of bird-related HP (BRHP). This study contends that in the first stages of BRHP there is a mixed Th1/Th2 immune response with increased levels of type 2-related dendritic cells, an eosinophilic inflammation due to IL-5-producing cells, reduced levels of B cells because of their differentiation into antibody-producing plasma cells and secretion of type 1 and type 2 cytokines such as TNF-α and IL-13. With progression of BRHP, although there is a Th1 response with IFN-γ, IL-1β and IL-12 secretion, the levels of cytokines seem to indicate a switch towards a Th2/Th17 mixed response with increased levels of IL-5, IL-6 and IL-23.

The second study (Article 1) aims to determine the role of KL-6 and YKL-40 as biomarkers in the diagnosis and prognosis of patients with HP. This study demonstrates that both serum KL-6 and YKL-40 levels seem to be capable of distinguishing HP patients from healthy individuals and from patients with idiopathic pulmonary fibrosis (IPF) at a range of 346 - 1441 U/ml and 55 - 121 ng/ml respectively. In addition, serum KL-6 may also be a predictor of HP disease progression because of

its negative correlation with total lung capacity and diffusing capacity of the lungs for carbon monoxide (DLCO) at 12 months of follow-up.

The objective of the third study is to evaluate the degree of sensitization to avian and fungal antigens and the potential risk of developing HP in a cohort of urban pest surveillance and control service workers. A high degree of sensitization to avian and fungal antigens is observed in the study population. In particular, workers involved in nest pruning have higher levels of specific parakeet and mucor IgGs and lower DLCO/VA (alveolar volume) values. In addition, this study identifies Ig Lambda chain and Apolipoprotein A-I as candidate proteins for distinguishing patients with HP from workers exposed to pigeons.

The last study (Article 2) focuses on the development and validation of a sensitive sandwich enzyme link immunoassay (ELISA) technique and a rapid immunochromatographic test (ICT) to detect pigeon antigens in environmental air samples. This study demonstrates that there is a good correlation between the two assays, although the ELISA method has a broader range for quantifying pigeon antigen (58.4 - 10112.2 ng/ml and 420 - 3360 ng/ml respectively). Even so, this study concludes that the ICT is rapid, simple to use and a valid alternative that does not require expensive equipment.

RESUMEN

La neumonitis por hipersensibilidad (NH) es una enfermedad pulmonar intersticial caracterizada por una inflamación broncoalveolar generalmente causada por la inhalación de proteínas aviares y fúngicas. Las vías inmunopatológicas implicadas en el desarrollo de la enfermedad tras la exposición al antígeno aún se desconocen. Además, el diagnóstico de NH sigue siendo un desafío debido a la ausencia de un estándar. De hecho, los pacientes con NH son diagnosticados a partir de una combinación de hallazgos clínicos, de imagen y de laboratorio. En esta línea, YKL-40 (quitinasa 3-like-1) y Krebs von den Lungen-6 (KL-6) son dos biomarcadores prometedores que pueden tener un papel importante en el manejo del diagnóstico de la NH. Asimismo, para establecer un correcto diagnóstico de NH, la identificación del antígeno es un paso fundamental. La determinación de anticuerpos IgG específicos es una técnica muy útil para identificar el antígeno desencadenante. Además, la detección ambiental del antígeno causal puede tener un papel importante a la hora de controlar el cese de la exposición antigénica, que es crucial para prevenir la progresión de la enfermedad y mejorar los síntomas respiratorios en pacientes con NH. Sin embargo, en la práctica clínica, no existen métodos disponibles para medir directamente la exposición al antígeno en el medio ambiente.

La presente tesis doctoral tiene como objetivo evaluar las vías inmunopatológicas que se activan en la NH, así como mejorar la precisión diagnóstica y el pronóstico de los pacientes. El primer estudio se centra en la respuesta inmunitaria celular y el patrón de citocinas implicados en un modelo murino de NH relacionada con aves (NHRA). Este estudio respalda que en los estadios iniciales de NHRA hay una respuesta inmune mixta Th1/Th2 con niveles aumentados de células dendríticas relacionadas con la respuesta tipo 2, una inflamación eosinofílica debido a las células secretoras de IL-5, niveles reducidos de células B debido a su diferenciación a células plasmáticas secretoras de anticuerpos y secreción de citocinas tipo 1 y tipo 2, como TNF-α e IL-13. Con la progresión de la enfermedad, aunque hay una respuesta Th1 con secreción de IFN-γ, IL-1β e IL-12, los niveles de citocinas parecen indicar un cambio hacia una respuesta mixta Th2/Th17 con niveles aumentados de IL-5, IL-6 e IL-23.

El segundo estudio (Artículo 1) tiene como objetivo determinar el papel de KL-6 e YKL-40 como biomarcadores en el diagnóstico y pronóstico de pacientes con NH. Este estudio demuestra que los niveles séricos de KL-6 e YKL-40 parecen distinguir a los pacientes con NH de los individuos sanos y de los pacientes con fibrosis pulmonar

idiopática (FPI) en un rango de 346-1441 U/ml y 55-121 ng/ml, respectivamente. Además, KL-6 en suero podría ser un predictor de progresión de enfermedad en NH debido a su correlación negativa con la capacidad pulmonar total y la capacidad de difusión de los pulmones para el monóxido de carbono (DLCO) a los 12 meses de seguimiento.

El objetivo del tercer estudio es evaluar el grado de sensibilización a antígenos aviares y fúngicos y el potencial de riesgo de desarrollar NH en una cohorte de trabajadores de servicios de vigilancia y control de plagas urbanas. En este estudio se observa un alto grado de sensibilización a antígenos aviarios y fúngicos en la población de estudio. Concretamente, los trabajadores implicados en la poda de nidos tienen niveles más altos de IgG específica a periquito y mucor y presentan una DLCO/VA más baja. Además, en este estudio se identifican la cadena Ig Lambda y la apolipoproteína A-I como proteínas candidatas para diferenciar a pacientes con NH de trabajadores expuestos a paloma.

El último estudio (Artículo 2) se centra en el desarrollo y validación de una técnica sensible de inmunoensayo ligado a enzimas (ELISA) y un test rápido de inmunocromatografía (ICT) para detectar antígenos de paloma en muestras de aire ambiental. Este estudio demuestra que existe una buena correlación entre ambos ensayos, aunque el método ELISA tiene un rango más amplio de cuantificación de antígeno de paloma (58,4 - 10112,2 ng/ml y 420 - 3360 ng/ml respectivamente). Aun así, este estudio concluye que el ICT es un test rápido, sencillo y una válida alternativa que no requiere del uso de equipamiento costoso.

1. INTRODUCTION

1.1 PATHOGENESIS OF HYPERSENSITIVITY PNEUMONITIS

1.1.1 DEFINITION

Hypersensitivity pneumonitis (HP) is an interstitial lung disease (ILD) affecting lung parenchyma and the small airways in genetically predisposed and sensitized individuals after recurrent inhalation of certain environmental antigens (1,2). A large variety of organic and inorganic causative antigens have been described, which are divided into six different categories: bacteria, fungi, animal proteins, plant proteins, low molecular weight chemicals and metals (3,4) .

1.1.2 SUBTYPES OF HP

Typically, HP has been divided into acute, subacute and chronic forms. However, this classification has fallen into disuse because the subacute form is particularly difficult to define; in fact, features of this type of HP overlap with those of the acute and the chronic form (5). Recently, another classification has been proposed based on clinical, radiological and pathological features of HP patients, which divides the condition into two categories: acute/inflammatory/non-fibrotic and chronic/fibrotic (6,7). The acute form, with a symptom duration not exceeding six months, is due to intermittent but high level antigen exposure, is characterized by cellular inflammation, and is often reversible with antigen avoidance. The chronic category, with a symptom duration exceeding six months, is caused by continuous and low dose antigen exposure, is characterized by fibrotic areas inside lungs, and is potentially reversible, at least to some extent (7).

1.1.3 IMMUNOLOGICAL MECHANISMS

The pathophysiological mechanisms involved in HP are poorly understood, although there is some evidence of the contribution of both humoral and cellular immune responses. Inhaled antigens are recognized by antigen-presenting cells (APCs) of the innate immune response (e.g., macrophages and dendritic cells, DCs) through different pattern recognition receptors (PRRs) such as TLRs (Toll-like receptors, mainly TLR6 and TLR9), NODs (nucleotide-binding oligomerization domain–like receptors) and dectins (8–10). Signaling via PRR leads to the activation of innate immune cell and the production of cytokines such as IL-1, IL-12, IFN-γ and TNFα (11,12). Moreover, APCs are able to phagocytose, process and present antigens in the context of MHC-I (major

histocompatibility complex class I) or MHC-II (major histocompatibility complex class II) molecules in the cell surface, leading to the recruitment of CD8+ and CD4+ T lymphocytes respectively (13). Activated T lymphocytes start to produce different cytokines such as IL-2, IFN-γ and TNFα, promoting the polarization of lymphocytes to Th1 cells. IFNγ and TNFα also induce the accumulation, activation and aggregation of macrophages, resulting in granuloma formation (14,15). The secretion of IL-8 by alveolar macrophages and of IL-17 by activated CD4+ lymphocytes induces neutrophil recruitment and activation, which intensify the inflammatory response (16,17). Previous studies have demonstrated the presence of dysfunctional T-regulatory (Treg) cells (FOXP3+CD4+) in HP unable to suppress the proliferative response of activated T-cells, which also play a role in the exaggerated immune response (18,19).

In addition, presentation of processed antigens by innate immune cells to B lymphocytes induces their differentiation into plasma cells, which produce immunoglobulins (Igs) that can bind to antigenic proteins forming immunocomplexes. These immunocomplexes can activate the classical pathway of the complement, promoting the recruitment of more macrophages (2,20). Activated lung macrophages secrete IL-1 and TNF but also upregulate the expression of adhesion molecules, leading to neutrophil recruitment in the alveolar spaces. Neutrophils are also recruited to the site of inflammation by the interaction of their receptors with the constant region (Fc) of Igs (21).

In later stages of HP, a relative switch of Th1 cells to Th2 seems to promote the maintenance of inflammation and fibrosis development with IL-4 and IL-13 as effector cytokines (22). The triggers of this switch are unknown but there is evidence that Treg cells suffer a decrease in FOXP3 expression, acquiring a phenotype of Th2 cells (23). The decrease in apoptosis of lymphocytes at these final stages also contributes to T cell persistence, activation, and accumulation in lung tissue. In fact, increases in CD4+ T cells and in the CD4+/CD8+ ratio have been observed in chronic HP. Apoptosis of alveolar epithelial cells and granulocytes has also been described, stimulating chemokine production by DCs and increasing the recruitment of immune cells in the lungs (22,24). The maintenance of these inflammatory patterns, together with the abnormal activation of fibroblasts, seems to contribute to pulmonary fibrosis. Specifically, there is evidence that fibrocytes are attracted to lung inflammatory sites by CXCL12-producing epithelial cells, and that these fibrocytes are able to induce the differentiation of fibroblasts into myofibroblasts (25). Alterations of the extracellular matrix (ECM) also take place during HP and may play a role in the immune response.

Some studies suggest that ECM components such as fibronectin or tenascin-C can bind to chemokines, growth factors, proteases and receptors on the immune cell surface, influencing cell phenotypes and their activation (26). In chronic HP, increased levels of metalloproteinases (MMP) have also been observed, especially MMP8 and MMP9 (27).

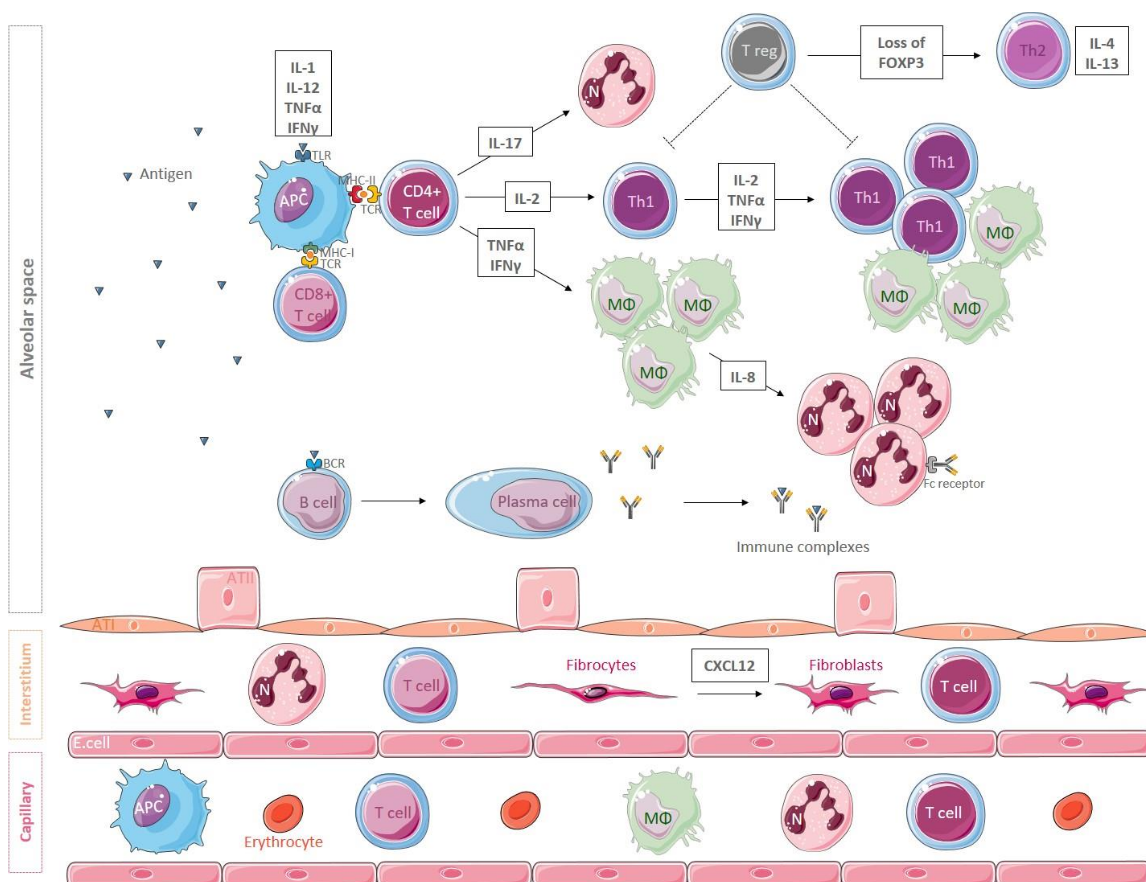

Figure 1. Immunopathology in HP. Following inhalation, the antigen is phagocytosed and degraded by APCs and coupled to MHC-II or MHC-I molecules. The antigen is then recognized by CD4+ T cells and CD8+ cells. A Th1 type differentiation of CD4+ T cells is initiated through the expression of cytokines such as IL-2, which drives the proliferation and differentiation of these cells. The secretion of IFNγ and TNFα by CD4+ and Th1 cells also induces the accumulation, activation and aggregation of macrophages. On the other hand, the suppressive activity of Treg cells is impaired, which facilitates the amplification of the inflammatory response. Treg cells express the transcription factor FOXP3 on the cell surface, and the decrease in FOXP3 expression reduces their suppressive function. Treg cells expressing decreased FOXP3 mainly become Th2 effectors, even in an environment that favors Th1 cells, which release cytokines such as IL-4 and IL-13. A B cell response against the antigens also occurs, leading to the production of specific antibodies and the formation of immunocomplexes. IL-17 is likely to be produced by some subpopulations of CD4+ T cells, and IL-8 is mainly secreted by alveolar macrophages; both cytokines are potent chemoattractants for neutrophil recruitment and

activation. Neutrophils express receptors for the constant region of IgG, favoring the recruitment of these cells to the site of inflammation and the interaction with antigen–antibody immunocomplexes, a process that occurs mainly during acute HP. Abbreviations: APC, antigen presenting cell; ATI, alveolar type 1 cell; ATII, alveolar type II cell; E. cell, endothelial cell; MΦ, macrophage; N, neutrophil.

Despite the existing evidence of the involvement of different immune cells in the development of HP, little is known about the immunological pathways involved in the progression of the disease. For this reason, and in an attempt to shed light on the immunomodulatory mechanisms of the different subtypes of HP, the first study of this thesis assesses the cellular immune response and the cytokine pattern involved in the disease via an analysis of lung tissue in a mouse model of BRHP.

1.2 DIAGNOSIS OF HYPERSENSITIVITY PNEUMONITIS

Epidemiological studies suggest that the prevalence of HP varies depending on the geographical area, local climate and the intensity of exposure to the causative antigen. According to a Spanish study, the incidence of ILD in its population between 2000 and 2001 was 7.6 per 100,000 persons/year, with idiopathic pulmonary fibrosis (IPF) accounting for 38.6%, sarcoidosis for 14.9% and HP for 6.6% (28). In the UK, the incidence of HP between 1991 and 2003 was 0.9 per 100,000 individuals/year (29) while a US study covering the period from 2004 to 2013 reported a yearly incidence of between 1.67 – 2.71 per 100,000 inhabitants (30). In India, the most common cause of ILD between 2012 and 2015 was HP (47.3%) followed by IPF (13.7%) (31).

Recent studies suggest that HP cases may be underdiagnosed and may represent up to 43% of ILDs classified as IPF (32). These diagnostic errors occur because HP and IPF present similar clinical, radiological and pathological features (33). The diagnosis of HP remains challenging because of the absence of a gold standard technique. In fact, HP patients are diagnosed on the basis of a combination of clinical, imaging and laboratory findings such as physical examination, suspicion of exposure to an antigenic source, determination of specific IgG antibodies against a causative antigen in serum, lung function impairment, compatible high resolution computed tomography (HRCT) scan, increase in lymphocytes in bronchoalveolar lavage (BAL), granulomatous reactions in lung biopsies and/or a positive specific inhalation challenge (SIC) (34–37).

1.2.1 CLINICAL ANAMNESIS AND PHYSICAL EXAMINATION

The first step in HP diagnosis is to obtain a detailed patient history in order to determine exposures at home, workplace, neighborhood, during hobbies, or in other environments frequently visited by the patient (7). In some cases, the clinical interview quickly elucidates the causative antigen and the source of exposure (Table 1). Usually, however, the triggering antigen is not easy to identify, especially in chronic cases where there may be unexpected exposure to an already known antigen. For this reason, the use of a standardized questionnaire regarding exposures including the amount and frequency of antigens that may be used by patients or encountered in daily life, is highly recommended (1,7). On occasion, the physical examination is completely normal, with non-specific clinical symptoms. However, the most typical symptoms are dyspnea, cough, chest tightness, fever, weight loss and inspiratory crackles on auscultation (4).

Table 1. Most prevalent causative antigens and exposure sources in HP (1,38).

