NON-INVASIVE METHODS TO STUDY LUNG INFLAMMATION IN WORK-RELATED ASTHMA

Métodos no invasivos de estudio de la inflamación bronquial en el asma relacionado con el trabajo

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para acceder al grado de Doctor en la
Facultat de Medicina de la
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Barcelona, 18 de abril de 2012
**List of abbreviations**

AHR: Airway hyper-reactivity
APC: Antigen presenting cell
ACQ: Asthma control questionnaire
ATP: Adenosine triphosphate
ATS: American Thoracic Society
BAL: Bronchoalveolar lavage
BF: Breathing frequency
Cys LT: Cysteinyl leukotriene
COPD: Chronic obstructive pulmonary disease
DC: Dendritic cell
DRR: Dose-response ratio
DTT: Dithiothreitol
EAR: Early-phase asthmatic reaction
EBC: Exhaled breath condensate
ELISA: Enzyme-linked immunosorbent assay
ERS: European Respiratory Society
FeNO: Fractional exhaled NO
FEV₁: Forced expiratory volume in one second
GEMA: Spanish guide for asthma management
GINA: Global initiative for asthma
GM-CSF: Granulocyte macrophage colony stimulating factor
HMW: High-molecular-weight
IFN-γ: Interferon-gamma
Ig: Immunoglobulin
IL: Interleukin
IS: Induced sputum
LABA: Long-acting beta agonist
LAR: Late-phase asthmatic reaction
LMW: Low-molecular-weight
LTB₄: Leukotriene B₄
MHCII: Major histocompatibility complex II
MV: Minute ventilation
NO: nitric oxide
NO₂⁻: Nitrite
NO₃⁻: Nitrate
NoOA: Non-occupational asthma
NWRA: Non-work-related asthma
OA: Occupational asthma
PBS: Phosphate-buffered saline
PC₂₀: Provocative concentration of methacholine inducing a 20% fall in FEV₁
PEF: Peak expiratory flow
RADS: Reactive airway dysfunction syndrome
ROC: Receiver-operating characteristic
SIC: Specific inhalation challenge
TCR: T-cell receptor
Th: T-helper lymphocyte
TNF-α: Tumor necrosis factor-alpha
TNF-β: Tumor necrosis factor-beta
VD: Variable “difference”
VOCs: Volatile organic compounds
Vt: Tidal volume
WEA: Work-exacerbated asthma
WHO: World Health Organization
WRA: Work-related asthma
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INTRODUCTION
1. Asthma

1.1. Definition and prevalence

Asthma is a complex chronic disorder of the airways which is mainly characterized by variable airway obstruction, resulting in airflow limitation, airway inflammation, non-specific airway hyper-reactivity (AHR), airway remodelling (injury-repair process) and reversibility, either spontaneously or as a result of treatment (1, 2). Classically, asthma has been considered as an illness associated with atopy and/or allergic reactions, which begins in infancy and may or may not persist into adulthood (3). However, there is increasing evidence that asthma is a multifactorial disease that displays heterogeneity and variability, can manifest at any age, and is constituted by overlapping separate syndromes probably with different causes and natural histories. This heterogeneity is influenced by factors such as age, sex, socio-economic status, race, and gene-gene and gene-environment interactions (1, 4).

The prevalence of asthma (and other local allergic conditions) increased markedly over the second half of the last century, especially in westernized societies, where today it poses a considerable disease burden on individuals and a major economic burden on healthcare systems and society as a whole (5, 6). Asthma is now one of the commonest chronic diseases and its prevalence in developed countries has risen to epidemic proportions, ranging from 1% to around 8% of the population, with a very heterogeneous distribution (7). World Health Organization (WHO) estimates that around 300 million people in the world currently have asthma (7, 8). The prevalence of asthma increases as communities adopt modern lifestyles and become urbanized (9); with the proportion of the world’s population living in urban areas projected to rise from 45% to 59% in 2025, over the next two decades the population of sufferers worldwide is likely to increase by an additional 100 millions (7). Evidence from recent epidemiological studies suggests that there are no signs of any decline in asthma prevalence (10).

Several factors have been proposed to account for the increasing prevalence of asthma (11, 12). The rapidity of this epidemiological shift cannot be explained by genetic changes alone (13); genetic changes in populations are too slow to have such an effect (11). Considering that the lung is one of the three major organs (along with the gut and
the skin) that are continuously exposed to the external environment, it is widely agreed that the environment is an important determinant of asthma pathogenesis (13). However, most information on the effects of environmental exposures on the risk of asthma comes from cross-sectional studies that do not take temporal changes into account, and robust data linking changes in the environment to changes in the prevalence and incidence of asthma over time are lacking (11).

Asthma also displays heterogeneity and variability in its clinical expression and the therapeutic responses in both adults and children, indicating that it may be a syndrome rather than a specific disease entity. It can manifest as a short single attack that disappears spontaneously, as a single more severe attack, or as successive crises over several days. Despite this marked heterogeneity, it is recognized to be a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role: in particular, mast cells, eosinophils, T-lymphocytes, neutrophils, and epithelial cells. In susceptible individuals, this inflammation causes typical clinical symptoms: recurrent episodes of wheezing, breathlessness, chest tightness and cough, particularly at night and/or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that often reverses, either spontaneously or with treatment. The inflammation also causes an associated increase in the existing AHR to a variety of stimuli. Furthermore, airway inflammation is associated with the development of airflow limitation as the result of bronchoconstriction, airway edema, mucus secretion, and airway wall remodeling (14). Clearly, then, all these symptoms represent a significant burden, not only in terms of morbidity and reduced quality of life of patients, but also in terms of healthcare costs (7). Childhood asthma causes many lost school days and emergency department visits, and may have a negative impact on sufferers’ academic achievement and social interaction, affecting their daily activities, schooling, family life and finances (8, 15). In the case of adult asthma, demographic characteristics such as poverty, low educational attainment, female gender, race and urban environments are related to a greater healthcare cost and to a reduction in quality of life and housework activities. The direct and indirect costs of this disease are principally associated with lower work productivity, especially due to the complete cessation of employment rather than to the reduction of hours or missed work days among subjects who remain employed (16-18).
1.2. Pathophysiology of asthma

Several risk factors for asthma have been evaluated in the context of disease inception: viral infections (19), environmental exposures [aeroallergens (20), pollution (21), tobacco smoke (22)], lifestyle [living on a farm (23), diet (24), antibiotic use (25)], comorbid conditions [atopic dermatitis (26), obesity (27)], and occupational exposures (28) among others. However, the exact cause of asthma remains uncertain. Taking all these risk factors into account, asthma can also be subdivided into allergic (atopic) and non-allergic asthma. In this thesis, however, we focus mainly on the best-known type: allergic or atopic asthma.

Allergic asthma is considered a type I hypersensitivity reaction, together with allergic rhinitis, which means that it is mediated by the production of immunoglobulin (Ig)E specific for otherwise harmless environmental substances, most of which are proteins. Currently, more than 1000 protein allergens have been sequenced, and the numbers of known allergens are steadily increasing. However, only a very small percentage of the total proteins from environmental sources that our immune system encounters elicit an allergic reaction due to molecular interactions between the allergen and its corresponding IgE antibody (29).

These environmental allergens evoke a biphasic response which consists of an acute or early-phase asthmatic reaction (EAR) characterized by constriction of airway smooth muscle cells, vascular leakage, mucus production, enhanced AHR and recruitment of inflammatory cells, lasting 30-60 min. This is followed 4-6 h later by the late-phase asthmatic reaction (LAR), which is characterized by excessive inflammation of the airways, resulting in structural changes collectively known as airway remodeling (30).

One of the first steps in the immune response is the recognition of the antigen or allergen by antigen presenting cells (APCs) present in the skin and mucosal sites (Figure 1) (31). These APCs may be macrophages, B-lymphocytes or dendritic cells (DCs). DCs are the most important APCs in the lung and are mainly recognized for their exceptional potential to generate a primary immune response and sensitization to aeroallergens, and they also play a crucial role in established inflammation (32, 33). A network of airway DCs is located immediately above and beneath the basement
membrane of the upper and lower airways, which enables the DCs to have direct contact with inhaled antigens (32). From this location, DCs have an immature phenotype, meaning that they have exceptional allergen uptake and processing capability, but lack the power to stimulate naïve T-lymphocytes. While they are migrating to the draining lymph nodes they will mature, losing the capacity to take up antigen and acquiring a professional APC phenotype expressing all the co-stimulatory molecules and chemokines needed to attract and stimulate naïve T-lymphocytes (32, 34). DCs transport the antigen from the mucosa to the draining lymph nodes of the lung, after degradation of the antigen into short immunogenic peptides and loading the peptides on major histocompatibility complex II (MHCII) molecules on their surface. DCs then migrate to T-lymphocyte rich areas of draining lymph nodes where naïve T-lymphocytes continuously pass by. T-lymphocyte activation is initiated when the T-cell receptor (TCR) and associated proteins recognize the peptide/MHCII complex on the DCs. This leads to the formation of an immunological synapse, with the consequent T-lymphocyte activation, division and differentiation. The T-cell side of the synapse is focused on CD3 and the TCR, which binds specifically to the peptide/MHCII complex, as well as CD4 molecules that stabilize the interaction (32, 33). Activation of the T-lymphocytes leads to the production of an array of type 2 helper (Th2) cytokines implicated in asthma pathogenesis, including interleukin (IL)-4 and IL-3 which are required to drive the isotype switch of B-lymphocytes and activate them. Activated B-lymphocytes become plasmatic cells producing allergen-specific IgE (2). Together with IL-9, these cytokines play an important role in mast cell development, mucus overproduction and AHR (30, 35).

Re-exposure to a previously met allergen leads to its cross-linking on mast cell-bound specific IgE, thus causing the activation of membrane and cytosolic pathways, which subsequently trigger the release of preformed mediators such as histamine, the synthesis of prostaglandins and leukotrienes, and the transcription of cytokines by mast cells. These mediators are responsible for the symptoms of the EAR and also for some aspects of the LAR (30, 35). Histamine induces constriction of the airway smooth muscle cells and endothelial cells, mucus secretion and vasodilation (2). Meanwhile, mast cells and Th2-lymphocytes produce and secrete other cytokines, such as IL-3, IL-5 and granulocyte macrophage colony stimulating factor (GM-CSF), which are critical for
eosinophil development and survival. The late-phase of the asthmatic reaction is characterized by excessive inflammation of the airways resulting in structural changes, where eosinophils are the central effector cells. Besides eosinophils, neutrophils, T-cells, macrophages, DCs and endothelial cells also play an important role in the LAR (30).

Figure 1. General mechanisms of allergic asthma.
Abbreviations: DC, dendritic cell; Th, T-helper lymphocyte; IL, interleukin; IgE, immunoglobulin E; GM-CSF, granulocyte macrophage colony stimulating factor (31).

Repetitive cycles of tissue damage and inflammatory cell recruitment lead to chronic inflammatory processes which will further result in structural airway remodeling. The chronic inflammation leads to structural changes in the airway architecture, including airway wall thickening, subepithelial fibrosis, increased vascularity, goblet cell hyperplasia, airway smooth muscle cell hyperplasia and hypertrophy and epithelial hypertrophy (2, 30, 35). This airway remodeling is a dynamic process that involves the laying down of extracellular matrix structures, which are also broken down by the action of tissue-degrading enzymes (32). The airway remodeling can mediate AHR through several mechanisms, including altered neural regulation or increased contractility of airway smooth muscle cells. In addition, airway remodeling can
Contribute to AHR through purely mechanical means (35). When asthma becomes a chronic disease, airway inflammation, AHR and airway remodeling persist, and here mast cells play an important role. It has been reported that mast cells are markedly increased in association with airway smooth muscle in both the large and the small airways (36). It is at this site that mast cells interact with airway smooth muscle cells through the action of a variety of lipid mediators, chemokines, cytokines, and enzymes that may cause AHR to constrictive stimuli and the proliferation of airway smooth muscle cells. Activated airway smooth muscle cells can produce stem cell factor and other chemokines, cytokines, and growth factors that may act in the recruitment, differentiation, and retention of mast cells (37). Thus, airway smooth muscle is partly dependent on mast cells for its survival and enhanced contractility, whereas mast cells are dependent on smooth muscle factors for their survival and activation (38). Mast cell infiltration of the airways in asthma is T-cell dependent, and Th2 cytokines from T-cells act in mast cell expansion from circulating and tissue precursors (37).