Antigen	Exposure Source
Bacteria	
Thermoactinomyces spp.	Moldy hay, straw, grain, sugar cane dust, mushrooms
Pseudomonas spp.	Detergents
Klebsiella spp.	Humidifiers, compost
Fungi and yeasts	
Aspergillus spp.	Flour, moldy cured meat, compost, hay, grain, cork, contaminated water reservoirs
Penicillium spp.	Moldy cured meat or cheese, hay, cork
Candida spp.	Contaminated fountains, humidifiers
Animal proteins	
Feathers	Birds (mostly pigeons and budgerigars), duvets, pillows
Serum and droppings	Birds (mostly pigeons and budgerigars), rats
Plant proteins	
Soy dust	Soy foods
Grain flour	Flour dust
Chemicals	
Isocyanates	Adhesives, paints, varnishes
Acid anhydrides	Spray paints, glues, adhesives, shoes, plastic material
Metals	
Cobalt	Hard metals, alloys
Zinc	Zinc welding

1.2.2 LUNG FUNCTION TESTS

Lung function analyses are not useful for differentiating between HP and other forms of ILD, although they do support the diagnosis of interstitial disease. The most common pattern in HP is a restrictive ventilator defect with reduced forced vital capacity (FVC) and total lung capacity (TLC), together with impaired gas exchange (reduced diffusing capacity of the lungs for carbon monoxide, DLCO, and/or hypoxemia on exercise or at rest) (4,39,40). A decrease in the FEV_1 (Forced expiratory volume in 1 second)/FVC ratio is occasionally detected, which is suggestive of an airflow obstruction due to bronchiolitis and emphysema (41). Of note, between 10% and 17% of patients have normal lung parameters, especially in the non-fibrotic phase of the disease (4).

1.2.3 HRCT SCAN

In non-fibrotic HP the radiological pattern is characterized by a diffuse, usually bilateral and symmetrical distribution of lung findings such as centrilobular nodules, ground-glass opacities, mosaic attenuation, air trapping and lung cysts (38,42). By contrast, fibrotic HP has a variable radiological appearance. In up to 50% of patients, the radiological findings are located mainly in the lower lobes with a subpleural or peribronchovascular distribution, although in some cases the mid- and upper lungs are the most affected zones (6,43,44). The most common findings include reticulation, traction bronchiectasis and honeycombing (45). However, centrilobular nodules, mosaic attenuation and/or bronchiolar obstruction are observed in some cases (46). About 30% of the patients with fibrotic HP exhibit a usual interstitial pneumonia (UIP) pattern. However, a nonspecific interstitial pneumonia (NSIP) pattern is also observed in 15% of cases (43). In patients with chronic farmer's lung, emphysematous forms may also be present (47).

1.2.4 BAL CELLULAR ANALYSES

Marked lymphocytosis is a typical finding in BAL samples of patients with HP. In fact, up to 80% of patients with HP have more than 20% of lymphocytes in BAL (40). However, some patients with fibrotic HP may exhibit a less pronounced lymphocytosis in BAL (48). In addition, increased levels of lymphocytes can also be found in sensitized but asymptomatic subjects (47) and in other forms of ILD such as sarcoidosis (49).

1.2.5 HISTOPATHOLOGY

Typical histopathological features of non-fibrotic HP are cellular interstitial pneumonia accentuated around small airways (predominantly small lymphocytes but also plasma cells and eosinophils), cellular bronchiolitis with lymphocytic infiltrate and poorly formed granulomas. Multinucleated giant cells in the interstitium and/or in the bronchiolar walls may also be observed (38,50). In fibrotic HP, the histological pattern is similar to that seen in other fibrotic ILD with bronchiolocentric inflammation, peribronchiolar fibrosis, bronchiolar epithelial hyperplasia and multinucleated giant cells or granulomas (51). In some cases, the fibrotic interstitial pneumonia pattern includes typical traits of UIP (with subpleural honeycombing and fibroblast foci) or fibrotic NSIP (with a diffuse and uniform distribution) (52,53).

1.2.6 BIOMARKERS

In recent decades, several types of circulating biomarkers have been studied in order to evaluate their diagnostic and prognostic potential in HP, especially those reflecting lung epithelial damage (e.g., SP-D and KL-6), immune system regulation (such as CCL17 and CXCL10) and extracellular matrix remodelling (e.g., YKL-40) (Figure 2). Among the biomarkers described, KL-6 and YKL-40 seem to be the most promising for routine clinical use because they can differentiate HP from other types of ILD and can give information about the progression of the disease through the analysis of less invasive samples.

Krebs von den Lungen-6 (KL-6) is a 200KDa glycoprotein classified as a human mucin, mainly expressed in type II pneumocytes and bronchiolar epithelium. In affected lungs, expression of this protein is increased due to the regeneration of type II pneumocytes and the destruction of the alveolar-capillary barrier. Air-blood barrier permeability is thus enhanced and the concentration of KL-6 in the blood stream is increased. It seems that KL-6 has chemotactic and anti-apoptotic effects on fibroblasts, promoting their proliferation and the progression of pulmonary fibrosis (54,55). According to some studies, serum KL-6 levels are higher in HP patients than in patients with other types of ILD such as IPF and sarcoidosis. In addition, KL-6 is increased in serum of HP patients with active disease and during acute exacerbations, while lower levels are associated with clinical improvement (56,57). There is also evidence of seasonal variations in serum KL-6 levels in patients with ILD due to fluctuating antigen exposure. Indeed, during the summer patients with fungi-related HP have higher concentrations of KL-6

than those with BRHP, IPF, NSIP, collagen vascular disease-associated interstitial pneumonia (CVD-IP) and combined pulmonary fibrosis and emphysema (CPFE). In contrast, in winter, patients with BRHP have the highest serum KL-6 levels (58).

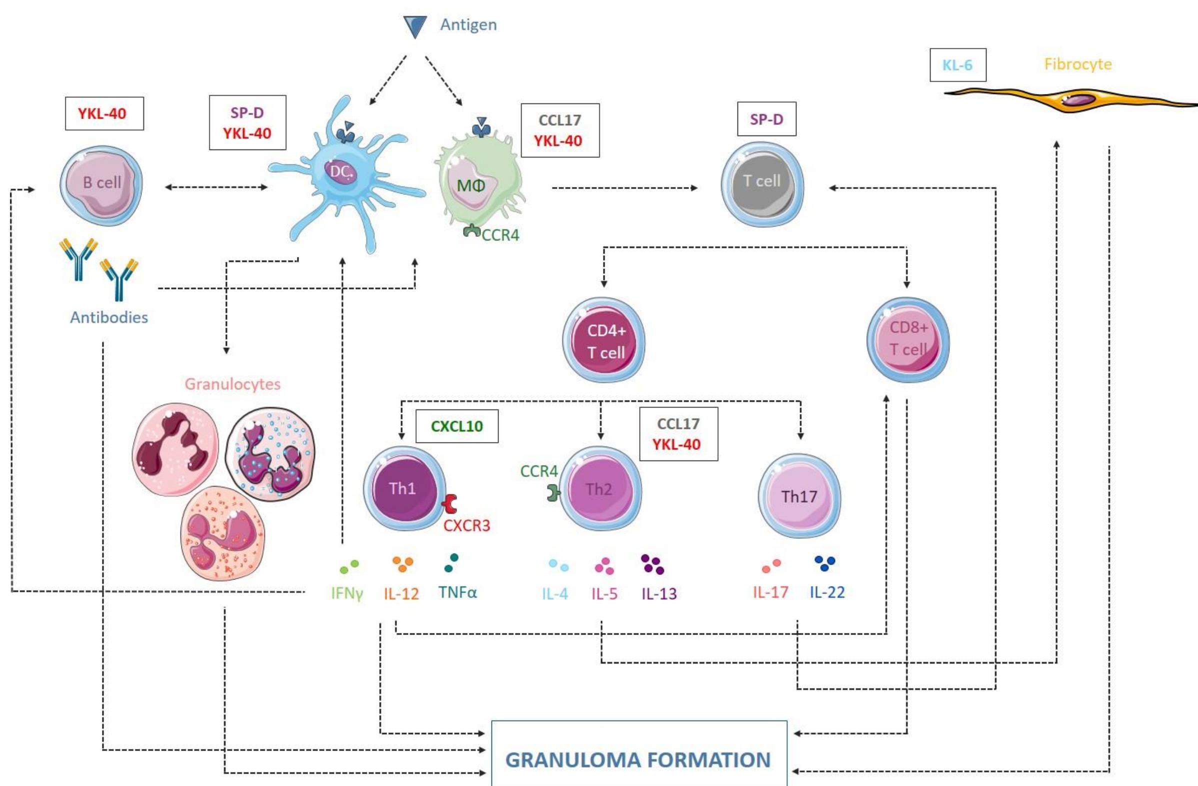

Figure 2. Biomarkers associated with HP. The main effector cells in HP disease are Th1, Th2 and Th17 lymphocytes. Several biomarkers have been associated with these immune cells: SP-D (which can regulate T cell and DC functions), CCL17 (involved in the recruitment of CCR4+ cells such as Th2 cells and macrophages), CXCL10 (which has a chemotactic effect on CXCR3+ cells like Th1 lymphocytes) and YKL-40 (associated with Th2 cytokine production and DC and macrophage activation). There is also evidence of the contribution of CD8+ T cells, fibrocytes, granulocytes and B cells in the formation of granulomatous structures in HP. The biomarkers associated with these cells are: KL-6 (which has a chemotactic and proliferative effect on fibroblasts) and YKL-40 (involved in allergen sensitization and IgE induction). The dotted lines indicate the effect after the activation of these cells (i.e., contribution to granuloma formation and/or activation of other immune cells).

YKL-40 is a 40 KDa human chitinase-like protein without enzymatic activity. It is primarily secreted by macrophages, neutrophils and epithelial cells but also by fibroblasts and other cells (59,60). There is evidence of its involvement in allergen sensitization, IgE induction, Th2 cytokine production, DC and macrophage activation, inhibition of apoptosis in inflammatory cells and ECM modulation during fibrogenesis (60,61). Only one published study has demonstrated that serum YKL-40 levels are

lower in HP than in other ILD like IPF, NSIP and cryptogenic organizing pneumonia (COP). In fact, in patients with HP, serum YKL-40 levels seem to be higher in males, smokers, patients not treated with corticosteroids and those with the acute form of the disease. In addition, HP patients with disease progression or who died have higher serum levels of this chitinase at baseline than those who remain stable and survived. Regarding BAL samples, this study also demonstrated that YKL-40 levels present greater increases in HP patients with the acute form of the disease than in patients with IPF (62).

Despite the evidence of the usefulness of KL6 and YKL-40 in the diagnosis and prognosis of HP, further research is needed before they can be considered in clinical practice and thus provide personalized precision medicine. For these reasons, Article 1 evaluates the diagnostic and predictive value of KL-6 and YKL-40 in patients diagnosed with HP.

1.2.7 DETERMINATION OF ANTIBODIES

Increased serum levels of specific IgG antibodies also support the diagnosis of HP. However, IgG determination merely provides evidence of antigenic sensitization (36); exposed but asymptomatic individuals may also exhibit high IgG levels against a specific antigen without having the disease. In fact, it has been demonstrated that up to 50% of healthy individuals exposed to birds can be sensitized to avian antigens (63), and that between 30-60% of those exposed to fungi can develop specific antibodies (64). In contrast, the absence of increased specific IgG levels does not rule out the diagnosis of HP because only a limited number of antigens are commercially available for routine testing. Antigen-specific IgG antibody determination in HP patients is a widely used technique that also contributes to the identification of the triggering antigen. However, cross-reaction may occur between different types of birds and fungi, thus complicating the identification of the antigen source involved in the patient's sensitization (65).

Today, several qualitative (e.g., precipitation, agglutination and immunoblots) and quantitative methods are available to determine specific IgG antibodies. The most frequently used quantitative tests are ELISA and ImmunoCAP (63,66) and the antigens tested can be obtained as commercial extracts or can be prepared from collected material (67,68). For both methods, specific IgG reference values have to be established for each antigen in healthy subjects (69). Although these techniques are

able to determine the source of exposure, the specific proteins causing HP pathology are still unknown. Recently, several groups have described antigenic substances that are found in bloom, serum, droppings (70) and/or intestinal mucin of various birds (71), such as IGLL-1 and ProE (72). Nevertheless, many other proteins triggering the disease remain to be discovered. For these reasons, the third study focuses on the analysis of specific IgG against avian and fungal proteins in serum samples of workers with high exposure to these antigens, in order to identify the antigenic proteins with diagnostic value for HP.

1.2.8 MEASUREMENT OF ANTIGEN EXPOSURE IN THE ENVIRONMENT

The identification of the causative antigen is essential for HP diagnosis, but in current clinical practice there are no methods available for directly measuring antigen exposure in the environment. A tool of this nature would help clinicians to determine whether a patient is being exposed to a specific antigen, and could thus help to establish a correct HP diagnosis.

Complete avoidance of the causative antigen is essential to improve respiratory symptoms and to prevent disease progression in acute and chronic HP. However, exposure removal is not always possible because it requires major changes in the patient's daily life (e.g., the occupational setting, hobbies, home environment) or the causative antigen may not have been identified (73). In some cases, patients experience disease progression in spite of avoiding antigen exposure and following treatment (especially corticosteroids) (74). However, there is evidence suggesting that these cases may be related to the persistence of exposure to low levels of antigen, mainly avian proteins (75).

Air measurements are crucial when controlling avian antigen exposure in specific environments (76). Previous studies have measured the concentration of avian proteins in collected dust and air samples by using direct, inhibition, antigen-capture or sandwich ELISA (77,78). All these techniques are costly and time-consuming and require access to sophisticated laboratories with specific equipment and staffed by experienced personnel, and so are not readily adaptable to field use. In order to enable legislators (and maybe also consumers themselves) to monitor the concentrations of antigenic proteins in the air in a straightforward way, Article 2 of this thesis focuses on the development of a rapid semiquantitative allergen estimation method, which is also portable and cheap.

1.2.9 SIC

When a particular antigen is suspected to be the cause of the disease, SIC can be used to confirm or rule out its involvement. The SIC is performed by exposing the patient directly to an antigen obtained from a specific environment, or to commercially available extracts (32). This test must be conducted at a specialized center by experienced personnel because the performance and the interpretation of the results obtained is not easy. In addition, the absence of a standardized method for conducting the test complicates its implementation and limits its use for the diagnosis of HP (79). Nevertheless, at our center (Hospital Universitari Vall d'Hebron, Barcelona, Spain) this test is considered a useful diagnostic tool in clinical practice and is frequently carried out when a specific antigen is suspected.

Despite the availability of all these techniques, the diagnostic criteria for HP are not well established among clinicians because multiple agents may be involved in the development of the disease, the pathophysiological mechanisms are not well understood, the histopathology can be variable, and the disease has different forms of presentation. All these factors make the diagnosis of the disease a difficult task. The prognosis of HP varies widely from patient to patient because it depends on the type of causative antigen, exposure duration, dose inhaled and the clinical form of the disease (80). Survival in HP patients depends mostly on the presence and extension of lung fibrosis, with a 5-year mortality of 27% and a median survival of 12.8 years. In fact, up to 41% of chronic HP patients present fibrosis progression, resulting in lung capacity loss and respiratory insufficiency, which may require lung transplantation (81). Therefore, prompt and accurate diagnosis is of vital importance.

The purpose of this thesis is to assess the immunopathological pathways that are activated in HP and to improve the diagnostic accuracy and prognosis of patients, through the detection of the causative antigen and the biomarkers released. In the first study, a mouse model of BRHP is described, and the role of the innate and adaptive immune responses are analysed. In Article 1, to better establish HP diagnosis and prevent disease progression, KL-6 and YKL-40 are evaluated as potential biomarkers in patients with HP. Then, in the third study, specific IgG are analysed in workers with high exposure to birds and fungi and also in HP patients in order to identify antigenic proteins with diagnostic value. Finally, in Article 2 a rapid estimation method is developed to detect pigeon allergen in air samples and ensure complete avoidance of antigen exposure. A chart of the studies in the thesis is shown in Figure 3.

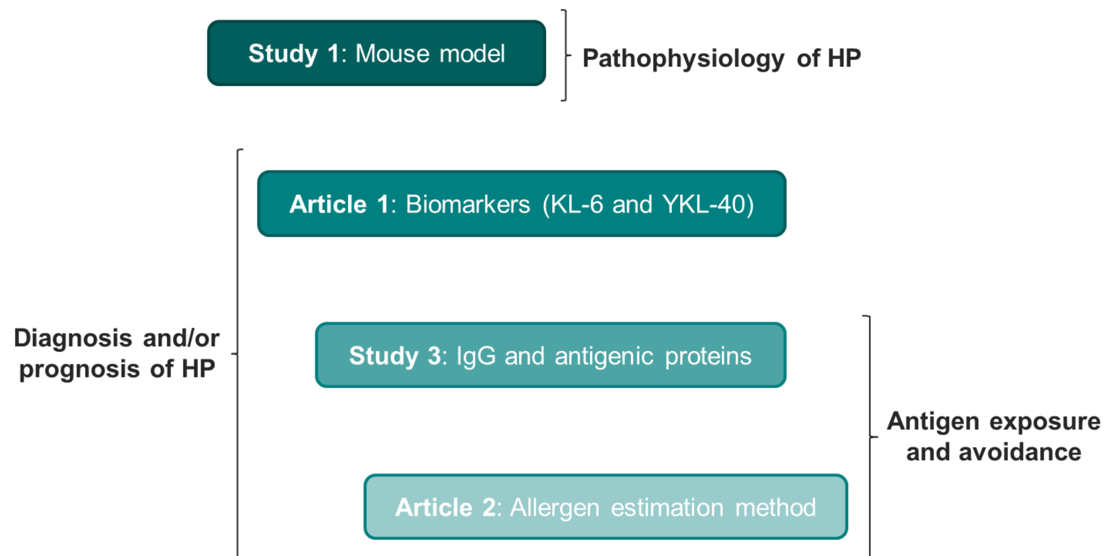

Figure 3. Diagram of the studies included in this thesis.

2. HYPOTHESIS

HYPOTHESIS

1. Both innate and adaptive immune responses are involved in the development of HP. The activation of different pathways contributes to disease progression and chronicity.

2. Identification and quantification of biomarkers and environmental antigen control are useful tools to establish a correct diagnosis, to predict disease progression, and to achieve complete avoidance of causative antigens.

3. OBJECTIVES

3.1 MAIN OBJECTIVE

To assess the immunopathological pathways that are activated in hypersensitivity pneumonitis to improve diagnostic accuracy and prognosis of patients, through the detection of the causal antigen and the released biomarkers.

3.2 SECONDARY OBJECTIVES

1. To assess the cellular immune response and the cytokine pattern involved in a murine model of bird-related hypersensitivity pneumonitis.

2. To determine the role of YKL-40 and KL-6 as biomarkers in the diagnosis and prognosis of patients with hypersensitivity pneumonitis.

3. To evaluate the degree of sensitization to avian and fungal antigens and the potential risk of developing hypersensitivity pneumonitis in a cohort of urban pest surveillance and control service workers.

4. To develop and validate a sensitive sandwich ELISA technique and a rapid ICT to detect pigeon antigens in environmental air samples.

4. COMPENDIUM OF PUBLICATIONS

4.1 ARTICLE 1. "YKL-40 AND KL-6 LEVELS IN SERUM AND SPUTUM OF PATIENTS DIAGNOSED WITH HYPERSENSITIVITY PNEUMONITIS"

Sánchez-Díez S, Munoz X, Ojanguren I, Romero-Mesones C, Espejo D, Villar A, et al. YKL-40 and KL-6 Levels in Serum and Sputum of Patients Diagnosed With Hypersensitivity Pneumonitis. J Allergy Clin Immunol Pract. 2022 Jul 2;S2213-2198(22)00654-7. doi: 10.1016/j.jaip.2022.06.031.