1.3. Asthma phenotypes and endotypes

Clinical guidelines reflect the heterogeneity of asthma by defining multiple levels of severity and dividing patient groups into categories or asthma phenotypes. A phenotype describes observable characteristics determined by the genotype and modulated by the environment, and in the context of asthma describes clinical, physiological, morphologic, and biochemical characteristics as well as the response to different treatments (39). In the last twenty years, asthma phenotypes have been defined on the basis of clinical or physiological characteristics (severity, age at onset, degree of obstruction, resistance to treatment), by asthma triggers (exercise, allergens, occupational allergens, aspirin-induced, menstruation) or on the basis of the type of inflammation (eosinophilic, neutrophilic or paucigranulopilic) (40). Recently, the asthma phenotype has been redefined using the cluster modelling method to identify unique groups (clusters) of asthmatic patients with the same disease characteristics (41-43). In this context, one asthma phenotype is becoming frequent: new-onset asthma during adulthood. Currently, data on its prevalence and incidence and also of its determinants are insufficient (44); in general, it is defined as a difficult asthma with poor control, despite high dose treatment (45), and a neutrophilic inflammation is most often seen (46). Multiple independent risk factors for the development of new-onset
asthma in adults have been proposed, such as female gender, obesity, lung function, AHR, nasal allergy, parental asthma, respiratory infections in early life, high-risk occupations, pollution and atopy; the last factor is responsible for only a small proportion of new-onset adult asthma cases (44).

Although asthma phenotypes are usually clinically relevant, in terms of presentation, triggers, and treatment response, they do not necessarily relate to or give any insight into the underlying disease processes. As a result, a new concept has been proposed: the asthma endotype (39). An endotype is a subtype of a condition, which is defined by a distinct functional or pathophysiological mechanism. Endotypes are thus a different form of classification from phenotypes and describe distinct disease entities with a defining aetiology and/or a consistent pathophysiological mechanism. It has previously been suggested that asthma is made up of several endotypes (47), and although the underlying mechanisms of many of the proposed endotypes are poorly understood at present, their definition will allow the identification of novel therapeutic targets and biomarkers that meet formal diagnostic and prognostic criteria. Furthermore, defining endotypes may help predict the response to treatment and thus facilitate improved management decisions with currently available treatments (39).

2. Work-related asthma

2.1. Definitions and prevalence

More than 300 years ago, the Italian physician Bernardino Ramazzini, recognized as the father of occupational medicine, published De Morbis Artificum Diatriba (Diseases of Workers) in which he described the effects of work on health for over 50 professions. This masterpiece provided the knowledge for the development of the modern occupational medicine (48, 49).

An occupational disease is one that arises from work or is aggravated by work. According to the WHO, an occupational disease is not characterized merely by the disease itself, but by a combination of a disease and an exposure, as well as an association between the two (50). Occupational and also environmental exposures increase the risk of respiratory diseases, which are predicted to become the third leading
cause of death by 2020, according to the WHO (51). The lungs are the primary target for a diverse spectrum of work-related dusts, gases, fumes and vapours. Depending on the concentration inhaled, the duration of exposure and their physical-chemical properties, these agents have the capacity to cause annoyance, irritation, corrosive changes and/or sensitization in the respiratory tract (52).

Work-related asthma (WRA) is the broad term that refers to asthma that is exacerbated or induced by inhalation exposures in the workplace (53). WRA, which includes occupational asthma (OA) as well as work-exacerbated asthma (WEA), has become one of the most prevalent occupational lung diseases (54, 55) (Figure 2). OA is defined as “a disease characterized by variable airflow limitation and/or hyperresponsiveness and/or inflammation due to causes and conditions attributable to a particular occupational environment and not to stimuli encountered outside the workplace” (56). OA refers to asthma occurring de novo caused by exposure in the workplace or the recurrence of previously quiescent asthma (i.e., asthma as a child or in the distant past that has been in remission) induced by either sensitization to a specific substance (sensitizer-induced OA) or by exposure to an inhaled irritant at work (irritant-induced OA) (53). WEA is defined as a worsening of pre-existing or concurrent asthma which is exacerbated by working conditions (aeroallergens, irritants, or exercise), regardless of frequency or duration of worsened asthma and regardless of whether there are permanent changes in severity, or as the development of asthma that had been present in childhood or earlier life and now recurs due to agents in the workplace (48, 57). For any individual, OA and WEA are not mutually exclusive, meaning that someone with OA may subsequently experience WEA, and vice versa (53, 57).
WRA is currently considered the most common form of chronic occupational lung disease and causes significant morbidity and disability. It is estimated that between 10% and 25% of all asthma cases that develop in adulthood are work-related or caused by occupational exposure (58, 59). The annual incidence of WRA, which is associated with a high rate of prolonged work disruption, is ~50 per million workers (60). However, the prevalence of WRA has not been well defined, due in part to variable definitions, diagnostic criteria, and work settings, as well as limited surveillance data (53). Reports from several surveillance programs have suggested that OA is one of the most common presentations of work-related respiratory pathologies in many industrialized countries (61, 62). Nevertheless, OA surveillance data vary widely in case capture, and thus underestimate the true extent of the problem. It has been suggested that in patients in whom WRA has been diagnosed the proportion with WEA ranges from about 10% to 50%, although this may be reflective of compensation practices as much as of true prevalence (53, 57).

About 250 specific occupational exposures are associated with asthma, such as animal handlers, laboratory research workers, bakers, grain handlers, health-care workers, detergent and pharmaceutical industry workers, textile workers, metal-refining workers,
woodworkers and many others (63). Only some of these exposures have been assessed in epidemiological studies and only a few studies have looked at OA in the general population (58, 64). The occupations and industries associated with WEA often differ from those associated with OA (57). The prevalence and onset of OA depends mainly on the causative agent to which the workers are exposed and the intensity of the exposure, and to some extent also on the distribution of host susceptibility factors, such as atopy, smoking status and the presence of occupational rhinitis (63, 65, 66). In addition, the large increase in the prevalence of asthma observed in most developed countries (67) in recent decades has pointed toward environmental factors being responsible in part for this increase, particularly changes in diet, such as decreased consumption of fresh fruit, vegetables and unprocessed food (68, 69).

2.2. Types of occupational asthma

Traditionally, two types of OA have been described, depending on whether or not a latency period occurs between the first exposure to the agent and the onset of asthma symptoms (Figure 2). The first type, ‘OA with latency’, is immunologically mediated (allergic, or sensitizer-induced OA) and is characterized by the fact that the development of sensitization against a workplace agent occurs only after a latency period that may vary from a few weeks to several years (70). Depending on the molecular weight of the offending agents, immunologically mediated OA can be further divided into two types according to whether the cause is a high-molecular-weight (HMW) agent, most of which induce OA via IgE-dependent mechanisms, or a low-molecular-weight (LMW) agent, many of which (though not all) appear to induce OA via unknown pathways that do not involve IgE-dependent mechanisms (71, 72).

The second type, ‘OA without latency’, is non-immunologically mediated OA – also called non-allergic OA or irritant-induced OA, and it is the reaction observed in patients whose illness is caused by exposure to irritant chemicals encountered in the workplace to which the host does not become sensitized. Until recently, non-immunologically mediated OA was represented by its most typical and clearest presentation, the reactive airways dysfunction syndrome (RADS), a persistent asthma syndrome characterized by the absence of a latency period and initiated by a single acute exposure to a non-specific irritant chemical at concentrations high enough to induce airway injury and
inflammation, typically as a result of an accident occurring in the workplace or of a situation with poor ventilation and limited air exchange (73, 74). Currently, however, some authors include under this heading individuals in whom asthma had a delayed onset after repeated exposures to low concentrations of irritants and is associated with latency in a similar manner to sensitiser-induced asthma (75). Although this entity, called low dose irritant-induced asthma (LDIIA), is not yet accepted by all the scientific community, it sets a diagnostic challenge in the sense of being able to differentiate between immunologically but not IgE-mediated OA caused by LMW agents and asthma induced by exposure to low concentrations of irritants, when two types of asthma appear after a latency period (76).

2.3. Agents causing OA

More than 350 agents have been reported to cause allergic or immunologically mediated OA. As mentioned above, they can be divided into two groups according to their molecular weight: HMW biological agents (> 5 kDa) such as proteins (of animal or vegetable origin), glycoproteins and polysaccharides, and LMW chemicals (< 5 kDa) such as synthetic chemicals, natural compounds, drugs and metals (77). Although there are fewer LMW chemicals than HMW agents in the lists of occupational respiratory sensitisers, LMW chemicals still represent an important subset of aetiologic agents, currently including approximately 100,000 individual chemical entities in the European Inventory of Existing Commercial Substances, and new LMW chemicals are frequently introduce into the market (77, 78). Isocyanates, acid anhydrides, plicatic acid (from Western red cedar), colophony fume, metals such as complex platinum salts, persulfate salts and some acrylates are just a few examples of important chemicals that induce OA. Recent data indicate that, in some countries, LMW chemicals account for more new cases of OA caused by sensitization than HMW agents (77).

Most HMW agents induce asthma by producing specific IgE antibodies, acting as complete antigen. For certain LMW agents (platinum salts, trimetillic anhydride, and other acid anyhidride) the development of OA is also accompanied by the production of specific IgE antibodies, probably acting as hapten and combining with a body protein to form functional antigens (Figure 3). Since LMW agents are non-immunogenic in their native state, it is assumed that they must first form a stable association with
proteins such as albumin, keratine or tubuline in order to initiate an immune response. These protein-hapten conjugates can be recognized and internalized by APCs. Like most HMW agents, these conjugates are then presented to T-lymphocytes, which initiate an immune response and, possibly, asthma via an IgE-mediated or other mechanisms. In subjects who develop irritant-induced asthma, alarm signals from damaged epithelial cells might in turn activate immunocompetent cells (72).

The airway inflammation process is similar in IgE and non-IgE-dependent asthma, as both types are characterized by the presence of eosinophils, lymphocytes, neutrophils, mast cells and typical features of airway remodeling. Airway inflammation is accompanied by a release of preformed and newly formed proinflammatory mediators, which contributes to the functional alterations of OA (72, 77, 79).

**Figure 3.** Schematic summary of possible mechanisms in occupational asthma. HMW agents and certain LMW agents are recognized by APCs and mount a CD4 type 2 immunologic response leading to production of specific IgE antibodies by IL-4/IL-13-stimulated B cells. Most LMW agents induce a mixed CD4/CD8 type 2/type1 immunologic response (72).
2.4. WRA diagnosis

The diagnosis of WRA is based on a detailed clinical and occupational history, also on the demonstration of sensitization when the mechanism is IgE-mediated, based on the finding of asthma, and finally based on the relationship between this asthma and the occupation (80). Specific inhalation challenge (SIC) is currently considered the reference standard method for the correct diagnosis of OA (53). However, several factors and diagnostic tests other than SIC have been proposed for OA diagnosis in the Spanish Guide for Asthma Management (GEMA) (80) and by other authors (81) (Figure 4).