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Original Article

YKL-40 and KL-6 Levels in Serum and Sputum of Patients Diagnosed With Hypersensitivity Pneumonitis

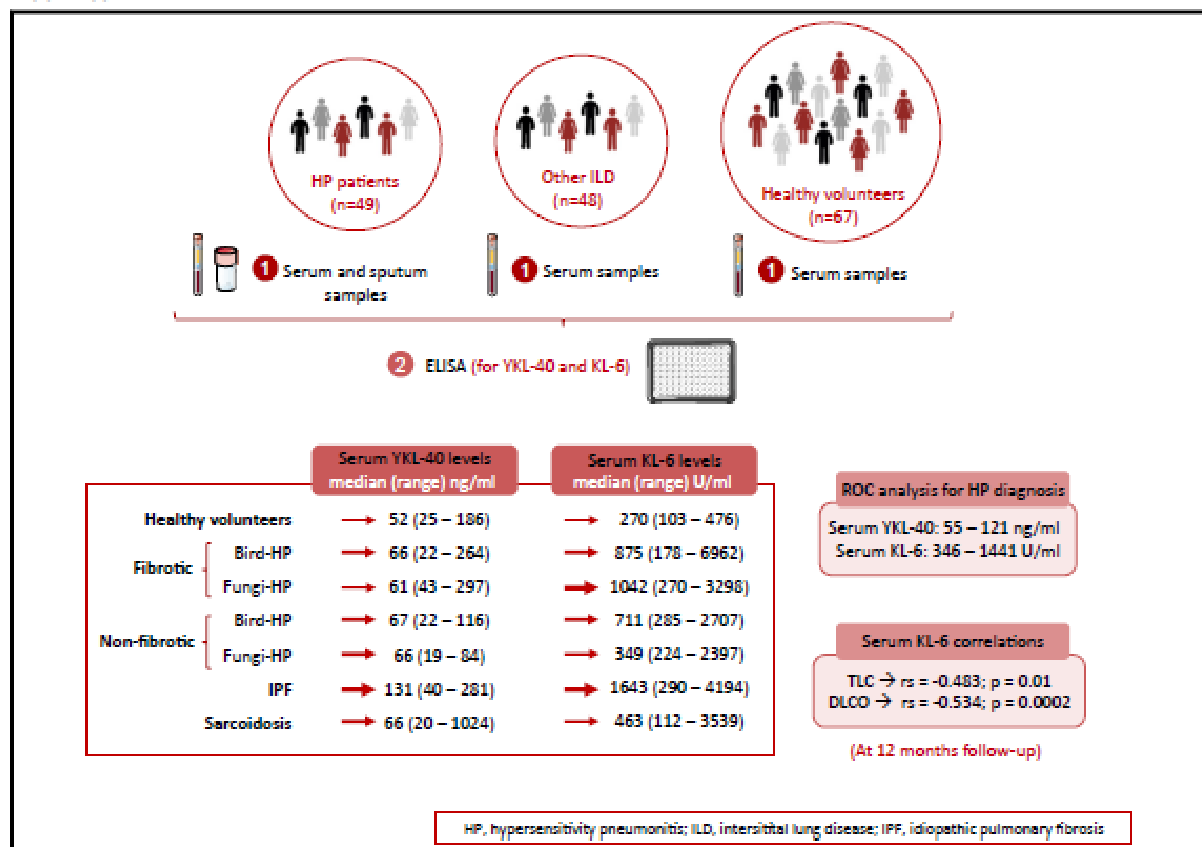
Silvia Sánchez-Díez, BSc[a,b], Xavier Munoz, MD, PhD[a,b,c], Iñigo Ojanguren, MD, PhD[a,b], Christian Romero-Mesones, MD[a], David Espejo, MD[a], Ana Villar, MD, PhD[a], Susana Gómez-Olles, PhD[a,b], and María-Jesús Cruz, PhD[a,b] *Barcelona and Madrid, Spain*

What is already known about this topic? Hypersensitivity pneumonitis (HP) cases may be underdiagnosed and may in fact represent up to 43% of the interstitial lung diseases classified as idiopathic pulmonary fibrosis (IPF).

What does this article add to our knowledge? Chitinase 3-like-1 (YKL-40) and Krebs von den Lungen-6 (KL-6) proteins seem to be capable of distinguishing patients with HP from healthy individuals and patients with IPF with adequate sensitivity and specificity: YKL-40 (sensitivity: >74%, specificity: >53%) and KL-6 (sensitivity: >70%, specificity: >52%), thus confirming their potential role as biomarkers. KL-6 may be a predictor of disease progression.

How does this study impact current management guidelines? Measurement of YKL-40 and KL-6 levels in patients with HP offers insights into the pathobiology of this disease and has the potential to improve diagnostic accuracy.

VISUAL SUMMARY



Abbreviations used
AUC-Area under the curve
BAL-Bronchoalveolar lavage
DLCO-Diffusing capacity of the lungs for carbon monoxide
FVC-Forced vital capacity
HP-Hypersensitivity pneumonitis
ILD-Interstitial lung disease
IPF-Idiopathic pulmonary fibrosis
KL-6-Krebs von den Lungen-6
NPV-Negative predictive value
PPV-Positive predictive value
ROC-Receiver operating characteristic
SIC-Specific inhalation challenge
TLC-Total lung capacity
YKL-40-Chitinase 3-like-1

BACKGROUND: YKL-40 (chitinase 3-like-1) and Krebs von den Lungen-6 (KL-6) are 2 promising biomarkers that may have an important role in the management of interstitial lung diseases (ILD).
OBJECTIVE: The aim of this study was to investigate the values of KL-6 and YKL-40 as biomarkers in the diagnosis and prognosis of patients with hypersensitivity pneumonitis (HP).
METHODS: A cross-sectional study conducted in 49 patients diagnosed with HP due to exposure to birds (n = 32) or fungi (n = 17), 48 patients with other ILD, and 67 healthy volunteers. Patients with HP were divided into fibrotic and nonfibrotic. Serum and sputum YKL-40 and KL-6 levels were determined using commercial enzyme-linked immunosorbent assay kits. Receiver operating characteristic (ROC) curves were used to determine the sensitivity and specificity of both biomarkers for the diagnosis of HP. Pulmonary function tests were performed in patients during follow-up.
RESULTS: KL-6 and YKL-40 levels were significantly higher in serum of patients with HP exposed to birds with a fibrotic pattern than in controls ($P < .0001$ and .0055, respectively). Serum KL-6 levels were also significantly higher in patients with fibrotic HP exposed to fungi compared with the control group ($P = .0001$). In patients with HP exposed to fungi, sputum KL-6 and YKL-40 levels were higher in those with a fibrotic pattern ($P = .0289$ and .016, respectively). ROC analysis showed that the range between 55-121 ng/mL for serum YKL-40 levels and 346-1441 U/mL for serum KL-6 levels had the best sensitivity and specificity for discriminating between patients with HP, healthy controls, and patients with idiopathic pulmonary fibrosis (IPF). In patients with HP, serum KL-6 levels correlated negatively with total lung capacity ($r = -0.485$; $P = .0103$) and diffusing capacity of the lungs for carbon monoxide ($r = -0.534$; $P = .0002$) at 12 months.
CONCLUSIONS: Both KL-6 and YKL-40 proteins seem to be capable of distinguishing patients with HP from healthy individuals and from patients with IPF. Their sensitivity and specificity confirm their potential role as biomarkers. KL-6 may also be a predictor of disease progression. **© 2022 American Academy of Allergy, Asthma & Immunology** **(J Allergy Clin Immunol Pract 2022;■:■-■)**

Key words: *Hypersensitivity pneumonitis; Biomarkers; Serum; Sputum*

Hypersensitivity pneumonitis (HP) is a type of interstitial lung disease (ILD) that occurs in genetically predisposed individuals after the recurrent inhalation of certain environmental antigens. A wide variety of causative antigens have been associated with HP, including avian proteins, fungi, bacteria, and chemical compounds.[1] This disease is characterized by inflammation of the bronchioles, alveoli, and lung interstitium,[2] and there are 2 different categories: acute/inflammatory and chronic/fibrotic. The acute form appears with intermittent but high-level antigen exposure, is often reversible with antigen avoidance, and is related to cellular inflammation. The chronic type appears with continuous but low-dose antigen exposure, is reversible only in some cases, and is characterized by fibrotic areas inside the lungs.[3]

Epidemiological studies estimate that the prevalence of HP in exposed subjects ranges between 0.5% and 7.5%.[4,5] However, recent studies suggest that HP cases may be underdiagnosed and may in fact represent up to 43% of the ILDs classified as idiopathic pulmonary fibrosis (IPF).[6] These diagnostic errors occur because HP and IPF may present similar clinical, radiological, and pathological features.[7] Indeed, the absence of a gold standard technique means that the diagnosis of HP remains challenging; in clinical practice, HP is diagnosed on the basis of a combination of clinical, imaging, and laboratory findings.[8-11] The specific inhalation challenge (SIC) is one of the most useful tools in the diagnosis of HP and in the identification of the causative antigen.[12,13]

Given the difficulty of establishing a well-defined HP diagnosis in the early stages of the disease and of differentiating this condition from other types of ILDs, the assessment of biomarkers in the diagnosis and prognosis of HP could be particularly useful. YKL-40, also known as chitinase 3-like-1, and Krebs von den Lungen-6 (KL-6) are 2 promising biomarkers that may play an important role in the diagnosis and prognosis of patients with HP.

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S. Sánchez-Díez is supported by CIBER, Spain, and M-J. Cruz is supported by the Miguel Servet Programme from the Instituto de Salud Carlos III (MSII17/00025). This project was supported by the Fundació Catalana de Pneumologia (FUCAP), Sociedad Española de Patología Respiratoria (SEPAR), Instituto de Salud Carlos III (PI18/00345), and Fondo Europeo de Desarrollo Regional (FEDER). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
Conflicts of interest: The authors declare that they have no relevant conflicts of interest.
Received for publication February 4, 2022; revised June 16, 2022; accepted for publication June 18, 2022.
Available online ■ ■
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2213-2198
© 2022 American Academy of Allergy, Asthma & Immunology
https://doi.org/10.1016/j.jaip.2022.06.031

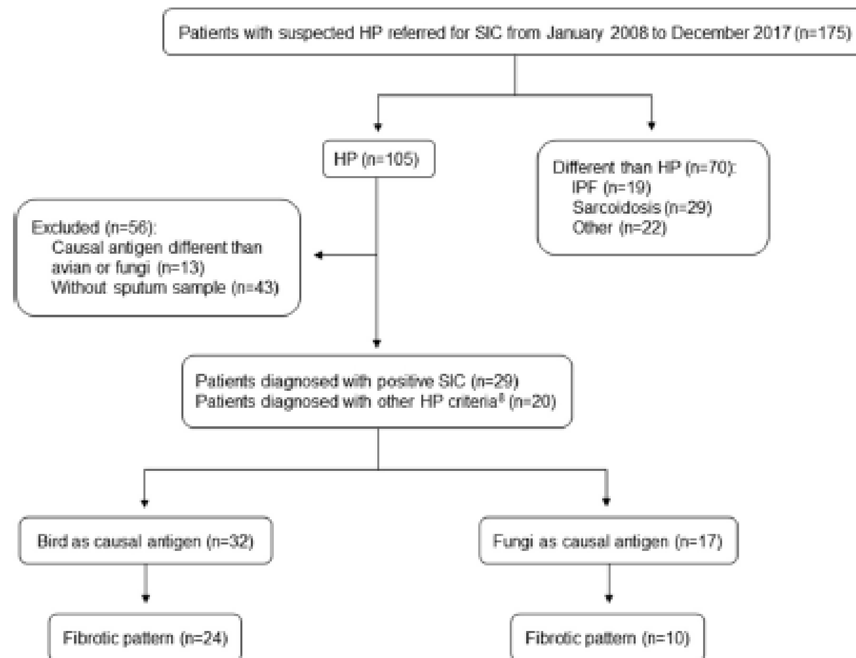

FIGURE 1. Flow chart of patient enrollment. *HP*, Hypersensitivity pneumonitis; *IPF*, idiopathic pulmonary fibrosis; *SIC*, specific inhalation challenge.

YKL-40 is a human chitinase-like protein mainly secreted by macrophages, neutrophils, and epithelial cells but also by fibroblasts and other cell types.[14,15] YKL-40 is involved in various biological functions, including the regulation of cell proliferation, adhesion, migration, and activation. There is evidence of its involvement in allergen sensitization, IgE induction, Th2 cytokine production, dendritic and macrophage activation, inflammatory cell apoptosis inhibition, and extracellular matrix modulation during fibrogenesis.[15,16] It seems that serum YKL-40 levels are lower in HP than in other ILDs, and that there is a negative correlation with pulmonary function (%DLCO, diffusing capacity of the lungs for carbon monoxide, and %FVC, forced vital capacity). In addition, serum levels of this chitinase are higher in patients with HP with disease progression than in those who remain stable.[17,18]

KL-6 is a human mucin primarily expressed in regenerating type II pneumocytes and bronchiolar epithelium. This glycoprotein has chemotactic and antiapoptotic effects on fibroblasts, promoting fibroblast proliferation and pulmonary fibrosis progression.[19,20] There is evidence of higher serum KL-6 levels in patients with HP than in patients with IPF and sarcoidosis.[21] Moreover, patients with HP with clinical improvement present reduced KL-6 levels, but this biomarker remains significantly higher during active disease.[22,23]

The identification and quantification of biomarkers may be useful tools for establishing a correct HP diagnosis and for predicting disease progression. In this regard, the aim of this study was to investigate the potential value of KL-6 and YKL-40 as biomarkers in the diagnosis and prognosis of patients with HP.

MATERIALS AND METHODS

Study population

We performed a cross-sectional study with follow-up in real life including all patients older than 18 years with suspected HP who were referred to our center (Hospital Universitari Vall d'Hebron, Barcelona, Spain) for SIC from January 2008 to December 2017. For all patients, data from the clinical history and physical examination were analyzed. Results from additional tests were also evaluated: specific serum IgG antibody determinations, chest radiograph, chest computed tomography, and pulmonary function testing (spirometry, static lung volumes, and carbon monoxide diffusing capacity). The definitive diagnosis of HP was established by 2 pulmonologists with extensive experience in diffuse ILD.

The diagnosis was made according to the criteria proposed by Schuyler et al[8] when at least 4 major and 2 minor criteria were present and if other conditions with similar characteristics were ruled out. The major criteria were symptoms consistent with HP, evidence of appropriate antigen exposure in medical history and/or detection of specific precipitins in serum and/or bronchoalveolar lavage (BAL), findings consistent with HP on chest plain films or chest computed tomography, lymphocytosis in BAL (when performed), histological changes consistent with HP, and/or positive SIC. The SIC was considered positive according to the criteria previously established by the group.[24] The minor criteria were bilateral basal crackles, decrease in DLCO, and/or arterial hypoxemia, at least after exercise.

Only patients with HP due to avian or fungal exposure as a final diagnosis, blood and sputum samples at baseline, and with 2 years of clinical follow-up since the first spirometry were considered for the study (Figure 1). In the study population, a fibrotic pattern was defined as upper and middle lobe fibrosis or a honeycomb

TABLE I. Demographic and lung function characteristics of the subjects included in the study

Characteristic	HP (n = 49)	IPF (n = 19)	Sarcoidosis (n = 29)	Controls (n = 67)
Sociodemographic and clinical data				
Age (y), median (range)	60°,* (32-81)	65°,+ (46-82)	44*,+ (29-76)	(31-65)
Sex (male), n (%)	21 (43)°	16 (84)°,+,´	14 (48)+	30 (45)´
Smoking habit, n (%)				
Smoker	5 (10)¥	0 (0)	1 (4)	0 (0)¥
Former smoker	19 (39)¥	9 (47)´	11 (38)§	0 (0)¥,´,§
Nonsmoker	25 (51)¥	10 (53)´	17 (59)§	67 (100)¥,´,§
Survival, n (%)	37 (76)°,¥	6 (43)°,´	21 (91)§	67 (100)¥,´,§
Pulmonary function test				
Baseline				
FVC (% predicted), median (IQR)	82 (25)°	67(12)°,+	88 (30)+	NA
TLC (% predicted), median (IQR)	83 (23)	63 (22)	91 (30)	NA
DLCO (% predicted), median (IQR)	56 (25)	45 (21)+	66 (23)+	NA
Exposure data				
Avian antigens, n (%)	32 (65)*	8 (42)	9 (31)*	NA
Fungal antigens, n (%)	17 (35)	7 (37)	11 (38)	NA
Neither avian nor fungal antigens, n (%)	0 (0)	4 (21)	9 (31)	NA
Radiological pattern				
Fibrotic, n (%)	34 (69)°,*	19 (100)°,+	7 (24)*,+	NA
Nonfibrotic, n (%)	15 (31)°,*	0 (0)°,+	22 (76)*,+	NA

Certain variables have missing values in some groups: HP (4 in survival, 21 in baseline TLC, and 2 in baseline DLCO); IPF (5 in survival and 9 in baseline TLC); sarcoidosis (6 in survival, 14 in baseline TLC, and 2 in baseline DLCO). Statistically significant differences ($P < .05$) between the groups are indicated with different symbols (°, *, +, ´, ¥, §).
DLCO, Diffusing capacity of the lungs for carbon monoxide; *FVC*, forced vital capacity; *HP*, hypersensitivity pneumonitis; *IPF*, idiopathic pulmonary fibrosis; *NA*, not applicable; *TLC*, total lung capacity.

radiological pattern and usual interstitial pneumonia or airway-centered fibrosis in the histological pattern.

Patients who were referred for SIC but were diagnosed with IPF or sarcoidosis were included as controls. Diagnosis of sarcoidosis was verified by transbronchial and/or surgical lung biopsy in all patients included in the study.[25] Healthy volunteers were included as a second control group and were matched for age range and gender, had never been diagnosed with respiratory diseases, and were nonsmokers. The samples of these healthy volunteers were obtained from the Vall d'Hebron Biobank (collection number C.0005458), and only serum samples were available for this population.

The study was approved by the local ethics committee (Hospital Vall d'Hebron Ethics Committee approval PR(AG)19/2005), and all subjects signed informed consent before participation.

Sample collection and sputum examination

Blood was extracted in all subjects, and after blood centrifugation (3000 rpm during 10 minutes), serum samples were obtained and stored at −80°C until analysis.

Sputum induction was performed in the HP group following Pizzichini et al's previous description.[26] Briefly, an aerosol of hypertonic saline generated by an OMRON ultrasonic nebulizer (Peróxidos Farmacéuticos S.A., Barcelona, Spain) was inhaled through a mouthpiece with a nose clip in place at increasing concentrations of 3%, 4%, and 5% for 7 minutes per concentration. Sputum samples were processed within 2 hours, as described by Pizzichini et al.[26]

YKL-40 and KL-6 analysis

YKL-40 levels were measured in serum and sputum samples by an YKL-40 enzyme immunoassay kit (Quidel, San Diego, Calif) in accordance with the manufacturer's instructions, as previously described.[18]

KL-6 levels were measured in serum and sputum samples by a KL-6 enzyme immunoassay kit (Sekisui Medical Co, Tokyo, Japan), as previously described[22] and in accordance with the manufacturer's instructions.

Follow-up study

The patients were seen as part of the usual clinical practice. Information on death during follow-up was obtained from the clinical records. Patients still alive were contacted by telephone and were offered the possibility of re-evaluation. Those who agreed underwent a complete pulmonary function study including forced spirometry, static pulmonary volumes, and DLCO 1 and 2 years after the first visit.