![Figure 4. Algorithm for the diagnosis of occupational asthma.](image)

Abbreviations: IgE, immunoglobulin E; OA: occupational asthma; PEF: peak expiratory flow; SIC: specific inhalation challenge; RADS: reactive airways dysfunction syndrome. * Measurements after 15 days at work and 15 days off work; sputum-analysis of changes in the eosinophils numbers. Adapted from GEMA 2009 (80).
The purpose of SIC is to explore, through a direct observational approach, the causal relationship between exposure to a test substance and an asthmatic reaction, and then aims to demonstrate this direct relationship (53, 82). Moreover, SIC allows precise identification of the agent causing OA when a subject is exposed to more than one agent in the workplace, which is particularly important for giving proper advice to affected workers and employers, and it is also an important tool for identifying new causal agents and for exploring the pathogenic mechanisms leading to asthmatic reactions (72, 82). SIC can also be included as the final confirmatory step in surveillance programs and epidemiological surveys of OA in high-risk workplaces. In addition, it can be used to assess the efficacy of preventive measures and protective devices. Combining SIC with quantitative assessment of airborne agents could help to determine the level that elicits reactions in already sensitized subjects, and could be used as a guide to establish permissible exposure levels at work (82). However, although SIC is a very good tool for establishing an accurate diagnosis, false-positive and false-negative responses to an SIC can occur (83-85). It is generally agreed that false positives are easy to control when the test is performed by expert staff, and that they do not in fact hinder correct diagnosis (86). False-negative SIC responses may occur in around 20% of cases (87) but their prevalence may be reduced by detecting changes in airway responsiveness before and after challenges (53, 88).

In the WRA field, differentiating OA from WEA is relevant for medical, preventive and medico-legal purposes. OA may require complete removal from workplace exposure, because persistence of exposure may result in the progressive worsening of asthma features and long-term functional impairment. These therapeutic and preventive options may have tremendous professional, financial and social consequences. In contrast, WEA could be managed at a lower societal cost by reducing the levels of irritants to acceptable limits at work and/or by optimizing anti-asthma treatment, having this considerable financial and psychosocial impact. According to some consensus statements (53, 54), the possibility that an individual with previous asthma will present OA cannot be excluded, nor can it be ruled out that a patient will develop non-occupational asthma during his/her working life and that this will be exacerbated by work. In this context, SIC may be useful to differentiate between OA and WEA, because a positive result confirms the diagnosis of OA. Nonetheless, when SIC is
negative, the diagnosis of WEA remains unclear. When WRA is confirmed, one can conclude that individuals with a negative SIC response have WEA (89). However, in clinical practice, SIC is often performed in subjects with asthma unrelated to the workplace and in patients who do not have asthma, leading to a false-positive WEA diagnosis (90). In these situations, it would be necessary to perform other tests in conjunction with SIC to try to help in establishing a definitive diagnosis.

2.5. Socio-economic impact of WRA and prevention strategies

Regarding socio-economic outcomes, the negative impact on the indirect cost of asthma can take many forms, including reduced workforce participation and employment rates, changes in employment or job duties as an adjustment to the asthmatic condition, asthma-related lost work days, and impaired work effectiveness while on the job (91). Wage-replacement costs for lost work days accounted for almost as high as medical care expenses, and impairment in self-reported work effectiveness has been associated with more severe asthma, marked AHR, and the presence of work-related respiratory symptoms (92). Studies investigating socio-economic outcomes in WRA have generally shown that OA and WEA patients report similar income loss, rates of unemployment, and partial work disability (93, 94). However, it has also been suggested that patients with WEA, unlike those with OA, can usually return to their workplace making certain adjustments to reduce exposure to likely airway irritants and/or with optimized asthma medication (95).

The magnitude of WRA is matched by the important opportunities for the primary prevention of new cases, which involves substituting asthma-inducing agents with harmless substances in order to prevent new OA cases or the reduction of exposures to levels that do not cause WEA (53). Secondary preventive measures include screening and surveillance of exposed workers (those with high-risk exposures or increased individual susceptibility) enabling early diagnosis and removal from harmful exposure, which is associated with a better prognosis (54). In this regard, complete elimination of the exposure is the most straight-forward approach to reducing the burden of disease and it seems logical that decreased exposure will lead to a reduced disease burden of OA, but the long-term health effects of reducing rather than eliminating exposure to the agent causing OA remain uncertain (60, 91). In case of removal from the workplace, it
has been reported that only 25% of workers who do so are symptom-free. The main prognostic factors identified so far are the duration of exposure, the severity of asthma at time of diagnosis, the presence of airway inflammation and specific IgE (96). Tertiary prevention acts once the illness has fully manifested itself and consists of measures aimed at softening the impact of long-term disease and disability by eliminating or reducing impairment, disability and handicap. It may involve re-assigning a worker to a different job, supplying personal respiratory equipment and providing anti-asthma medication (57, 60). Prevention is intimately linked to both the diagnosis and the treatment of disease. The diagnosis of a single case of OA among a group sharing similar exposures offers the possibility of preventing new asthma or the progression of subclinical illness to frank disease. In this context, it is important that employers recognize the problem and develop an appropriate policy regarding hazardous agents. It is their responsibility to educate workers on the risks of the agents used. Employers should also provide regularly health surveillances and perform exposure monitoring (97).

3. Non-invasive methods to study airway inflammation

Airway inflammation is a dominant feature of asthma and a hallmark of its pathophysiology, which is associated with AHR and airway remodelling. Airway inflammation has typically been assessed by histological examination of lung tissue using bronchial biopsy, and by bronchoalveolar lavage (BAL) and/or bronchial washing. However, these techniques are invasive, which means that repeated measurements are not possible, and they require experienced, skilled pathologists for tissue examination, which limits their clinical application. More recently, several potential diagnostic tools for evaluating airway inflammation in a less invasive manner have been developed and validated, such as induced sputum (IS) and analysis of exhaled breath (98, 99).

3.1. Induced sputum

IS analysis is a reproducible, valid, responsive, and non-invasive method for studying airway inflammation. It comprises two steps: sputum induction, and sputum processing, both of which are well validated. In the first stage, sputum production is induced by
inhalation of hypertonic saline solution using an ultrasonic nebulizer (100, 101). After induction, sputum samples need to be processed within 2 h to ensure optimal retrieval of information on both inflammatory cells and inflammatory mediators present in the airways. The principal output is the differential inflammatory cell count based on the counting of major cell types in the cellular phase: eosinophils, neutrophils, lymphocytes, macrophages, and epithelial cells (99). Besides this cellular phase, the sputum fluid phase or sputum supernatant can be used to measure a variety of soluble inflammatory biomarkers, such as eosinophil-derived proteins, tryptase, myeloperoxidase, albumin, fibrinogen, nitric oxide (NO) derivatives, leukotrienes, cytokines, chemokines, and stress-related proteins (102).

This method has been widely used to assess airway inflammation in several respiratory diseases: chronic obstructive pulmonary disease COPD (103), bronchiectasis (104), sarcoidosis (105), interstitial lung diseases (106) and, especially, asthma. IS has been used to investigate airway inflammation in stable asthma and in acute exacerbations of asthma in both adults and children, and the application of its analysis has allowed repeated measurements of airway inflammation in large numbers of asthmatic patients, as well as the identification and characterization of asthma phenotypes (41, 43). Compared with healthy subjects, asthmatics have increased sputum cell counts for eosinophils and neutrophils as well as for markers of inflammatory cell activation (100). Eosinophilic airway inflammation has been shown to predict worse asthma outcomes, and there is also evidence that the use of sputum eosinophils improves asthma management by decreasing the number of exacerbations (107, 108). Although sputum eosinophilia is a typical feature of asthma, non-eosinophilic asthma, or sputum neutrophilia, is also common, accounting for 25% to 55% of corticosteroid-naïve asthmatics. Its identification is important, as it is associated with a poor response to corticosteroids, whereas asthmatics with sputum eosinophilia respond favourably to this treatment (109, 110).

In WRA, analysis of sputum cells may be useful in the investigation of the effects of occupational agents on experimental exposures because it provides direct information on the type, intensity, and time course of airway inflammation, being a direct reflection of the disease (111). In subjects with suspected OA, some studies have attempted to
evaluate the usefulness of IS when workers are exposed in the workplace and when IS is performed in the context of SIC. Analysis of the sputum inflammatory profile to support the diagnosis of OA in the workplace has produced useful findings. In this regard, monitoring of the functional and inflammatory changes during periods at and away from the workplace has demonstrated that subjects with OA seem to show predominantly an eosinophilic airway inflammation after exposure to the causal agent when at work (112). In addition, a recent study has shown that an increase in sputum eosinophil count greater than 2% when at work compared to periods away from work, in conjunction with serial peak expiratory flow (PEF) monitoring, is useful for improving the diagnosis of OA (113). Although a sputum eosinophilia seems to be the most relevant inflammatory profile in OA when at work, it is important to note that a neutrophilic airway inflammation has also been described (114, 115). However, the significance of this sputum neutrophilia remains unclear at present.

As mentioned above, the current gold-standard method for diagnosing OA is the performance of a SIC with the suspected agent. Although SIC tests are reliable, they can be falsely positive or negative (83-87). The addition of a non-invasive measure, like IS, to SIC is therefore likely to improve the diagnosis of OA. In fact, it has been postulated that the increases in sputum inflammatory cells observed following SIC in subjects without OA may be an accurate parameter for predicting the development of an asthmatic response to subsequent challenges with a sensitivity of 67% and a specificity of 97%, referring specially when an eosinophilic inflammation is present (116). Furthermore, evaluating airway inflammation preceding and following SIC after removal from exposure at workplace has proved useful for asthma follow-up, showing a rapid decrease in eosinophilic airway inflammation followed by an improvement of AHR within the first six months after the removal in workers with OA, and subjects with a non-eosinophilic asthmatic reaction during SIC seem to have a poorer prognosis than subjects with eosinophilic airway inflammation (117).

SIC may also be useful for trying to determine which is the mechanism responsible for OA (72), in particular if analysis of sputum cell counts is performed preceding and following SIC in the laboratory (118-120). In this context, few studies in the literature have evaluated the performance of IS in conjunction with SIC to several occupational
agents in order to identify the mechanisms involved in the development of this disease. Some of them are case reports documenting changes in airway inflammation following SIC to a particular LMW agent (121-123), and others are clinical series evaluating OA diagnosis regardless of the type of causative agent (116, 118, 119, 124); only two studies have taken into account the nature of occupational agents (120, 125). In this context, an increase in sputum eosinophils has been observed after SIC to a large number of HMW agents but also to certain LMW agents: isocyanates, acrylates, red cedar, exotic woods, eugenol, persulfate salts, manganese and styrene (120, 122, 126). However, an increase in sputum neutrophils has also been reported after asthmatic reactions induced by HMW agents (125) as well as following SIC to certain LMW agents such as isocyanates and welding fumes (121, 123). Thus, changes in airway inflammatory profile seem to differ depending on the causative agent, but the mechanisms that influence the type of airway inflammation remain unclear, especially in the case of LMW-induced asthma.

For WEA, the mechanisms responsible for the changes in sputum inflammatory profile due to exposure to occupational agents are even less clear than in the case of OA. There are a limited number of studies that have looked at airway inflammatory changes in subjects with WEA after exposure to the offending agent, predominantly sensitizers. In this sense, after exposure in the workplace, subjects with WEA have shown no change in sputum inflammatory profile (112) or a neutrophilic airway inflammation (113). After exposure in the laboratory to specific occupational sensitizers, it has been reported that the majority of subjects with WEA have shown no change in airway inflammation, and a few have shown an increase in sputum eosinophilia (118, 127).

### 3.2. Exhaled breath condensate

A more recent method for assessing airway inflammation in a non-invasive way is the analysis of exhaled breath condensate (EBC). EBC is the liquid phase of exhaled air and is sampled non-invasively by cooling or freezing exhaled air with the use of special condensing devices, allowing the collection of condensed water vapor, non-volatile substances that are released in aerosol particles and volatile substances in a gas phase (128). It is a simple technique requiring the subject to breathe tidally and its non-invasive nature gives the opportunity for repeated measurements that can be made in the
same person, and valuable information can be gathered for the assessment of airway inflammation (129). EBC collection does not disturb the underlying disease process, making it a useful approach for cross-day variation studies, for epidemiological and pharmacological therapy investigation and for longitudinal studies that monitor airway inflammation (111, 130). Although the collection procedure is not well standardized, there is emerging evidence that abnormalities in EBC composition may reflect biochemical changes in airway lining fluid (129).