Statistical analysis

The normal distribution of the data was evaluated with the Shapiro-Wilk test. Qualitative variables are expressed as absolute numbers and their corresponding percentages. For quantitative variables, nonparametric data are shown as medians and ranges or interquartile ranges, whereas parametric data are expressed as means and standard deviations. Comparisons between the groups were performed using the χ^2 test for categorical variables (or the Fisher exact test when one of the expected effects was less than 5) and the Student's t test or Mann-Whitney test for continuous variables, as appropriate. Multiple comparisons between the groups were performed using 1-way analysis of variance (with the Bonferroni correction) or the Kruskal-Wallis test (with Dunn's correction). Multivariate regression models were performed to assess the association between serum or sputum biomarker levels

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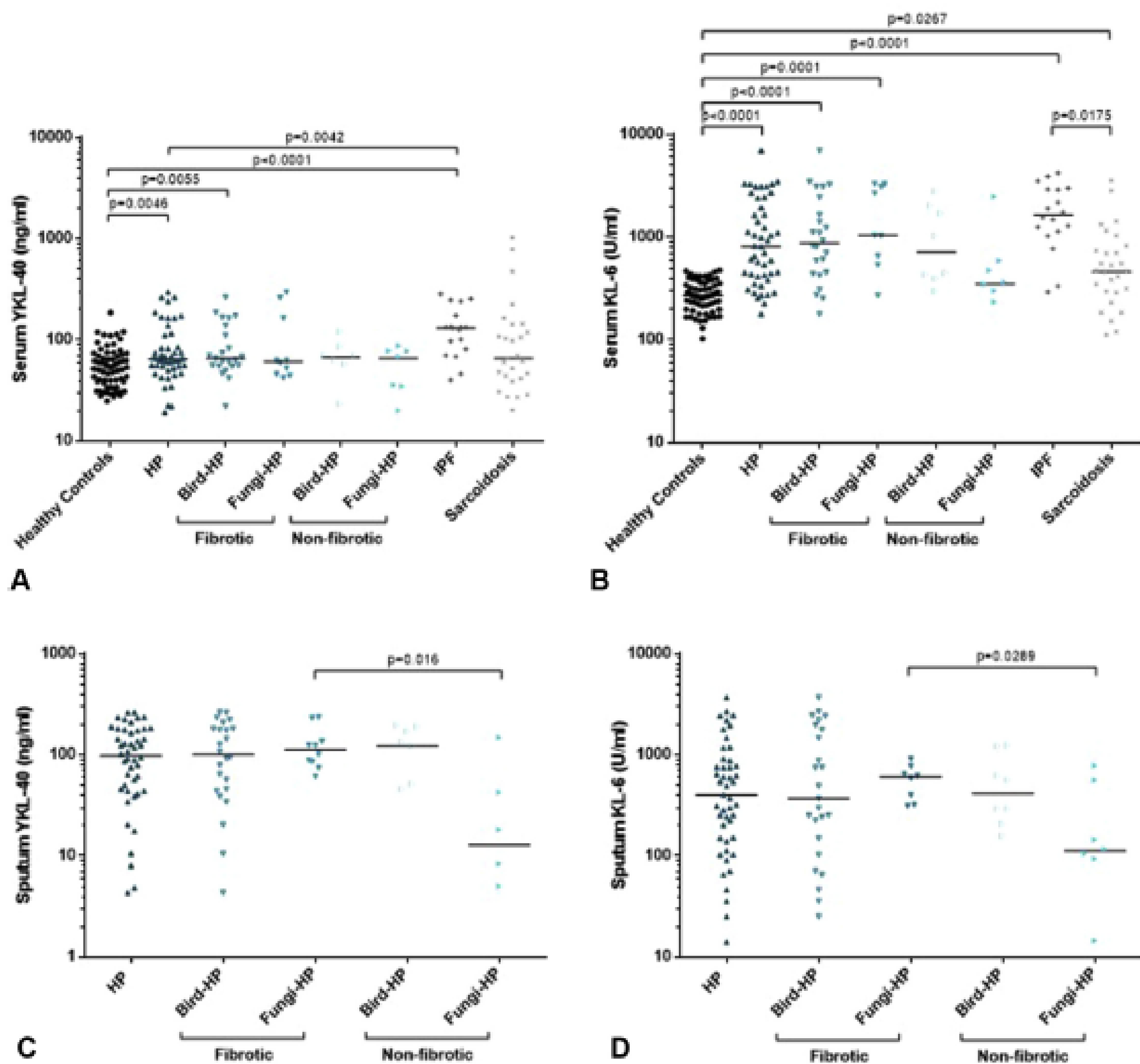

FIGURE 2. (**A**) Serum and (**C**) sputum levels of (**B**) YKL-40 and (**D**) KL-6 in the study population. The Kruskal-Wallis test (with Dunn's correction) was used to determine the statistical significance between the groups. *HP,* Hypersensitivity pneumonitis; *IPF,* idiopathic pulmonary fibrosis; *KL-6*, Krebs von den Lungen-6; *YKL-40*, chitinase 3-like-1.

and the other covariates (group, age, sex, smoking habit, survival, and radiological pattern). Correlations between variables were calculated with the Pearson correlation coefficient (r) or the Spearman correlation coefficient (r_s). Receiver operating characteristic (ROC) curves were used to determine the cutoff points of YKL-40 and KL-6 in serum samples as diagnostic markers to discriminate patients with HP from healthy controls or patients with IPF. HP diagnosis was used as the gold standard for the ROC curves. Analyses were conducted using GraphPad Prism 6 for Windows (version 6.01; GraphPad Software Inc, San Diego, Calif) and IBM SPSS Statistics (version 26; IBM Corporation, Armonk, NY). Differences with a *P* value of <.05 (2-tailed) were considered to be significant.

RESULTS

Characteristics of the study population

Demographic and clinical characteristics of the subjects included in the study are shown in Table I. The participants comprised 49 patients with HP, 48 patients with other ILD (19 IPF and 29 sarcoidosis), and 67 healthy volunteers (healthy controls). There was a significant difference between the median age of patients in the HP and sarcoidosis groups (median [range]) (HP: 60 [32-81] years; sarcoidosis: 44 [29-76] years; $P = .0094$) and between patients with HP and IPF (65 [46-82] years; $P = .0121$). Significant differences were also observed in the median age between patients with IPF and sarcoidosis ($P = .0004$). Of the 49 patients with HP, 37 remained alive at the end of the

TABLE II. Association between serum biomarker levels and type of ILD according to multivariate regression analysis

	Serum YKL-40 levels (ng/mL)			Serum KL-6 levels (U/mL)		
Covariates	**B coefficient**	**95% CI**		**B coefficient**	**95% CI**	
(Intercept)	118,095	−92,980	329,170	955,945	−304,147	2,216,036
Sex	−35,062	−71,187	1062	−77,899	−316,948	161,150
Smoking habit	−2619	−103,127	97,889	−274,692	−875,129	325,746
Survival	−61,981	−128,110	4148	−37,991	−461,648	385,666
Radiological pattern	117,379	55,232	179,526	686,833	288,085	1,085,580
HP	−32,722	−91,790	26,346	423,630	43,837	803,423
IPF	−40,145	−129,312	49,021	534,592	−44,689	1113,873
Sarcoidosis	100,136	47,169	153,103	270,665	−74,619	615,948

CI, Confidence interval; *HP*, hypersensitivity pneumonitis; *IPF*, idiopathic pulmonary fibrosis; *KL-6*, Krebs von den Lungen-6; *YKL-40*, chitinase 3-like-1.

study, 8 died because of respiratory illness after 8 years (mean) of the diagnosis, and 4 were lost to follow-up. The IPF group had the lowest survival rate at 43%, compared with 82% in patients with HP ($P = .0127$). Birds were the causative antigen in 65% of the cases with HP, and fungi in the remaining 35%. Chest imaging and/or histology classified 69% of patients with HP as fibrotic. Among patients with HP with a fibrotic pattern, 24 were exposed to avian antigens and 10 to fungi.

Levels of YKL-40 and KL-6 in serum and induced sputum

Serum YKL-40 and KL-6 levels were significantly higher in patients with HP than in healthy controls (HP YKL-40: 65 [19-297] ng/mL; healthy controls YKL-40: 52 [25-186] ng/mL; $P = .0046$) (HP KL-6: 814 [178-6962] U/mL; healthy controls: 270 [103-476] U/mL; $P < .0001$) (Figure 2, *A* and *B*). Significant differences were also observed for both biomarkers in serum samples when dividing patients with HP according to a fibrotic pattern and birds as a causative antigen (fibrotic HP with avian exposure YKL-40: 66 [22-264] ng/mL; healthy controls YKL-40: 52 [25-186] ng/mL; $P = .0055$) (HP KL-6: 875 [178-6962] U/mL; healthy controls: 270 [103-476] U/mL; $P < .0001$). Serum KL-6 levels were also significantly higher in patients with fibrotic HP exposed to fungi (fibrotic HP with fungal exposure KL-6: 1042 [270-3298] U/mL; healthy controls: 270 [103-476] U/mL; $P = .0001$) (Figure 2, *A* and *B*).

Moreover, there were significant differences in serum YKL-40 and KL-6 levels between the healthy controls and patients with IPF (IPF YKL-40: 131 [40-281] ng/mL; healthy controls YKL-40: 52 [25-186] ng/mL; $P < .0001$) (IPF KL-6: 1106 [290-4194] U/mL; healthy controls: 270 [103-476] U/mL; $P < .0001$) (Figure 2, *A* and *B*), in serum YKL-40 between patients with HP and IPF (HP YKL-40: 65 [19-297] ng/mL; IPF YKL-40: 131 [40-281] ng/mL; $P = .0042$) (Figure 2, *A*), and in serum KL-6 in IPF and sarcoidosis groups (IPF KL-6: 1106 [290-4194] U/mL; sarcoidosis: 290 [112-3539] U/mL; $P = .0175$) (Figure 2, *B*).

A negative association was observed between serum YKL-40 levels and patients with HP or IPF when adjusting for the different covariates, whereas the association was positive for patients with sarcoidosis. In regard to serum KL-6 levels, a positive association was observed in all groups (Table II).

Among patients with HP with fungi as a causative antigen, sputum YKL-40 and KL-6 levels were significantly higher in the ones with a lung fibrotic pattern (fibrotic HP YKL-40: 113.3 [61.55-238] ng/mL; nonfibrotic HP YKL-40: 12.83 [0-144] ng/mL; $P = .016$) (fibrotic HP KL-6: 375 [25.32-3729] U/mL; nonfibrotic HP KL-6: 112.5 [14.10-753.2] U/mL; $P = .0289$) (Figure 2, *C* and *D*). Sputum YKL-40 levels correlated positively with sputum KL-6 levels ($r = 0.497$; $P = .0004$). No significant associations were observed between the different covariates analyzed and the biomarker levels in the different groups.

In patients with HP, serum KL-6 levels correlated negatively with total lung capacity (TLC; $r = -0.401$; $P = .0343$) and DLCO ($r = -0.534$; $P = .0001$) at baseline (Figure 3, *A* and *B*, respectively). Moreover, a negative correlation was observed between KL-6 levels in sputum samples and baseline DLCO in patients with HP ($r = -0.463$; $P = .0014$) (Figure 3, *C*). There were no significant correlations between sputum KL-6 levels and other lung parameters. No statistically significant correlations were observed between pulmonary function parameters and YKL-40 levels in serum or sputum samples.

Predictive value of baseline serum YKL-40 and KL-6 for HP diagnosis

ROC analysis showed that a cutoff of >55 ng/mL for serum YKL-40 levels had the best sensitivity (75%), specificity (54%), and accuracy (63%) for discriminating between patients with HP and healthy controls (area under the curve [AUC]: 0.655; positive predictive value [PPV]: 55%, negative predictive value [NPV]: 75%; $P = .005$) (Figure 4, *A*), and that a cutoff of <121 ng/mL for serum YKL-40 showed the best sensitivity (79%), specificity (63%), and accuracy (75%) for discriminating between patients with HP and IPF (AUC: 0.741; PPV: 84%, NPV: 54%; $P = .002$) (Figure 4, *B*).

For serum KL-6 levels, a cutoff of >346 U/mL had the best sensitivity (81%), specificity (71%), and accuracy (66%) for discriminating between patients with HP and healthy controls (AUC: 0.883; PPV: 67%, NPV: 84%; $P < .0001$) (Figure 4, *C*), and a cutoff of <1441 U/mL showed the best sensitivity (71%), specificity (63%), and accuracy (67%) for discriminating between patients with HP and IPF (AUC: 0.702; PPV: 83%, NPV: 46%; $P = .011$) (Figure 4, *D*). The discriminatory capacity of YKL-40 and KL-6 was not affected by confounders when HP diagnosis was compared with IPF or healthy controls in ROC curves.

Follow-up

Patients with HP who died had higher levels of KL-6 at baseline in sputum samples compared with patients who survived (median [range]) (exitus: 835.6 [225.4-3729] U/mL; alive: 323.9 [14.1-2697] U/mL; $P = .0478$). No significant differences were

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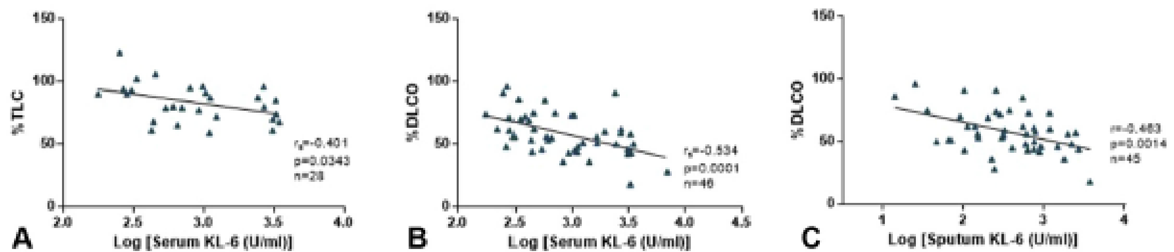

FIGURE 3. Correlations between serum KL-6 levels and baseline (**A**) TLC or (**B**) DLCO and (**C**) sputum KL-6 levels and baseline DLCO in patients with HP. (**A, B**) The Spearman correlation coefficient and (**C**) the Pearson correlation coefficient were used, as appropriate, as statistic tests for data analyses. *DLCO,* Diffusing capacity of the lungs for carbon monoxide; *HP,* hypersensitivity pneumonitis; *KL-6,* Krebs von den Lungen-6; *TLC,* total lung capacity.

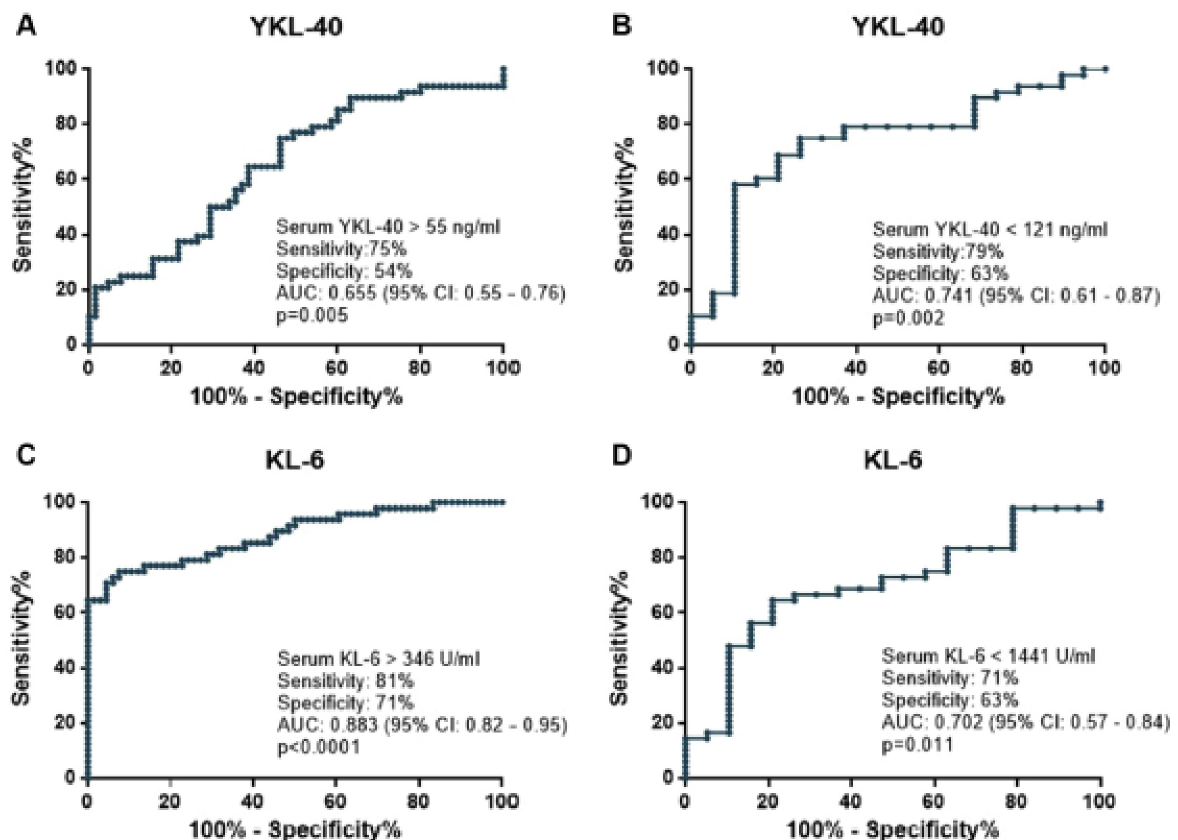

FIGURE 4. ROC curves for HP diagnosis according to serum (**A, B**) YKL-40 or (**C, D**) KL-6 levels. In (**A**) and (**C**), the biomarker levels are compared between patients with HP and the control group, and in (**B**) and (**D**) between patients with HP and patients with IPF. *AUC,* Area under the curve; *CI,* confidence interval; *HP,* hypersensitivity pneumonitis; *IPF,* idiopathic pulmonary fibrosis; *KL-6,* Krebs von den Lungen-6; *ROC,* receiver operating characteristic; *YKL-40,* chitinase 3-like-1.

observed in serum KL-6 levels or serum/sputum YKL-40 levels according to survival.

Dividing patients with HP according to serum KL-6 cutoff (<346 or >346 U/mL), no significant differences were observed in FVC values between the groups either at baseline or during the follow-up (Figure 5, *A*). For TLC, the only significant differences between the groups were observed at baseline (<346 U/mL: 93.8 ± 4.92; >346: 78.91 ± 12.94; $P = .0222$) (Figure 5, *B*). DLCO differed significantly between the groups at baseline (<346 U/mL: 71.67 ± 16.55; >346: 54.68 ± 15.22; $P =$

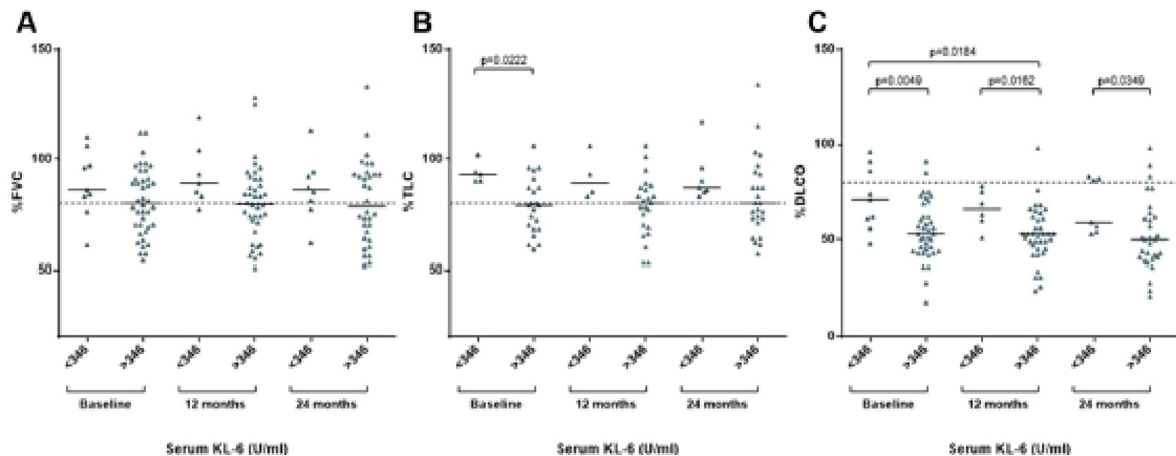

FIGURE 5. Lung function follow-up in patients with HP according to serum KL-6 cutoff estimated with ROC analysis. **(A)** %FVC. The dotted line indicates the 80% of FVC. **(B)** %TLC. The dotted line indicates the 90% of FVC. **(C)** %DLCO. The dotted line indicates 80% of DLCO. A cutoff of 346 U/mL is estimated with ROC analysis for KL-6 in serum samples of patients with HP. The Student *t* test (for FVC and DLCO) or the Mann-Whitney test (for TLC and DLCO) was used when comparing the statistical significance between the groups at the same time point. Analysis of variance with the Bonferroni correction (for FVC) or the Kruskal-Wallis test with Dunn's correction (for TLC and DLCO) was used when comparing the groups at different time points. *DLCO*, Diffusing capacity of the lungs for carbon monoxide; *FVC*, forced vital capacity; *KL-6*, Krebs von den Lungen-6; *ROC*, receiver operating characteristic; *TLC*, total lung capacity.

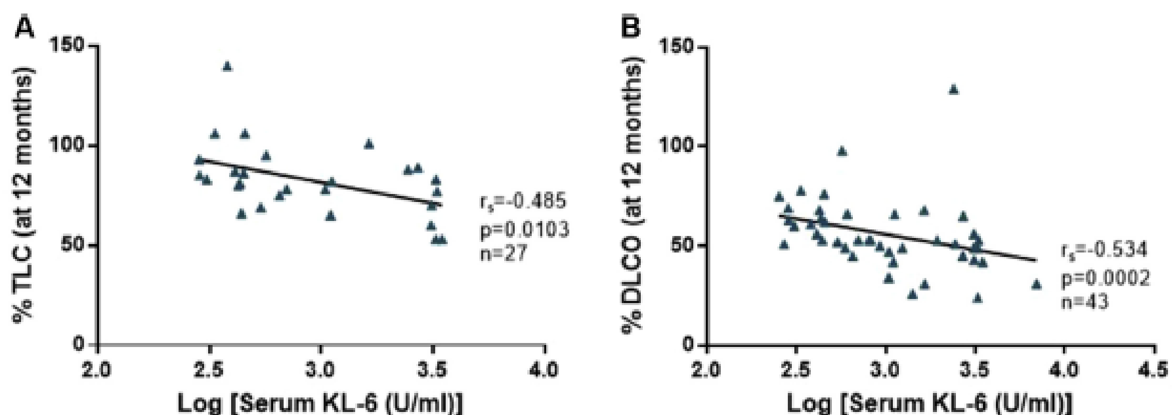

FIGURE 6. Correlation between baseline serum KL-6 levels and **(A)** TLC or **(B)** DLCO measured at 12 months' follow-up. Spearman's correlation coefficient was used as the statistic test for data analyses. *DLCO*, Diffusing capacity of the lungs for carbon monoxide; *KL-6*, Krebs von den Lungen-6; *TLC*, total lung capacity.

.0049), after 12 months (<346 U/mL: 66.0 ± 10.04; >346: 52.51 ± 14.23; $P = .0162$), and after 24 months of follow-up (<346 U/mL: 67.0 ± 14.18; >346: 52.57 ± 18.57; $P = .0349$) (Figure 5, *C*). In patients with HP, serum KL-6 levels correlated negatively with TLC ($r = -0.485$; $P = .0103$) and DLCO ($r = -0.534$; $P = .0002$) at 12 months (Figure 6, *A* and *B*, respectively).

DISCUSSION

HP is a syndrome caused by an exaggerated immune response to the inhalation of a variety of antigen particles found in the environment. In the United States, the yearly incidence rate of HP ranges from 1.67 to 2.71 per 100,000 persons.[27] A clear diagnosis of HP can be difficult to confirm, and the pathogenic determinants of onset and progression of the disease remain unresolved.[28] At present, there are no biomarkers able to accurately predict the presence or absence of HP and the progression of the disease; therefore, KL-6 or YKL-40 might contribute to our insights into disease progression and prognosis.

The results of the study showed that YKL-40 and KL-6 levels were high in serum of patients with HP, especially in those presenting a fibrotic pattern. Both proteins seem to be capable of distinguishing patients with HP from healthy individuals and

patients with IPF with adequate sensitivity and specificity, thus confirming their potential role as biomarkers. Moreover, KL-6 may be a predictor of disease progression.

Indeed, we observed that serum levels of KL-6 are high in patients with HP who present a fibrotic pattern. KL-6 has already been proposed as a potential biomarker of fibrotic lung involvement,[29] and in fact, high serum KL-6 is indicative of lung remodeling.[30] Increased serum KL-6 levels in various ILDs are thought to result from the regeneration of type II alveolar epithelial cells and to enhance permeability after destruction of the air-blood barrier.[31] For patients with HP, results similar to ours have been reported in the literature. In the study by Takahashi et al,[32] serum KL-6 levels were significantly higher in patients with farmer's lung disease than in farmers without the disease. Other studies have shown that KL-6 is associated with acute symptoms of early-stage HP in pigeon fanciers[33] and that changes in the serum KL-6 level during short-term strict antigen avoidance could predict prognosis in patients with fibrotic bird-related HP.[34] Other authors have studied the role of this biomarker in discriminating between different ILDs. In this context, a study carried out in patients with sarcoidosis, IPF, and HP reported significant differences in the levels of KL-6 in the 3 groups, the highest levels being observed in patients with IPF and the lowest in those with sarcoidosis.[35] By contrast, in a retrospective study of 92 patients with HP, Okamoto et al[21] observed that serum KL-6 was higher in chronic HP than in IPF. Our findings highlight that a serum level of KL-6 >346 U/mL can discriminate between patients with HP and healthy controls and that levels >1441 U/mL can discriminate between HP and IPF.

To the best of our knowledge, this is the first study to analyze KL-6 levels in sputum samples of patients with HP. We found sputum KL-6 levels to be significantly higher in patients with HP exposed to fungi with a lung fibrotic pattern than in those without fibrosis. In this connection, a previous study[36] concluded that patients with IPF had higher levels of KL-6 in sputum samples than healthy controls.

Nevertheless, in the literature, KL-6 has been associated more with the evolution and prognosis of fibrotic lung disease than with diagnosis.[22,34,37] Changes in concentrations of serum KL-6 with time were strongly associated with ptrogression of ILD and survival.[34,37] Indeed, this protein may also be a candidate biomarker for early progression. Satoh et al[22] reported that disease progression was significantly faster in patients with ILDs whose KL-6 levels were 1000 U/mL or above at the initial measurement than in patients whose KL-6 levels were below this figure. In the present study, we observed that KL-6 levels in patients with HP correlated negatively with TLC and DLCO at 12 months. In this context, in a study carried out in patients with IPF, Guiot et al[36] reported that sputum KL-6 levels were inversely correlated with TLC. Early identification of patients at high risk of suffering a deterioration in lung function or of developing pulmonary fibrosis could allow clinicians to consider additional pharmacological therapies.

To the best of our knowledge, only one previous study has described YKL-40 levels in serum and BAL in patients with HP,[17] and our study is the first to analyze this biomarker in sputum samples as well. The results presented here are in line with those obtained by Long et al.[17] These authors quantified the values of YKL-40 in serum and BAL in 72 patients with HP, reporting similar results to ours regarding the sensitivity and specificity of this biomarker for the diagnosis of the disease. However, they observed that patients with HP whose disease progressed or who died had had higher baseline YKL-40 levels than those who remained stable and survived; in the present study, no relationship was found between this biomarker and disease progression. The differences found in the present study in the serum levels of YKL-40 in patients with a fibrotic pattern were observed only in patients exposed to birds, not in those exposed to fungi. According to the adjusted model, sarcoidosis disease has a positive association with serum YKL-40 levels. These results are in line with those obtained by Uysal et al[38] and Johansen et al.[39] These authors concluded that individuals with chronic sarcoidosis and with active disease had higher levels of YKL-40 in serum than the control group. A systematic review recently published[40] also reported that patients with sarcoidosis had the highest serum YKL-40 levels compared with patients with different types of ILD.

Although we did not have a healthy control group for comparison of the results obtained in sputum, we observed that in patients with HP exposed to fungi, the levels of YKL-40 were lower in those who did not have a fibrotic pattern. In this regard, the results of previous studies by our group analyzing cellular inflammation in induced sputum of patients with HP prompted the hypothesis that different antigens may cause the development of HP via different pathophysiological mechanisms.[41] Moreover, although KL-6 is expressed exclusively on type 2 pneumocytes, YKL-40 is produced by a variety of cell types, including airway epithelial cells, macrophages, neutrophils, monocytes, vascular smooth muscle cells, and chondrocytes. Therefore, the KL-6 values found derive directly from the synthesis of this biomarker by pneumocytes, whereas YKL-40 levels may be derived from other nonpulmonary cells. This means that the values found for YKL-40 may be less specific for lung damage and may express mechanisms of other kinds.

Our study has several limitations. First, for the analysis of biomarkers in sputum, we did not have a healthy control group, which could make the interpretation of the results difficult. Future studies should aim to study the differences in these biomarkers in sputum between patients with HP and healthy individuals. Second, the fact that patients were recruited among individuals attending to a referral center for SIC may have introduced a bias. In any case, these are patients in whom the diagnosis is difficult to establish, and so access to other diagnostic biomarkers is urgently needed. Moreover, as this is a cross-sectional study with follow-up in real life, some of the pathological reports and analyses were lacking. Another limitation of this study was the absence of association between the biomarker levels and the potential confounding variable introduced by smoking, probably because of the small size of smoker patients included in the study. In this sense, future research studies could be designed to assess the influence of smoking in the levels of these biomarkers. In addition, patients with HP diagnosis may present different degrees of progression of fibrosis, and this differential evolution may affect the ROC curve results when comparing patients with HP and IPF because the results may overlap between the groups. Moreover, it is possible that these biomarkers may reflect pulmonary fibrosis rather than being a specific marker for HP. The validity of the ROC analyses might be limited because of the sample size. In this sense, further studies with other cohorts with larger sample sizes are necessary to validate the diagnostic and predictive value of these biomarkers. In addition, the nonstatistically significant differences

between the groups could also be associated with the sample size. Finally, as notable seasonal variations in KL-6 and YKL-40 levels have been shown in some chronic HP, the time of serum collection may affect the results.[42]

CONCLUSIONS

The measurement of YKL-40 and KL-6 levels in patients with HP offers insights into the pathobiology of this disease and has the potential to improve diagnostic accuracy. The measurement of these biomarkers could help pulmonologists to identify patients with fibrotic HP who are likely to show rapid progression. Nevertheless, the study was not designed to assess the progression of disease for a given biomarker level and further prospective studies with larger samples are needed to validate the reliability and clinical utility of YKL-40 and KL-6 as biomarkers of disease severity and prognosis of HP.

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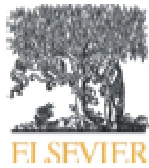
4.2 ARTICLE 2. “A RAPID TEST FOR THE ENVIRONMENTAL DETECTION OF PIGEON ANTIGEN”

Sánchez-Díez S, Cruz MJ, Álvarez-Simón D, Montalvo T, Muñoz X, Hoet PM, et al. A rapid test for the environmental detection of pigeon antigen. Sci Total Environ. 2021 Sep 20;788. doi:10.1016/j.scitotenv.2021.147789

Science of the Total Environment 788 (2021) 147789



Contents lists available at ScienceDirect

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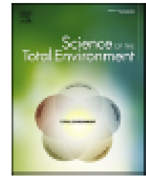

A rapid test for the environmental detection of pigeon antigen

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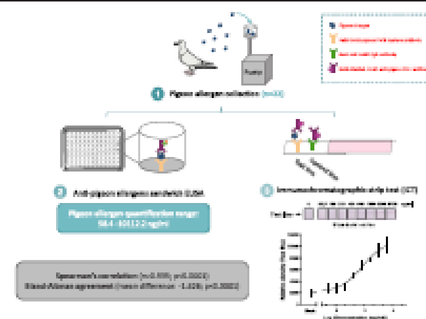
HIGHLIGHTS

- Demonstrating antigen exposure is critical in the diagnosis and management of hypersensitivity pneumonitis.
- An immunochromatographic test is compared with an ELISA method for the measurement of pigeon antigens in the environment.
- The immunochromatographic test described was highly consistent with the validated ELISA and does not require specific skills.
- This method may prove to be a useful tool for predicting the progression of bird-related hypersensitivity pneumonitis.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 11 March 2021
Received in revised form 11 May 2021
Accepted 11 May 2021
Available online 15 May 2021

Editor: Lotfi Aleya

Keywords:
Hypersensitivity pneumonitis
Immunochromatographic test
Pigeon antigen
Strip assay

ABSTRACT

Introduction: Avoidance of inhaled bird antigens is essential to prevent hypersensitivity pneumonitis disease progression. The aim of the present study was to develop a sandwich enzyme link immunoassay (ELISA) and an immunochromatographic test (ICT) and compare their ability to detect pigeon antigens in environmental samples.
Methods: An amplified sandwich ELISA using pigeon serum as a calibration standard and a ICT using gold-labeled anti-pigeon serum antibodies for the rapid detection of pigeon antigens in environmental samples were developed. Twenty-two different airborne samples were collected and analysed using both methods. Strip density values obtained with ICT were calculated and compared with the concentrations determined by the ELISA method for pigeon antigens. Strips results were also visually analysed by five independent evaluators.
Results: The ELISA method to quantify pigeon antigen had a broader range (58.4 and 10,112.2 ng/ml), compared to the ICT assay (420 to 3360 ng/ml). A kappa index of 0.736 ($p < 0.0001$) was obtained between the observers evaluating the ICT strips. The results of the ELISA and the relative density of the ICT showed a highly significant correlation (rs:0.935; $p < 0.0001$). Bland-Altman plot also confirmed excellent agreement between the two methods (mean difference: −1.626; $p < 0.0001$).
Conclusions: Since there was a good correlation between both assays, we can conclude that the rapid and simple ICT assay is a good and valid alternative, which does not require expensive equipment, for the validated ELISA technique.
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https://doi.org/10.1016/j.scitotenv.2021.147789
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1. Introduction

Hypersensitivity pneumonitis (HP) is an interstitial lung disease characterized by inflammation in bronchioles and alveoli (Selman, 2004). It occurs in some genetically predisposed individuals after recurrent inhalation of particular environmental antigens, usually avian and fungal proteins. HP is characterized by fever, dyspnea, chest tightness/discomfort and/or cough (Quirce et al., 2016), and it has recently been classified into two different forms: an acute form characterized by inflammation and a chronic form characterized by fibrosis, according to the intensity and frequency of antigen exposure, clinical evaluation, pulmonary function tests and radiologic findings (Lacasse et al., 2009; Vasakova et al., 2017).

Bird-related hypersensitivity pneumonitis (BRHP) occurs after inhalation exposure to avian antigens. It can be caused by either direct exposure, i.e., through breeding birds, or indirect exposure, i.e., due to the presence of nearby dovecotes, flocks of pigeons in parks, feather duvets, and so on (Shirai et al., 2017; Morell et al., 2008). It has been demonstrated that the most common antigenic sources are droppings, feathers and blooms (Vasakova et al., 2019; Raghu et al., 2020). Next to pigeon antigens, other types of avian antigen such as those associated with parrots, parakeets, chickens, ducks, etc., can cause BRHP (Kokkarinen et al., 1994). Currently, it seems that the prevalence of BRHP is underestimated. A Japanese epidemiological study conducted between 2000 and 2009 revealed that 60% of chronic cases of HP were caused by avian antigens (Okamoto et al., 2013), and it has been shown that, in some patients, this disease can be caused by exposure to feather duvets or pillows (Morell et al., 2013).

For patients with BRHP, complete avoidance of inhaled avian antigens is essential to improve the respiratory symptoms and prevent disease progression (Vourlekis et al., 2004). Some BRHP cases possibly become chronic because of the persistence of their exposure to low levels of antigen (Borderías et al., 2010). To control exposure to avian antigen levels in the environment, air measurements are essential for these patients (Agache and Rogozea, 2013). Previous studies have measured the concentration of avian antigens in collected dust and air samples by using direct, inhibition or sandwich enzyme-linked immunoassays (ELISA) (Craig et al., 1992; Tsutsui et al., 2015), while others used an antigen-capture ELISA with signal amplification in order to improve sensitivity and detect small amounts of antigen (Kuramochi et al., 2010). All these techniques are costly and time-consuming and require access to laboratories with sophisticated equipment and trained personnel, and so they are not readily adaptable to field use. Therefore, considerable efforts are being made to develop easy-to-use analytical methods that are able to guide legislation on the concentration of these proteins in the air, and may also allow consumers to monitor their levels.

The immunochromatographic test (ICT) is a rapid semiquantitative allergen estimation method, easy to perform and interpret, portable, and cheap. In this test, a sample with labeled specific antibodies is mixed and incubated with a strip. Then, the immunocomplexes formed migrate by capillarity through the nitrocellulose membrane and bind to the line of immobilized specific capture antibodies if the sample contains the specific antigen (Bogdanovic et al., 2006; Tsay et al., 2002; Álvarez-Simon et al., 2014).

The aim of the present study was to develop and validate a sensitive sandwich enzyme link immunoassay technique and a rapid ICT to detect pigeon antigens in environmental air samples and to determine whether the results obtained with the two methods were comparable.

2. Material and methods

2.1. Preparation of rabbit anti-pigeon polyclonal antibodies and colloidal gold labeling

Anti-pigeon polyclonal antibodies were produced by Kaneka Eurogentec S.A., Seraing, Belgium. In Brief, a commercial pigeon serum (Rockland Immunochemicals Inc., Limerick, Ireland), with a protein concentration of 15.5 mg/ml determined previously by the Bicinchoninic acid (BCA) method (Pierce Chemical Co., Rockford, USA), was subcutaneous injected into two New Zealand white rabbits. Three booster injections were administered at 4-week intervals. The use of the animals for this purpose was approved by the firm's Animal Ethics Committee.

The IgG fraction of the polyclonal antisera of both rabbits was isolated on immobilized Protein A–agarose columns (Pierce Chemical Co., Rockford, USA), and then eluted to Excellulose columns (Pierce Chemical Co., Rockford, USA) to desalt and exchange the buffer to phosphate buffer saline (PBS). Purified and desalt IgG fractions from each rabbit were named Pol J and Pol K. The protein concentration of the IgG fraction of the sera was 7.7 mg/ml (Pol J) and 6.4 mg/ml (Pol K), as determined by the BCA method.

A portion of rabbit anti-pigeon polyclonal antibody from one rabbit (Pol J) was biotinylated using the EZ links Sulfo-NHS-LC-Biotinylation Kit (Pierce Chemical Co.) according to the manufacturer's instructions. Another portion of Pol J was labeled with colloidal gold by BBI Solutions (British Biocell International, Cardiff, UK). Briefly, the antibody was conjugated to a 40 nm gold colloid using passive adsorption and was stable at least for 10 months. Then, the conjugate was blocked with bovine serum albumin (BSA) (Pierce Chemical Co., Rockford, USA) and the resulting conjugate was concentrated by ultrafiltration to give a final optical density (OD) (520 nm) of 10 and suspended in a 2 mM disodium tetraborate buffer pH 7.2, containing 0.095% sodium azide.