Because EBC has no cellular components, the evaluation and quantification of airway inflammation is based on metabolic products released by cells from the lungs or substances originating from inflammatory reactions in the airway mucosa (128). Compounds identified in EBC, known as EBC biomarkers, include adenosine, ammonia, hydrogen peroxide, isoprostanes, leukotrienes, nitrogen oxides, peptides, cytokines, protons and various ions. However, according to the American Thoracic Society and the European Respiratory Society, none of these biomarkers have been standardized and validated sufficiently for clinical use (130). Even though EBC is currently a research tool, accumulating increased evidence suggests that it has the potential of becoming a validated method able to provide valuable information on the understanding of the pathways propagating airway inflammation (129).

Given its non-invasiveness, EBC has been widely used to investigate the underlying mechanisms of several respiratory and respiratory-related diseases in both adults and children, such as cystic fibrosis (131), acute lung injury (132), gastro-oesophageal reflux disease (133), obstructive sleep apnea syndrome (134), bronchiectasis (135), as well as the two main chronic inflammatory disorders, COPD and asthma (128). The most commonly reported indicator of airway inflammation is EBC pH. It has been suggested that EBC acidification could be representative of an airway pH homeostatic alteration that underlies some or much of the pathology of lung diseases, including airway inflammation. In this regard, EBC pH is a simple, robust, reproducible, measurable on-site and inexpensive assay showing a relevant and integrative marker of airway inflammation, but it has not been evaluated prospectively as a guide for treatment (136). To enhance the stability of EBC pH readings, de-aeration with a CO₂ free gas has been proposed, and the most widely accepted data for EBC pH values are
those after de-aeration. In healthy subjects, EBC pH after de-aeration has a mean pH of 7.7, with a range of normal considered to be from 7.4 to 8.8 (130). When EBC pH is lower than normal, this means that more volatile acid and/or less non-volatile base has been delivered to and captured in the EBC, primarily because more acid and less base has been volatilized from an acidic airway (137). Endogenous airway acidification, as assessed by pH in EBC, is implicated in the pathophysiology of several respiratory diseases. Indeed, EBC pH has been found to be substantially lower than normal in patients with COPD, cystic fibrosis, and bronchiectasis and during acute asthmatic exacerbations (138-140).

Asthma is the chronic inflammatory disease in which EBC has been most widely used, especially EBC pH. It has been reported that patients with acute asthma have substantially lower EBC pH than healthy subjects, and the lower values of EBC pH found during asthmatic exacerbations seem to normalize with anti-inflammatory therapy and remission of the exacerbation (139). EBC pH remains within normal limits when the asthmatic inflammatory process is well controlled, but EBC pH decreases in the presence of sputum eosinophilia, suggesting that it is probably a consequence of the eosinophilic inflammation and not its cause (138). Furthermore, it has been reported that EBC pH is significantly correlated with sputum eosinophilia in moderate asthmatics as well as with parameters expressing oxidative stress and NO metabolism, suggesting that its routine measurement in clinical practice may be of great value, especially in case of eosinophilic asthma phenotype (138). Apart from EBC pH, other biomarkers in EBC have been reported to be higher in patients with asthma compared with healthy individuals, such as adenosine concentration and several markers of oxidative stress: hydrogen peroxide, NO-related products, isoprostanes and leukotrienes (141). In spite of these promising findings in asthma, EBC is currently considered as a research tool, due to the lack of appropriate standardization and the absence of reference values for all these biomarkers (142).

The role of EBC in WRA have been evaluating in fewer studies comparing to those evaluating IS, and most of the studies performed have been conducted for evaluating EBC biomarkers of exposure and effect in occupational settings. It has been shown that toxic metals, trace elements and specific chemical substances can be detected in the
EBC of exposed workers, suggesting that it can be used to gather extremely useful information concerning the target tissue levels and doses of pneumotoxic compounds (143). EBC has also been proposed as a suitable medium for investigating biomarkers of effect, which reflect the biochemical reactions of airway fluid to outdoor chemical substances. Biomarkers related to asthma pathogenesis and its severity can be extrapolated and used for the diagnosis and monitoring of OA. Indeed, increased levels of oxidative stress biomarkers have been reported in EBC of hairdressers exposed to chemical agents with potentially irritant and sensitizing effects on the airways, without impairment in respiratory function (144). There are few studies that have assessed the role of EBC pH as a means of airway inflammation and the results obtained so far are conflicting. An association has been found between the duration of work and decreased pH and ammonia levels in the EBC of grain workers in situations of chronic exposure at work, suggesting that airway acidity and oxidative stress are distinct components of airway pathophysiology (145), whereas another study found increased pH values in workers exposed to welding fumes (146). One recent assessment of the utility of EBC pH during periods at work and off work carried out by our group found that a decrease of 0.4 EBC units in individuals with suspected OA between two weeks at work and two weeks off work has a specificity of 90% for definitive OA diagnosis, indicating that this parameter could be incorporated in the diagnostic work-up of OA (147).

Information on the role of EBC pH in the context of SIC is still lacking, and so, little is known about the degree to which EBC acidifies in patients with OA. In this context, one study found no association between asthmatic reactions induced by isocyanates and EBC acidification after realization of SIC (148), and another study monitored leukotrienes and 8-isoprostane in EBC before and after SIC, but the results cannot be considered conclusive (149). Although it has been shown that numerous biomarkers are detectable in EBC and that this approach can be used in both clinical and occupational settings, it still has substantial limitations which currently preclude its use as a routine means of biomonitoring in the workplace (150).