2.2. Sample collection and analysis

Twenty-two air samples were collected from two different dovecotes in the province of Barcelona with a medium-volume automated air sampler (Flite 3, Vertex technics S.L., Barcelona, Spain) containing nitrocellulose filters with a 0.65 μm pore size (Millipore S.A.S., Molsheim, France). The automated air sampler had a pole 115 cm high with a support at the top for the nitrocellulose filter. Dust was collected at a flow of 12 l/min during 1 h, 2 h, 4 h, 8 h, 12 h or 24 h. Air samples of environments without avian antigens were also collected at the same flow for 1 h, 2 h, 4 h or 8 h, for use as negative controls. Antigens were extracted from the filters in 1.5 ml PBS/0.2% BSA/0.5% Tween-20 (pH 7.4) buffer overnight at 4 °C. Filters were discarded and the eluates were stored at −20 °C until being analysed in parallel by the anti-pigeon sandwich ELISA and the ICT.

2.3. Characterization of pigeon serum and air samples by 1D and 2D electrophoresis

For 1D electrophoresis performance, Laemmli sample buffer 4× (Bio-Rad, Madrid, Spain) was diluted in 2-mercaptoethanol (Sigma-Aldrich Quimica S.L, Madrid, Spain) according to the manufacturer's instructions. Pigeon serum (850 μg protein) or air sample (60 ng protein) were mixed with the previous mixture at a 3:1 proportion and heated for 5 min at 90 °C in the water bath. Subsequently, samples were loaded onto preparative Criterion TGX Stain-Free gels 4–20% precast polyacrylamide gel (Bio-Rad, Madrid, Spain) according to the manufacturer's manual, and electrophoresis was performed at 200 V for 40 min.

For 2D electrophoresis, pigeon serum (100 μg protein) was mixed with rehydration solution (pure H_2O/8.5 M Urea/2% Chaps/0.5% immobilized pH gradient (IPG) buffer/0.002% Bromophenol blue) and loaded onto IPG strips (IPG strip, pH 3–10, 7 cm, General Electric Healthcare, Boston, USA). Isoelectric focusing (IEF) was then carried out using the IPGphor IEF system (General Electric Healthcare, Boston, USA) following the manufacturer's instructions. IPG strips were then equilibrated and loaded onto Mini-PROTEAN TGX Stain-Free gels 4–20% (7 cm, IPG/prep, Bio-Rad, Madrid, Spain) and run at 200 V for 30 min.

In both cases, Precision Plus Protein™ Unstained Standards (Bio-Rad, Madrid, Spain) were used as molecular weight (MW) markers with a MW ranging from 10 to 250 kDa. After 1D and 2D electrophoresis, gels were imaged in a Gel Doc EZ Imager (Bio-Rad, Madrid, Spain)

and proteins were stained with Coomassie brilliant blue (Bio-Rad, Madrid, Spain) or blotted to a membrane.

2.4. Immunoblotting of pigeon serum

1D and 2D gels of pigeon serum were blotted onto polyvinylidene difluoride (PVDF) membranes using the Trans-Blot Turbo transfer system (Bio-Rad, Madrid, Spain). Then, membranes were washed with PBS and blocked with 5% nonfat dry milk (Bio-Rad, Madrid, Spain) for 1 h at room temperature (RT) under gentle shaking. After three washing steps with PBS/0.05 T (PBS with 0.05% Tween-20), membranes were incubated with rabbit anti-pigeon polyclonal antibody Pol J or Pol K diluted 1/5000 in PBS for 2 h at RT with gentle shaking. After three washing steps with PBS/0.05 T, membranes were incubated with goat anti-rabbit IgG-HRP (H + L, SouthernBiotech, Birmingham, USA) diluted 1/20,000 in PBS for 1 h at RT. Membranes were then washed three times with PBS/0.05 T and incubated with the enzyme substrate for 5 min at RT (Kit Clarity Western ECL Substrate, Bio-Rad, Madrid, Spain), according to the supplier's recommendations. Image acquisition was carried out with the Odyssey Fc Imaging System and the Image Studio Lite software (LI-COR, Nebraska, USA).

2.5. Sandwich ELISA for pigeon antigens

Wells of high-binding microtiter plates (Costar, Cambridge, USA) were coated overnight at 4 °C with Pol K (0.1 μg protein/well) in PBS buffer (pH 7.4). The wells were blocked (200 μl/well) with PBS/1% BSA/0.05% Tween-20 for 1 h at 37 °C. After washing three times with PBS/0.1 T (PBS with 0.1% Tween 20), plates were incubated for 1 h at 37 °C with 100 μl/well of filter elutes, negative controls (eluate of a white filter) and pigeon antigen standards containing 6.7–27,500 ng protein/ml. After three washes with PBS/0.1 T, plates were incubated for 1 h at 37 °C with 100 μl/well of Pol J biotinylated at 1:15,000 dilution in PBS/1% BSA/0.05% Tween-20. Streptavidin (Sigma-Aldrich Quimica S. L, Madrid, Spain) at 1/10,000 dilution in PBS/1% BSA/0.05% Tween-20 was added (100 μl/well) after washing again three times with PBS/0.1 T. After streptavidin incubation for 1 h at 37 °C, wells were washed three times and the reaction was developed with 30% H_2O_2 and 3,3′,5,5′-tetramethylbenzidine in acetate buffer (pH 5.5) at 25 °C in the dark. After 20 min, the reaction was stopped with 2 M H_2SO_4 and read at 450 nm with a plate reader (Infinite M Nano, Tecan, Männedorf, Switzerland). The standard curve was constructed from seven data points using a four-parameter logistic curve fit. Results are expressed in ng/ml, referring to the protein content of the standard preparation. Magellan software (Tecan Austria GmbH, Grödig, Austria) was used to calculate the results.

The lower limit of detection (LOD) was defined as the concentration of the lowest point on the standard curve, with the optical density (OD) value at this point having to be at least 0.05 above the blank. The lower limit of quantification (LOQ) was defined, according to the curve fitting function, as the concentration that gave the OD of the lowest point on the standard curve plus 20% of the highest point on the standard curve. The upper LOQ was defined as the concentration that gave 80% of the OD at the highest point on the standard curve. Following these premises, the determinations were largely limited to the log-linear part of the curve.

The inter-assay coefficient of variation (CV) was determined by analysing two points of the standard curve in duplicate on 10 different days. Thus, the CV of each point was calculated as the standard deviation of the mean of 10 measurements in duplicate (SD/mean x 100).

2.6. Specificity of the ELISA

Cross-reactivity of the ELISA assay towards other types of bird protein was tested using serum of small parrot, parakeet and parrot. Sera from those birds were obtained by centrifugation of collected blood samples. Subsequently, protein concentration of sera was determined by the BCA method (Pierce Chemical Co., Rockford, USA). Characterization of the different avian sera was performed by 1D and 2D electrophoresis following the protocol described above.

2.7. Immunochromatographic strip test

The capture antibody Pol K (960 ng/strip) was applied to direct cast backed nitrocellulose nitrate membranes (Unisart 1UN95E CN95, Sartorius Stedim Biotech, Goettingen, Germany) in a line format at 3 mm from the application end of the strip (Test line). At 6 mm, a goat antirabbit IgG antibody (Southern Biotechnology Associates, Inc., Birmingham, USA) was bound at 600 ng/strip to provide a positive control (Control line). Membranes were dried for 1 h at 37 °C and assembled as follows: on an adhesive support, an absorbent pad was layered and the membrane was placed overlapping it by 2 mm and cut into individual strips of 4 mm in width.

After optimization, 20 μl of environmental samples or pigeon serum standards were mixed 1:1 in a microtiter plate well with gold-labeled anti-pigeon Pol J antibody solution with an OD of 4. After forward pipetting, the gold-labeled Pol J antibody was allowed to react during 10 min at RT with pigeon antigens of the samples to form a complex. Then, a test strip was dipped in each well for 30 min at RT to allow antigen-antibody complexes to diffuse across the nitrocellulose membrane. These complexes reacted with the specific anti-pigeon Pol K antibodies immobilized on the test line forming a pink-purple line. The excess of colloidal dye antibody conjugate migrated further and reacted with the goat anti-rabbit IgG antibody in the control line.

Strips were scanned with a scanner (HP Scanjet G4050, Hewlett-Packard Enterprise, California, USA) and test line intensities were analysed by densitometry with ImageJ 1.52a (National Institutes of Health, USA). To determine the limit of detection of the strip assay, pigeon serum (Rockland Immunochemicals Inc., Limerick, Ireland) with a protein concentration of 27.5 mg/ml, previously determined by the BCA method, was used in a series of concentrations from 52.5 to 6720 ng/ml and tested in triplicate. In parallel, test lines of environmental samples were also read with the naked eye by five independent observers who classified the strips as negative (52.5 ng/ml), positive (420 ng/ml) and double positive (3360 ng/ml) comparing their color intensity with a 3-point standard curve (Fig. 1).

2.8. Statistical analysis

The Shapiro-Wilk normality test was calculated for pigeon aeroallergen levels measured by the ELISA method and for relative density determined from immunochromatographic strips. Spearman's rank correlation coefficient (rs) was calculated between pigeon aeroallergen levels and strip density values since all data showed a non-normal distribution. Bland-Altman analysis was performed to determine the agreement between the two assays. After logit transformation of the data, the difference between the paired measurements was plotted against the mean of the measurements. The immunochromatographic strip results were visually analysed by five independent evaluators, and Fleiss' Kappa test was used to calculate the degree of agreement between the different raters.

Statistical analyses were performed using GraphPad Prism 6 for Windows (version 6.01, GraphPad Software Inc., San Diego, California, USA) and IBM SPSS Statistics (version 26, IBM Corporation, Armonk, New York, USA). Differences with a p-value <0.05 (two-tailed) were considered to be significant.

3. Results

3.1. Characterization of pigeon serum and immunoblotting

Fig. 2A shows the different protein bands separated by 1D electrophoresis from pigeon serum (Fig. 2A, lane 2) and one of the air sample

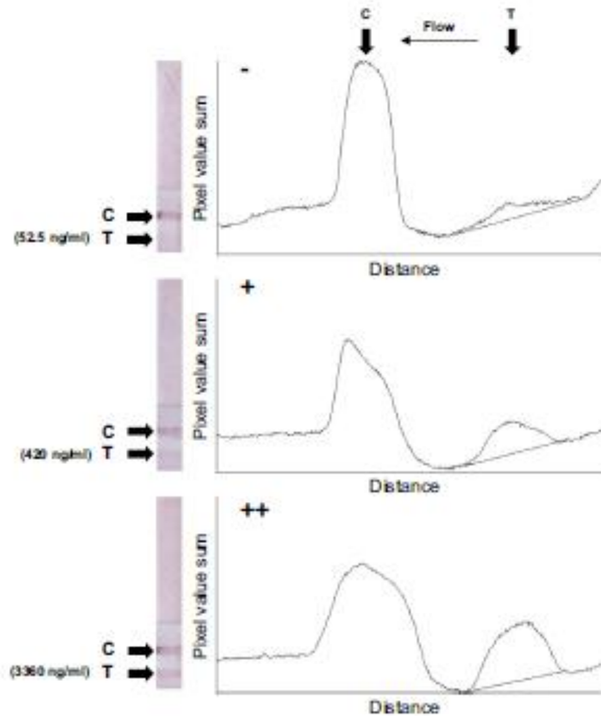

Fig. 1. Three-point standard curve of pigeon serum proteins to evaluate the test line of the environmental strips as negative (52.5 ng/ml), positive (420 ng/ml) or double positive (3360 ng/ml). The figure shows the scanning of each standard curve strip and their densitometry analyses. C, control line; T, test line.

(Fig. 2A, lane 3) collected from the dovecotes. Different bands were observed with molecular weights ranging from 10 to 250 KDa. Some bands were found in common between the serum and the air sample, mostly between 25 and 65 kDa.

Fig. 2B (lane 2, 3 and 4) shows different spots of proteins from pigeon serum separated by molecular weight and isoelectric point in two dimensions. The observed proteins were mostly in the range of 20 to 25 KDa and 50 to 75 KDa.

Immunoblotting in one and two dimensions showed that the polyclonal antibodies Pol K and Pol J were able to detect a wide range of proteins from pigeon serum (Fig. 2A, lane 4 and 5, and 2 B, lane 3 and 4) and from the air sample (Fig. 2A, line 6 and 7). Serum from a patient diagnosed with BRHP due to pigeon exposure showed a similar pattern of recognition to those of the polyclonal antibodies Pol K and Pol J (Fig. 2A, line 8 and 9).

3.2. Characterization of the ELISA method

The sandwich ELISA against pigeon proteins was able to detect a range of concentrations between 6.7 and 27,500 ng/ml in the standard curve. However, the lower and upper LOQ determined the working range of concentrations between 58.4 and 10,112.2 ng/ml. The standard curve was formed by seven different concentrations of pigeon serum proteins that followed a sigmoidal shape (Fig. 3A. The inter-CV using two different points of the standard curve was 2.76% and 7.35% respectively. In the negative controls the antigen concentration was undetectable. As regards the specificity of the method, the sandwich ELISA showed some cross-reactivity with small parrot, parakeet and parrot. Fifty percent of the OD of the highest point on the pigeon standard curve was reached at protein concentrations of 54,344, 74,806 and 172,175 ng/ml for small parrot, parakeet and parrot respectively (Fig. 3A). One and two-dimensional electrophoresis showed that these birds had some protein bands/spots of similar size and/or charge with pigeon that might explain this cross-reactivity (Fig. 3B).

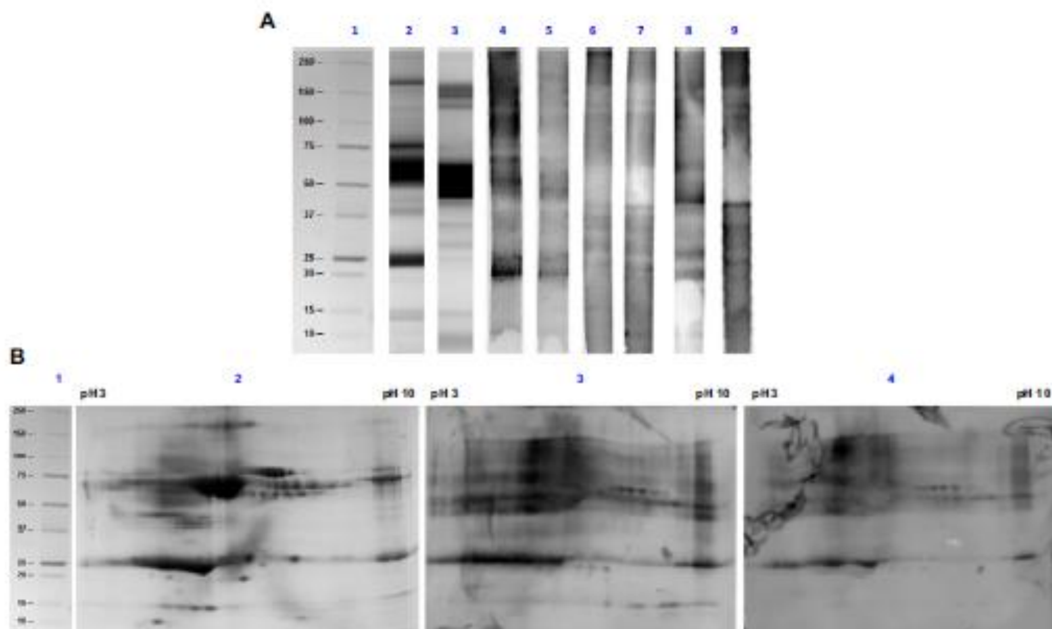

Fig. 2. Protein composition of pigeon serum and an air sample eluate collected from the dovecote by one and two dimensional electrophoresis and Western blots with antibodies Pol K and Pol J. 2A) Lane 1, a molecular weight size marker in KDa; lane 2, Coomassie-stained 1D gel of pigeon serum; lane 3, Coomassie-stained 1D gel of an 8 h air sample; lane 4 and 5, immunoblots with Pol K and Pol J against pigeon serum, respectively; lane 6 and 7, 8 h air sample immunoblot with Pol K and Pol J, respectively; lane 8 and 9, pigeon immunoblot and air sample immunoblot, respectively, with serum of a patient with BRHP due to pigeon exposure. 2B) Lane 1, molecular weight marker in KDa; lane 2, Coomassie-stained 2D gel of pigeon serum; lane 3 and 4, Pol K and Pol J immunoblots against pigeon serum, respectively.

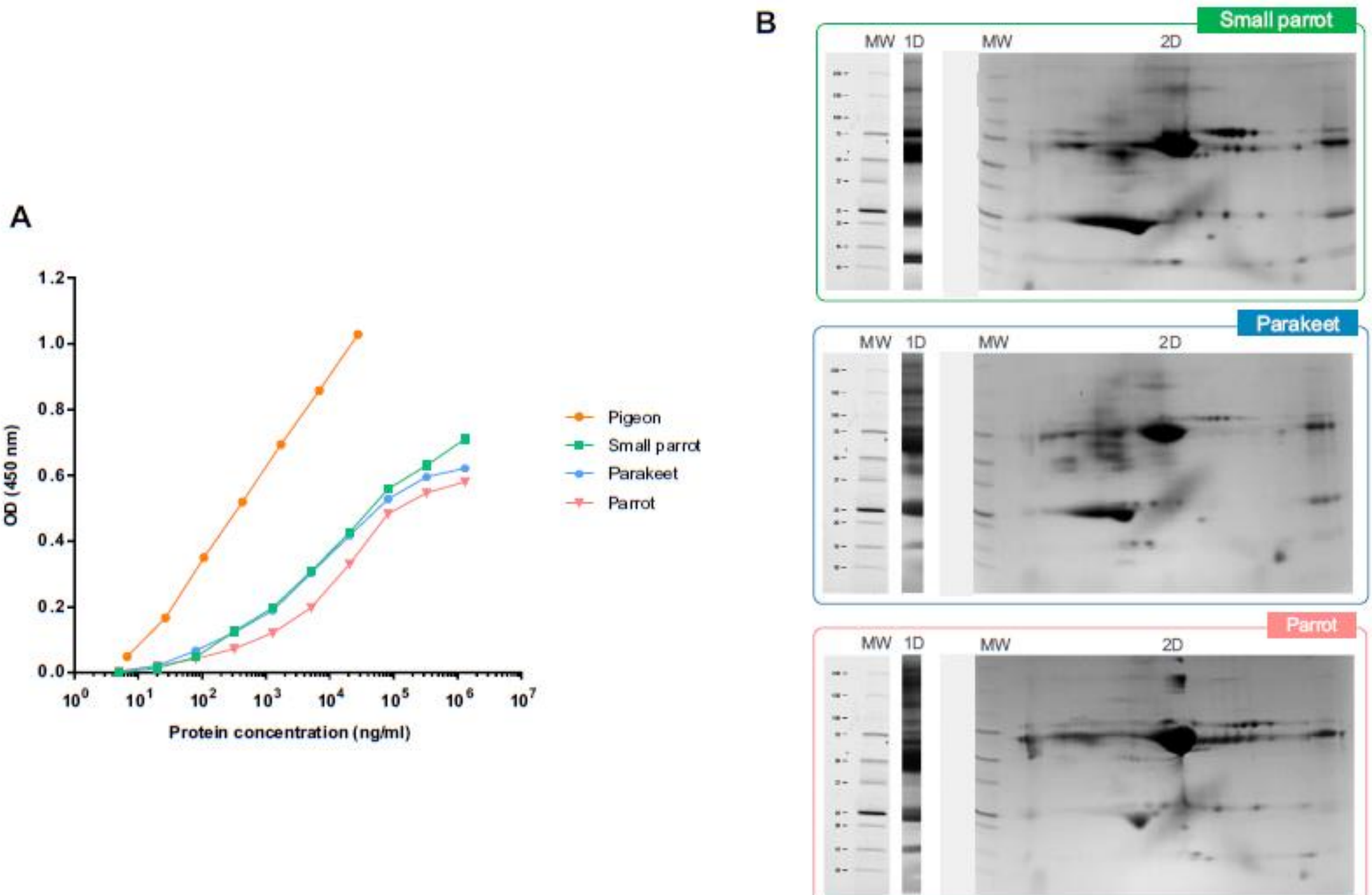

Fig. 3. Protein composition of bird sera (small parrot, parakeet and parrot serum) by one and two-dimensional electrophoresis (3A) and cross-reactivity of these sera with a sandwich ELISA for pigeon antigen detection (3B).

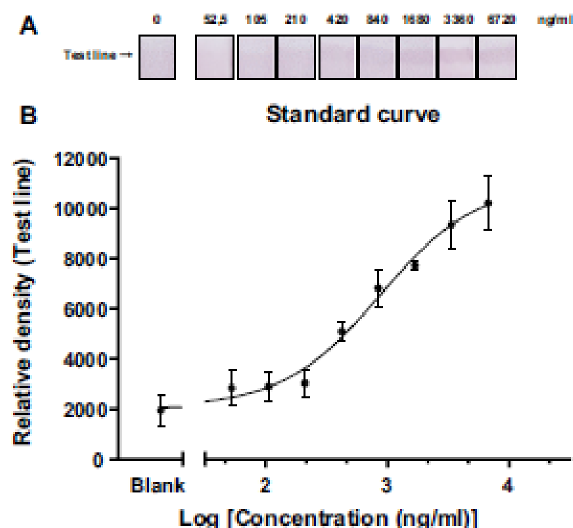

Fig. 4. Immunochromatographic strip assay standard curve to determine the lower and upper limits of detection. 4A) Scanning of the test line of each standard curve strip. 4B) Graph of relative density results (mean ± SD) at each concentration of pigeon serum proteins using a four parameter logistic curve.

3.3. Characterization of the immunochromatographic strip assay

The rapid test was able to detect a range of concentrations from 420 to 3360 ng/ml (Fig. 4). The lower LOD (420 ng/ml) was considered as the minimum amount of pigeon serum proteins from the standard curve giving a well-defined pink-purple band at the test line (Fig. 4A). The upper LOD (3360 ng/ml) was considered as the maximum amount of pigeon serum proteins producing a more intense pink-purple band at the test line compared to the previous concentration (Fig. 4A). In the negative controls the antigen concentration was undetectable.

3.4. Inter-reader agreement of immunochromatographic strip assay results

According to the observers' interpretations of the strips as negative, positive or double positive, a kappa index of 0.574 (CI 0.479–0.668; $p < 0.0001$) was obtained. However, when considering positive and double positive results as a single category the kappa index increased (0.736, CI 0.605–0.867, $p < 0.0001$). The observers were able to detect visually pigeon allergens in a concentration range of 141 to 3360 ng/ml in air sample eluates.

3.5. Comparison between ELISA and immunochromatographic strip assay

A strong correlation was observed between the concentration determined by the ELISA method and the relative density results of the strip assay with a r_s coefficient of 0.935 (CI 0.843–0.974; $p < 0.0001$) (Fig. 5A). The Bland-Altman plot also confirmed a good agreement between the methods with a mean difference of −1.626 (CI -1.988 to −1.264; $p < 0.0001$) (Fig. 5B).

4. Discussion

We developed and validated two sensitive methods to detect pigeon antigens in environmental air samples: an ELISA and an immunochromatographic strip assay. Although other studies describing the quantification of avian antigens have been reported (Craig et al., 1992; Tsutsui et al., 2015; Kuramochi et al., 2010), to our knowledge, this is the first in which an ICT is presented and compared with an ELISA method for the measurement of pigeon antigens in the environment. The study demonstrates that the methods have enough sensitivity, specificity, reproducibility, and applicability for the detection of environmental exposure to pigeon antigens.

Chronic HP is difficult to identify and is often misdiagnosed as idiopathic pulmonary fibrosis (Raghu et al., 2020; Morell et al., 2013). Therefore, demonstrating antigen exposure and evaluating the effects of allergen avoidance measures are critical in the diagnosis and management of this disease. Tsutsui et al. reported that the concentration of avian antigen present in household dust correlated with the prognosis of chronic bird-related HP (Tsutsui et al., 2015). Chronic HP has been assumed to progress as a result of persistent exposure to very small amounts of antigen. Moreover, avian antigens were reported to persist in the patient's house six months after removal of all birds (Morell et al., 2008; Craig et al., 1992). Therefore, it is important to examine the environmental levels of antigen after diagnosis and during antigen avoidance in order to facilitate the control of this disease.

Environmental monitoring of airborne antigens is a time-consuming process that commonly comprises three steps: sampling aeroallergens on a filter, elution of the allergens, and determination of allergen levels by an allergen-specific ELISA (Gómez-Ollés et al., 2006). Here, we first obtained polyclonal antibodies with high specific reactivity to pigeon serum antigens and developed a validated ELISA method using those antibodies. Second, we developed and validated a novel ICT using the same antibodies used in the ELISA method. Previous studies have proposed that ICT may be a valid alternative to the ELISA, as it speeds up the last step of the process (Bogdanovic et al., 2006; Tsay et al., 2002; Álvarez-Simon et al., 2014).

The antigenic components associated with the development of BRHP was described using biochemical analyses and immunoassays. The main antigenic substances suspected were immunoglobulins, serum albumin,

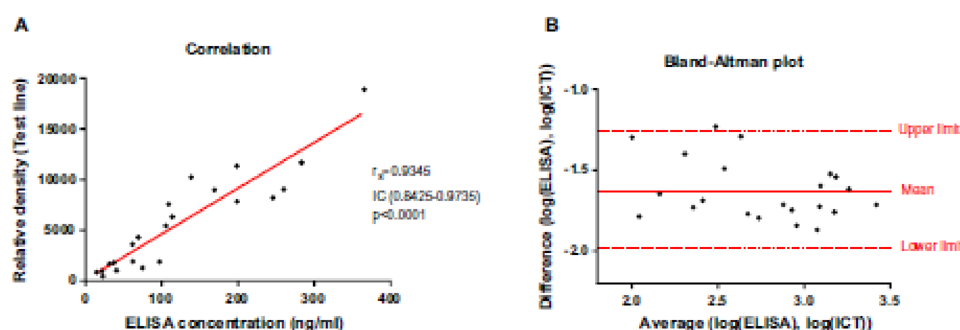

Fig. 5. Comparison of pigeon aeroallergen concentration determined by ELISA method and density values calculated with the immunochromatographic strip assay. 5A) Spearman's rank correlation. 5B) Bland-Altman plot.

and intestinal mucin, from droppings, blooms and pigeon sera (Shirai et al., 2017; de Beer et al., 1990; Rouzet et al., 2017). Owing to the difficulty of obtaining antigenic extracts from intestinal mucin or bloom, pigeon serum is the antigen source most often used to measure specific antibodies in this type of HP (Rodrigo et al., 2000). The immunoblotting results of the present study demonstrated that the polyclonal antibodies used in the methods developed were able to detect the relevant pigeon antigens and that most of the antigenic proteins identified were also abundant in droppings and blooms (Rouzet et al., 2017). Rouzet et al. (2017) found proteins with a molecular weight range between 20 and 200 kD in bird dropping extracts, and that IGLL-1 (Immunoglobulin lambda-like polypeptide-1, 24.5 KDa) and ProE (Proproteinase E, 20.5 KDa) were the antigenic proteins with the most clinical interest as biomarkers for BRHP diagnosis. In this regard, Shirai et al. (2017) in an immunoblot analysis of sera from patients with BRHP, identified multiple immunoreactive bands in pigeon serum at various molecular weights, especially IGLL-1, similar to those obtained in the present study.

Moreover, the cross-reactivity study with other birds demonstrated elevated specificity (Fig. 3). Sera of small parrot, parakeet and parrot seem to cross-react slightly with pigeon serum, probably because they have common sera proteins (as observed in the 2D electrophoresis). Another possible reason was suggested by Rouzet et al. (2014). in a study designed to determine the specificity of immunogenic proteins of the droppings of three bird species; those authors reported that although no proteins were found common to all extracts, cross-reactions to avian antigens from the three species of bird were observed.

The sandwich ELISA using these polyclonal antibodies has a high sensitivity and specificity and has demonstrated its value for measuring pigeon antigens, even at relatively low concentrations. In this study the levels of pigeon antigens were assessed with respect to a reference preparation (pigeon antigen standard) containing 6.7–27,500 ng of protein/ml. This standard proved to be a suitable reference preparation for the measurement of pigeon exposure, as shown by the results of the SDS-PAGE gel (Fig. 2) and the comparison of the proteins of this standard with the air sample eluates collected from the dovecote. These assessments demonstrated that this standard is composed of the proteins contained in the air.

Although the ELISA is a very useful technique for detecting soluble antigens and meets most of the main requirements for analysing pigeon antigens (i.e., reproducibility, good specificity and acceptable sensitivity), it is a time-consuming process that requires skilled personnel and expensive laboratory equipment. Therefore, cost-efficient, less labor-intensive technological procedures that can be performed by non-specialist laboratory staff are needed in order to monitor pigeon antigen levels. ICT can provide fast qualitative or even semi-quantitative results within minutes with very good analytical sensitivity. Furthermore, the characteristics of their design and construction make them especially suitable for field testing and for use by non-laboratory-skilled personnel (Bogdanovic et al., 2006; Tsay et al., 2002). Fast antigen monitoring systems has been shown to be highly beneficial to diminishing exposure (Tsutsui et al., 2015; Kuramochi et al., 2010), therefore, rapid immunoassay for the assessment of pigeon antigens would be a very useful tool.

The visual interpretation of the ICT showed high levels of sensitivity and specificity, comparable to those recorded with other rapid assays measuring other aeroallergens (Bogdanovic et al., 2006; Tsay et al., 2002; Álvarez-Simon et al., 2014; Koets et al., 2011). Furthermore, the line intensity (analysed by densitometry, an objective measurement) of samples categorized as negative was significantly lower than that of positive and double positive samples. The LOD, determined as the minimum amount of allergen capable of producing a clearly visible signal in the test line of the standard in the rapid immunoassay, was 420 ng/ml. Unexpectedly, in the visual analysis of our test results, the independent observers detected pigeon antigen concentrations below the theoretical LOD determined for the assay; depending on the rater, the actual LOD of the assay with real field samples ranged from 141 to 416 ng/ml. This observation has also been reported in other studies: for example, Tsay et al. (2002) graded the line intensity of some samples analysed as medium or high even although the allergen levels analysed by ELISA were below the theoretical LOD of the rapid assay. Similarly, in the studies by Bogdanovic et al. (2006) and Álvarez-Simon et al. (2014), some samples with allergen levels below the sensitivity of the rapid test were classified as positive. Moreover, visual interpretation of rapid assays is inherently subjective, and depends on factors such as the rater's color perception. Therefore, it is important to assess inter-rater agreement. In this study, the agreement was graded as substantial, according to the classification recommended by Landis and Koch (1977).

The main advantage of this ICT is the speed of the analysis, and the fact that it can be performed in a non-laboratory environments. Although speed may not be decisive in environmental monitoring, it may constitute a real advantage for industrial hygiene monitoring, as some of the sampling procedures widely used in occupational hygiene take only a short time (for example, surface wipe sampling, dust sampling or bulk sampling). In this context, some studies have assessed the environmental measurement of avian antigen in dust (Craig et al., 1992; Kuramochi et al., 2010; Sema et al., 2018; Curtis et al., 2002). For example, Craig et al. (1992) reported that avian antigen could be detected in dust samples for prolonged periods of time after bird removal and environmental clean-up. Curtis et al. (2002) also reported that pigeon antigen was found in dust and air samples from a pigeon-infected school.

One limitation of these methods is the problem of discriminating between safe and unsafe environments. In this sense, data on environmental levels of pigeon antigen to which patients with bird-related hypersensitivity pneumonitis are exposed were lacking in the present study. It may be difficult to establish thresholds for allergen exposures, due to inter-individual variations in susceptibility to both sensitization and disease elicitation. These differences may be caused by genetic variations, age-dependent effects, or lifestyle factors (Maciag and Phipatanakul, 2020). Smoking, for example, may promote sensitization due to an adjuvant effect (Carlos et al., 2009). Moreover, different proteins have different sensitization potencies (Krutz et al., 2020). In any case, avoiding exposure to causal agents is crucial in HP management and is a decisive factor in prognosis, because progression can broadly be prevented with appropriate antigen avoidance. Moreover, identification of the offending antigen source at work is important for the determination of antigen-specific IgG antibodies.

In conclusion, we developed an ICT to monitor pigeon aeroallergen levels. The strip assay described was rapid, simple, sensitive, highly consistent with the validated ELISA and does not require expensive equipment or specific skills. This method for monitoring levels of pigeon antigen may prove to be a useful and versatile tool for predicting the progression of bird-related hypersensitivity pneumonitis.

Funding

SSD is researcher supported by CIBER (Instituto de Salud Carlos III, Spain), and MJC is a researcher supported by the Miguel Servet programme of the Instituto de Salud Carlos III (MSII17/00025). This project was supported by the Fundació Catalana de Pneumologia (FUCAP), Sociedad Española de Patología Respiratoria (SEPAR), FIS PI15/01954 (Instituto de Salud Carlos III), Fondo Europeo de Desarrollo Regional (FEDER) and European Union's Horizon 2020 research and innovation programme under grant agreement No 874707. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

CRediT authorship contribution statement

Silvia Sánchez-Díez: Conceptualization, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **María-Jesús Cruz:** Conceptualization, Funding acquisition, Project administration, Writing – original draft, Writing – review & editing. **Daniel Álvarez-**

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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5. OVERALL SUMMARY OF RESULTS

OVERALL SUMMARY OF RESULTS

This thesis comprised four different studies. Briefly, the first study contended that in the first stages of BRHP there was a mixed Th1/Th2 immune response with increased levels of CD11b+Ly6C- DCs, an eosinophilic inflammation due to IL-5-producing cells, reduced levels of B cells because of their differentiation into antibody-producing plasma cells, and secretion of type 1 and type 2 cytokines such as TNF-α and IL-13. With progression of BRHP, although there was a Th1 response with IFN-γ, IL-1β and IL-12 secretion, the cytokine levels seemed to indicate a switch towards a mixed Th2/Th17 response with increased levels of IL-5, IL-6 and IL-23.

Table 1. Leukocyte population and cytokine pattern in lung tissue in the first stages and with progression of BRHP.

	First stages of BRHP	Progression of BRHP
Macrophages		
Alveolar	↓	↓
Interstitial	↑	↑
Monocytes		
Inflammatory	↓	↓
Resident	↑	↑
Dendritic cells		
CD11b+Ly6C-	↑	↑
CD11b-Ly6C-	↓	↓
Other cells		
Eosinophils	↑	NS
Neutrophils	↓	NS
B cells	↓	↑
T cells	↑	NS
Th1 cytokines		
IFN-γ	↑	↑
TNF-α	↑	NS
IL-1β	↑	↑
IL-12 (p70	↑	↑
Th2 cytokines		
IL-5	↑	↑
IL-6	↑	↑
IL-10	↑	↑
IL-13	↑	NS
Th17 cytokines		
IL-17	NS	NS
IL-23	NS	↑

↑, increased; ↓, decreased; NS, no statistically significant differences.

In the second study (Article 1), both serum KL-6 and YKL-40 levels seemed to be capable of distinguishing HP patients either from healthy individuals or from patients with IPF at ranges of 346 - 1441 U/ml and 55 - 121 ng/ml respectively. Moreover, serum KL-6 may also be a predictor of HP disease progression because of its negative correlation with TLC and DLCO at 12 months of follow-up.

In the third study, a high degree of sensitization to avian and fungal antigens was observed in a cohort of urban pest surveillance and control service workers. In particular, workers involved in nest pruning had high levels of specific parakeet and mucor IgGs and low DLCO/VA values. In addition, Ig Lambda chain and Apolipoprotein A-I were identified as candidate proteins for distinguishing patients with HP from workers exposed to pigeons.

Finally, the last study (Article 2) demonstrated a good correlation between the ELISA and the ICT developed, although the ELISA method had a broader range for quantifying pigeon antigen (58.4 - 10112.2 ng/ml and 420 - 3360 ng/ml respectively). Even so, this study showed that ICT was rapid, simple to use, and a valid alternative to ELISA that did not require expensive equipment.

6. OVERALL SUMMARY OF DISCUSSION

OVERALL SUMMARY OF DISCUSSION

The present thesis aims to provide insights into different aspects of HP. First of all, it assesses the pathophysiology of the disease by analysing the innate and adaptive immune responses in a murine model. In addition, it attempts to improve the diagnostic accuracy and prognosis of patients with HP. In this regard, the usefulness of two proteins (YKL-40 and KL-6) is determined through the analysis of biological samples of patients with HP or with other ILDs (e.g., IPF and sarcoidosis) and healthy controls. Moreover, two different ways of measuring antigen exposure are proposed: the determination of specific IgG antibodies in serum samples, and the monitoring of pigeon antigens in environmental samples. In particular, specific IgG of workers with high exposure to birds and fungi and also of HP patients are analysed in order to identify antigenic proteins with diagnostic value, and a rapid estimation method is developed to detect environmental pigeon allergen and avoid HP progression by controlling antigen exposure. Regarding the pathophysiology of the disease, this thesis contends that in the first stages of BRHP there is a mixed Th1/Th2 immune response with increased levels of cDC2, eosinophilic inflammation, and secretion of Th1 and Th2 cytokines such as TNF-α and IL-13. With progression of BRHP, although there is a Th1 response with IFN-γ, IL-1β and IL-12 secretion, the levels of cytokines seem to indicate a switch towards a mixed Th2/Th17 response with increased levels of IL-5, IL-6 and IL-23. With regard to the usefulness of YKL-40 and KL-6 in HP diagnosis and prognosis, serum levels of both proteins seem to be capable of distinguishing HP patients from healthy individuals and from patients with IPF. In addition, KL-6 may also have prognostic value because of its negative correlation with TLC and DLCO at 12 months of follow-up. The present thesis also identifies Lambda chain and Apolipoprotein A-I as candidate proteins in HP diagnosis for distinguishing patients from individuals exposed to pigeons. In addition, it demonstrates the existence of a high degree of sensitization to avian and fungal antigens in workers with substantial exposure to these antigens. In particular, workers involved in nest pruning have higher levels of specific parakeet and mucor IgGs and lower DLCO/VA values. Finally, the ICT developed seems to be a rapid, simple-to-use assay that is a valid alternative to the ELISA technique for pigeon allergen measurement in air samples, although ELISA has a broader range for quantifying pigeon antigen (58.4 - 10112.2 ng/ml and 420 - 3360 ng/ml respectively).

The immunopathological pathways involved in HP after antigen exposure are still unknown, although there is some evidence of contributions of both humoral and cellular immune responses. Antigen presentation by innate immune cells to B lymphocytes

induces the production of Igs (3,82). The decreased levels of B cells observed in our murine model in the onset of BRHP support the idea that activated B lymphocytes are being differentiated into plasma cells that secrete antibodies against the inhaled antigen. In fact, Igs have been reported to be involved in early phases of the disease, forming antigen-antibody complexes that can activate the classical pathway of the complement and promoting the recruitment of more macrophages and contributing to tissue injury (2). Activated lung macrophages secrete IL-1 and TNF but also upregulate the expression of adhesion molecules, leading to neutrophil recruitment in the alveolar spaces. These granulocytes are also recruited to the site of inflammation by the interaction of their receptors with the constant region of Igs (21). Indeed, in our murine model both groups, short and long-term exposure to pigeon serum, exhibited increased neutrophils counts in BAL samples.

According to the literature, in HP antigen-presenting cells also interact with T cells, leading to the secretion of different cytokines such as IL-12, IFN-γ and TNFα and stimulating the polarization of lymphocytes to Th1 cells (83). In fact, we found significantly increased levels of these types of cytokines in both groups of the disease compared with the control groups, although the secretion of these Th1 cytokines was more evident in the first stages of the disease. Regarding IFNγ and TNFα secretion, these two cytokines have been reported to induce the accumulation, activation and aggregation of macrophages, resulting in granuloma formation (14,15). In fact, in our murine model of 3 weeks of exposure, increased levels of TNFα were detected and the histopathological analysis also confirmed the presence of giant cells in lung tissue. The results of the murine model provided further evidence that pigeon serum exposure reduces alveolar macrophages and inflammatory monocytes while increasing resident monocytes and interstitial macrophages in the onset and the progression of the disease. These findings suggest that alveolar macrophages undergo subsequent apoptosis and that inflammatory monocytes are recruited to lung tissue from the vasculature to differentiate into interstitial macrophages. Subsequently, interstitial macrophages proliferate and migrate into the alveolar space to restore this depleted population (84,85).

At the later stages of HP, a relative switch of Th1 cells to Th2 has been reported to promote the maintenance of inflammation, with IL-13 being one of the effector cytokines (14,22). In the first stages of the disease our results demonstrated significant increases in IL-13 and IL-5, two cytokines involved in eosinophil activation, proliferation, and survival (86–88). In fact, the main difference detected between both

models of the disease was that eosinophil recruitment was much more evident in the onset of HP. The increased eosinophilia in early stages of the disease may be related to the short time elapsed (24 h) between the last antigen exposure and the lung sample acquisition due to the activation of IL-5-producing CD4+ T cells (89,90). In fact, a previous study by our group carried out in patients with HP also demonstrated an eosinophilic inflammation pattern in induced sputum samples after SIC to avian antigens (91).

Certain studies have demonstrated the presence in this disease of Treg cells with an impaired function, which also play a role in the exaggerated immune response (18,19). In our study, the effect of pigeon serum over CD11b-Ly6C- DCs was also evident, with reduced levels in both groups of the disease. These type 1 classical DCs induce tolerance to inhaled antigens, phagocytize apoptotic epithelial cells and cross-present them to CD8+ T cells in the lung-draining bronchial lymph node (92,93). Therefore, our findings suggested that pigeon serum induces lung epithelial damage and decreases airway tolerance, leading to an exaggerated immune response. Another subset of DCs that seemed to play an important role in our murine model were CD11b+Ly6C- DCs. These cDC2 are specialized in MHC class II-mediated antigen presentation and have previously been reported to have the ability to trigger Th2 cell-mediated immune response to inhaled allergens (94). However, cDC2 can also promote Th1 and Th17 cell responses in specific contexts via the secretion of pro-inflammatory cytokines such as IL-12 and IL-23 (95,96). In the present study, an increase in IL-12 was observed in both groups of HP while IL-23 levels rose only with HP progression. These results supported the hypothesis that with progression of the disease a Th17 immune response is activated by CD11b+Ly6C- DCs, while in the onset of HP these cells promote a Th1 response with the released cytokines. The secretion of IL-17 by lymphocytes, macrophages, and neutrophils has also been reported to induce a Th17 immune response in HP, intensifying the inflammatory response and contributing to chronic inflammation and lung fibrosis (16,17,97). In our study, no significant differences were observed in IL-17 levels between the groups. However, with progression of the disease we found increased levels of IL-23, which is a critical cytokine in the development of chronic inflammation by the activation of IL-17-producing T cells and neutrophil recruitment (98–100).

Decreasing apoptosis of lymphocytes in the final stage of the disease has also been reported to contribute to T cell persistence, activation, and accumulation in lung tissue (24). In this context, the lymphocyte count observed in the murine model was higher in

BAL of long-term exposed mice, and increased numbers of lymphocytes have already been reported to be a typical finding in BAL samples of patients with HP, especially in the chronic form (40,49,101).

Despite the evidence of the involvement of different immune cells in the development and progression of HP, the current knowledge is insufficient to apply these new insights in the diagnostic procedure. Data from European countries indicate that HP represents 4-15% of all interstitial diseases (102) with an incidence rate of 0.9 cases per 100,000 persons/year (29). However, recent studies suggest that HP cases may be underdiagnosed and may represent up to 43% of ILDs classified as IPF. These diagnostic errors occur because HP and IPF present similar clinical, radiological and pathological features (33). Indeed, the absence of a gold standard technique means that the diagnosis of HP remains challenging. The assessment of biomarkers in the diagnosis and prognosis of HP could be particularly useful in routine clinical practice given the difficulty of establishing a well-defined HP diagnosis in early stages of the disease and of differentiating this condition from other types of ILDs. In this connection, YKL-40 and KL-6 are two promising biomarkers that may have an important role in the management of HP diagnosis and progression.

KL-6 is a human mucin, primarily expressed in regenerating type II pneumocytes and the bronchiolar epithelium, with chemotactic and anti-apoptotic effects on fibroblasts. Therefore, KL-6 is a glycoprotein involved in fibroblast proliferation and pulmonary fibrosis progression (54,55). For its part, YKL-40 is a human chitinase-like protein mainly secreted by macrophages, neutrophils and epithelial cells but also by fibroblasts and other cells (59,60). This chitinase-like protein is involved in various biological functions, including the regulation of cell proliferation, adhesion, migration and activation, but there is also evidence of its involvement in allergen sensitization, IgE induction, Th2 cytokine production, DC and macrophage activation, inhibition of apoptosis in inflammatory cells and ECM modulation during fibrogenesis (59,60).

Previous reports have shown that KL-6 is able to discriminate between different ILDs. In this regard, Bergantini et al (103) demonstrated that patients with sarcoidosis, IPF and HP present significant differences in serum KL-6 levels, with the highest levels being observed in patients with IPF and the lowest in those with sarcoidosis. By contrast, in a retrospective study of 92 patients with HP, Okamoto et al (56) found serum KL-6 levels to be higher in chronic HP than in IPF. Our findings highlighted that a level of KL-6 > 346 U/ml can discriminate between HP patients and healthy controls, while levels > 1441 U/ml can differentiate HP from IPF. In addition, our results

demonstrated that serum KL-6 levels are higher in patients with HP who present a fibrotic pattern. In fact, KL-6 has already been proposed as a potential biomarker of fibrotic lung involvement (104) and high serum levels of KL-6 are indicative of lung remodeling and active disease (105). In this regard, Ji et al (106) demonstrated that KL-6 is associated with acute symptoms of early-stage HP in pigeon fanciers.

Nevertheless, in the literature, KL-6 has been more frequently associated with the evolution and prognosis of fibrotic lung disease than with diagnosis (107–109). Hanzawa et al (109) demonstrated that changes in serum KL-6 level during short-term strict antigen avoidance could predict prognosis in patients with fibrotic BRHP. In another study, Satoh et al (107) reported that disease progression was significantly faster in patients with ILDs whose KL-6 levels were 1,000 U/ml or above at the initial measurement. Moreover, in that study HP patients with clinical improvement exhibited reduced KL-6 levels during follow-up compared with baseline values. Our results highlighted that KL-6 levels in HP patients correlated negatively with TLC and DLCO values after 12 months of follow-up. Early identification of patients at high risk of suffering a deterioration in lung function or of developing pulmonary fibrosis could allow clinicians to consider additional pharmacological therapies.

Regarding YKL-40, only one previous study has described YKL-40 levels in serum and BAL samples of patients with HP (62), and our study is the first to analyse this biomarker in sputum samples. In the study just mentioned, Long et al (62) assessed 72 patients with HP, reporting similar results to ours regarding the sensitivity and specificity of YKL-40 for the diagnosis of the disease. However, they observed that HP patients with disease progression or who died presented higher baseline YKL-40 levels than those who remained stable and survived. In our study, no relationship was found between this biomarker and disease progression; however, significant differences were observed in serum YKL-40 levels between patients with a fibrotic pattern who were exposed to birds and healthy controls. Although we did not have a healthy control group for comparison of the results obtained in sputum, we observed that patients with HP exposed to fungi presented lower levels of YKL-40 than those that did not have a fibrotic pattern. In this regard, a previous study by our group prompted the hypothesis that different antigens may cause the development of HP via different pathophysiological mechanisms, after analysing the cellular inflammation in induced sputum of patients with HP (91).

In patients with HP disease the most frequent finding in lung function tests is a restrictive ventilatory pattern or an alteration in gas exchange, consisting of a decrease

in DLCO values (6). We found that DLCO/VA values may also play a role as a biomarker in the progression of HP disease. In particular, workers involved in nest pruning had low DLCO/VA values; several studies have highlighted that a reduction in this lung parameter may reflect qualitative impairment of the alveolar-capillary membrane and may be associated with different abnormal conditions, diffuse alveolar-capillary disease, reduced alveolar surface or capillary density or diminished hemoglobin levels (118,119). In addition, low levels of DLCO/VA values may predict DLCO reduction over time and also disease progression (118). Although no cases of HP were diagnosed in the individuals in the nest pruning group, these lowered levels of DLCO/VA may indicate a greater predisposition to the development of HP disease.

Although biomarker determination in biological samples could be of interest for HP management, the essential step in establishing a correct HP diagnosis is to prove an existing exposure but also to identify a causative antigen. This allows complete avoidance of the inciting antigen in order to improve respiratory symptoms and to prevent HP progression. In this context, specific IgG antibody determinations and environmental antigen monitoring are useful techniques.

Epidemiological studies have already documented a relationship between exposure to organic antigens and the level of serum-specific IgG antibodies against these antigens (110) and/or the prevalence of HP (111–113). In fact, increased serum levels of specific IgG antibodies confirm the existence of sensitization to a specific antigen after a previous exposure. In our study, workers engaged in nest pruning exhibited high levels of specific IgG to avian and fungal antigens and, given their job description, probably had the highest exposure to these antigens. In this connectoin, in a study carried out in 41 patients suffering from HP, Miyagawa et al (114) concluded that sera from all patients had high titers of antibodies against a specific fungus. Rodrigo et al (63) also demonstrated that patients who developed pigeon breeder's disease synthesized high levels of IgG antibodies against pigeon proteins. However, IgG determination merely provides evidence of antigenic sensitization; exposed but asymptomatic individuals may also present high IgG levels against a specific antigen without having the disease (40,115,116). In fact, there are studies demonstrating that up to 50% of healthy individuals exposed to birds may be sensitized to avian antigens (63). In this regard, Erkinjuntti-Pekkanen et al (64) concluded that not only farmer's lung patients but also between 30-60% of control farmers developed specific antibodies against different microbes tested. In fact, individuals in our study with a work activity

other than nest pruning also exhibited increased levels of specific IgGs against birds and fungi.

The specific proteins that cause HP pathology are still unknown and could be of diagnostic value for discriminating between exposed but asymptomatic individuals and patients suffering from the disease. In fact, the main limitation of specific IgG measurements is the unavailability of validated antigen preparations for most agents causing HP. Recently, several groups have described antigenic substances that are found in bloom, serum, droppings and/or intestinal mucin of various birds that might be used as diagnostic markers of disease (70,71,117). For instance, Shirai et al (117) found that patients with BRHP presented high serum IgG antibodies against a protein contained in pigeon serum, IGLL-1, compared to control subjects. Rouzet et al (72) also identified IGLL-1 as a biomarker of disease and also ProE in pigeon bloom and droppings when comparing sera from patients with bird fancier's lung, asymptomatic exposed controls, and healthy volunteers. Our study also identified Ig Lambda chain and Apolipoprotein A-I as candidate proteins for distinguishing HP patients from workers exposed to pigeons.

Decreasing titers of specific IgG antibodies correlate with less exposure to a specific antigen, and complete avoidance of the causative antigen is crucial to prevent disease progression and improve respiratory symptoms in patients with HP. Tsutsui et al (78) reported that the concentration of avian antigen present in household dust correlated with the prognosis of chronic BRHP. Indeed, CR HP has been assumed to progress as a result of persistent exposure to very small amounts of antigen. In this context, avian antigens have been reported to persist in patients' houses six months after removal of all birds (40,77). Therefore, environmental measurements of the causative antigen may have an important role in controlling cessation of exposure after diagnosis and during antigen avoidance.

In the literature, several studies have described the quantification of avian antigens in air or house dust samples using a sandwich ELISA technique with antibodies against pigeon dropping extract (78,120). Our results demonstrated that the polyclonal antibodies used in the ELISA developed were able to detect serum antigenic proteins also abundant in droppings and blooms (72). In this regard, Rouzet et al (72) found proteins with a MW range between 20 and 200 kD in bird dropping extracts, IGLL-1 and ProE being the antigenic proteins with most clinical interest as biomarkers for BRHP diagnosis. In an immunoblot analysis of sera from patients with BRHP, Shirai et al (117) identified multiple immunoreactive bands in pigeon serum at various MWs,

especially IGLL-1, similar to those obtained in the present study. Our findings also highlighted slight cross-reactivity between pigeon serum and sera of small parrot, parakeet and parrot, probably because they have common serum proteins. Another possible reason was suggested by Rouzet et al (121) in a study designed to determine the specificity of immunogenic proteins in droppings of three bird species. Specifically, those authors observed cross-reactions to avian antigens from the three species of bird, although no proteins were found that were common to all extracts.

The sandwich ELISA developed had a high sensitivity and specificity and demonstrated its value for measuring pigeon antigens, even at relatively low concentrations, from 6.7 to 27,500 ng of protein/ml. However, it is a time-consuming technique that requires skilled personnel and expensive laboratory equipment. Therefore, cost-efficient, less labor-intensive technological procedures that can be performed by non-specialist laboratory staff are needed in order to monitor pigeon antigen levels. Previous studies have proposed ICT as a valid alternative to ELISA, as it can provide fast qualitative or even semi-quantitative results within minutes with very good analytical sensitivity. Furthermore, the characteristics of their design and construction make these tests especially suitable for field testing and for use by non-laboratory-skilled personnel (122,123). Therefore, rapid immunoassays for the monitoring of pigeon antigens in air would be highly beneficial tools for reducing exposure in HP patients.

The ICT developed was able to detect a range of concentrations from 420 to 3360 ng/ml of pigeon proteins, and the visual interpretation of the test lines showed high levels of sensitivity and specificity, comparable to those recorded with other rapid assays measuring other aeroallergens (123–126). Unexpectedly, in the visual analysis of our test results, the independent observers detected pigeon antigen concentrations below the theoretical LOD determined for the assay. In this context, Tsay et al (123) graded the line intensity of some samples analysed as medium or high, even though the allergen levels analysed by the ELISA were below the theoretical LOD of the rapid assay. Similarly, in the studies of Bogdanovic et al (124) and Álvarez-Simon et al (125), some samples with allergen levels below the sensitivity of the rapid test were classified as positive.

The main advantages of ICT are the fact that it can be performed in non-laboratory environments and also the speed of the analysis. In fact, for industrial hygiene monitoring it may constitute a real advantage because some of the sampling procedures widely used in occupational hygiene take only a short time (e.g., surface wipe sampling, dust sampling or bulk sampling). Certain studies have assessed the

environmental measurement of avian antigen in dust (77,84,120,127). For instance, Craig et al. (77) reported that avian antigens could be detected in dust samples for prolonged periods of time after bird removal and environmental clean-up, and Curtis et al. (127) found pigeon antigen in dust and air samples from a pigeon-infected school.

7. CONCLUSIONS

CONCLUSIONS

The main conclusions obtained are:

- In the first stages of BRHP there is a mixed Th1/Th2 immune response characterized by eosinophilic infiltration, Th2-related DC recruitment, and a Th1/Th2 cytokine pattern.
- With progression of the disease, although there is a Th1 response, the levels of cytokines seem to indicate a switch towards a mixed Th2/Th17 response with a notable recruitment of lymphocytes, macrophages and Th2-related DCs and the release of Th2 and Th17 cytokines.
- YKL-40 and KL-6 seem to discriminate between HP patients, healthy individuals and IPF patients with adequate sensitivity and specificity, thus confirming their potential role as biomarkers and their usefulness for improving diagnostic accuracy.
- Individuals exposed to birds and fungi exhibit a high degree of sensitization to these antigens; higher exposure is associated with higher levels of specific IgGs.
- Two pigeon proteins (Ig Lambda chain and Apolipoprotein A-I) may play a role in the development of pathological differences between HP patients and exposed individuals, and DLCO/VA values may have a predictive role in HP progression.
- The strip assay developed is rapid, simple to use, sensitive, highly consistent with the validated ELISA, and does not require expensive equipment or specific skills to monitor pigeon aeroallergens.

8. FUTURE LINES OF RESEARCH

FUTURE LINES OF RESEARCH

The studies that comprise this doctoral thesis highlight the complexity of the immunopathological mechanisms involved in both forms of HP disease. Even so, several candidate biomarkers and methods have been identified for improving diagnostic and/or prognostic accuracy.

In the first study a switch towards a mixed Th2/Th17 response was detected with progression of BRHP, with a notable recruitment of lymphocytes, macrophages and cDC2 and the release of Th2 and Th17 cytokines. However, further studies are necessary in animal models to broaden our knowledge of the immune pathways involved in lung fibrosis, and thus to promote the design of pharmacological products and possible treatments for HP disease.

With regard to the first article, YKL-40 and KL-6 seem to have the potential to improve diagnostic and/or prognostic accuracy in HP disease. Nevertheless, further studies with external cohorts and larger samples are needed in order to validate the reliability and clinical utility of these biomarkers in the disease severity assessment and prognosis in HP.

In the third study, Ig Lambda chain and Apolipoprotein A-I were identified as candidate proteins to distinguish HP patients from workers exposed to pigeons. In future studies these two proteins should be synthesized as recombinant antigens in vitro and assessed in an ELISA with serum of exposed individuals and patients with HP due to pigeon. In this way, the involvement of these two proteins in HP pathology and their diagnostic value could be confirmed. In this same chapter, DLCO/VA values were identified as candidate biomarkers for predicting HP progression. Further studies are necessary in order to confirm the predictive value of DLCO/VA as a biomarker in the progression and development of HP disease.

The second article describes the development of a rapid, simple-to-use and sensitive test for the detection of pigeon aeroallergen levels that does not require expensive equipment or specific skills. For these reasons, this technique has a significant potential for the direct monitoring of pigeon antigens in different environments (home, workplace or other frequented places) of patients with a suggestive anamnesis. Therefore, the commercialization of this type of test in the future should help clinicians to identify the causative antigen and establish a correct HP diagnosis.

Taken together, all these proposals should help to provide insights into the immunological mechanisms involved in the development of fibrotic HP and into possible treatments. They are also likely to improve diagnosis and prognosis, thus raising patients' quality of life and preventing disease progression.

9. BIBLIOGRAPHY

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