**3.3. Other non-invasive methods**

Besides IS and EBC, in the last few years there has been a great deal of interest in the analysis of breath constituents for purposes of monitoring oxidative stress and airway
inflammation. NO is the one that has attracted the most attention. In the lung, endogenous NO influences outcomes in airway disease, pulmonary hypertension, lung injury and infection, and changes in NO levels have been reported in airway inflammatory diseases (151). Fractional concentration of exhaled NO (FeNO) is the most extensively studied exhaled biomarker and increased levels of FeNO have been documented in steroid-naïve patients with asthma (152). It has even been postulated that FeNO measurement might be used as an additional diagnostic tool for the screening of patients with a suspected diagnosis of asthma (153), and due to its usefulness in asthma diagnosis some asthma management guidelines have incorporated it in the diagnostic process (80). Furthermore, it has been shown that anti-inflammatory therapy reduces FeNO in asthmatic patients; it has also been suggested that FeNO is specifically a marker for eosinophilic inflammation and that increased levels of FeNO may reflect uncontrolled asthma (141, 154). Nevertheless, some authors have reported that FeNO measurement may be a useful alternative for asthma control (155), while others have concluded that the addition of FeNO as an indicator of control of asthma resulted in increased doses of treatment, without clinically important improvements in symptomatic asthma control (156). In this context, the clinical usefulness of this biomarker remains an issue because the direct association with airway inflammation is still unclear (157, 158).

Some studies have examined the usefulness of FeNO in the investigation of OA, but the results are inconsistent due to the low specificity of this biomarker compared to IS and to several confounding factors that influence the results (111). In this context, it has been suggested that increased levels of FeNO could be related with occupational agents inducing IgE-dependent asthma (159). Nonetheless, although FeNO is highlighted as a new perspective in the diagnosis of OA, further prospective studies are required to confirm its utility in occupational settings (160).

Finally, it has recently been postulated that airway inflammation seems to influence the processes responsible for producing volatile organic compounds (VOCs) by oxidative stress and lipid peroxidation in exhaled breath. Currently, two main methods are used to detect and analyse VOC profiles: polymer-based gas sensor array, the “electronic nose”, and gas chromatography-mass spectrometry (141). It has been suggested that VOCs
measurement may be useful for asthma diagnosis, phenotyping and management, since it identifies patients who could benefit from personalized therapeutic strategies (161). In addition, it has been noted that assessment of VOC profiles using integrative analysis by electronic nose can distinguish between patients with COPD and those with asthma (162). However, VOCs are still at an experimental stage and robust validation in a large population of asthmatics is necessary, as well as longitudinal assessment of inter-individual variation of VOC patterns (141).
References


Introduction


Introduction


HYPOTHESIS AND OBJECTIVES
Asthma is a heterogeneous inflammatory disorder of the airways, associated with airflow obstruction and airway hyper-reactivity (AHR) which vary in severity across the spectrum of the disease. Although asthma is considered a chronic inflammatory disorder, evaluation and therapy guidance are mainly based on clinical symptoms and lung function tests which remain the cornerstones of clinical practice. Work-related asthma (WRA) is a chronic inflammatory disorder of the airways due to occupational exposures and represents up to 25% of all asthma cases in working-aged populations. In recent years there has been a growing interest in the non-invasive assessment and monitoring of airway inflammation in order to understand the pathophysiological mechanisms of inflammatory airway diseases such as asthma and WRA.

Hypothesis: the study of cellularity and biomarkers in induced sputum (IS) and exhaled breath condensate (EBC) will aid the evaluation of subjects with suspected WRA, both to identify the pathophysiological mechanisms and to establish diagnosis.

The research presented in this thesis has the following broad aim: to analyse the usefulness of non-invasive methods (IS and EBC) for the study of airway inflammation in WRA.

We focus on the following main issues:

- **IS and well-controlled asthma**: We determine the type and degree of airway inflammation present in patients with well-controlled mild or moderate asthma, and the relationship between this airway inflammation and the degree of AHR (Chapter 1).

- **EBC normal data**: We first standardize EBC collection and establish reference values in a healthy adult population for specific markers of airway inflammation (EBC pH, 8-isoprostane concentration and nitrogen oxide values). We thus define normal ranges for these biomarkers in different age groups (Chapter 2).
Hypothesis and objectives

- IS and WRA: To establish the inflammatory profile of subjects with suspected occupational asthma, we analyse sputum inflammatory cell types at baseline and following a specific inhalation challenge (SIC) to occupational agents. We also investigate the role of biomarkers such as Th1/Th2 response cytokines and leukotriene B₄ in asthma pathogenesis (Chapter 3).

- EBC and WRA: To analyse the role of EBC in WRA we measure EBC pH, a marker of endogenous airway acidification, and nitrite/nitrate concentrations, markers of nitrosative stress, before and after realization of SIC in individuals with suspected WRA who are exposed to occupational agents. We then evaluate whether these changes in EBC biomarkers are useful to distinguish between different types of WRA (Chapter 4).
BRONCHIAL INFLAMMATION AND HYPERRESPONSIVENESS IN WELL-CONTROLLED ASTHMA

Muñoz X, Sánchez-Vidaurre S, Roca O, Torres F, Morell F and Cruz MJ

Clinical and Experimental Allergy (In press). IF: 4.195
Abstract

Background: Little research has been devoted to the characteristics of bronchial inflammation in patients with stable, well-controlled asthma.

Objective: The aim of this study was to assess the degree and type of airway inflammation and investigate the relationship between inflammation and bronchial hyperresponsiveness in patients with well controlled asthma.

Material and methods: A cross-sectional study was conducted in 84 adult patients (43 men, mean age 43 years) with documented well-controlled asthma. Induced sputum samples were obtained and cell types determined by differential cell count. Spirometry and methacholine challenge testing were performed. Asthma Control Questionnaire (ACQ) was used to assess symptoms. Patients were included if their ACQ score was < 0.75.

Results: A total of 59 patients had persistent bronchial inflammation: 28 cases were considered eosinophilic, 28 neutrophilic, and 3 mixed. Median (range) percentage of eosinophils was 4% (0-64) in patients testing positive to methacholine challenge (n=66) and 1% (0-3) in those testing negative (n=18) (p=0.003). A positive correlation was found between eosinophil percentage and the methacholine dose/response ratio (r=0.477, p=0.0001). The geometric mean (95% CI) of the methacholine PC_{20} was 1.74 mg/mL (1.04-2.93) in patients with eosinophilic inflammation and 4.14 mg/mL (2.5-6.84) in neutrophilic inflammation (p=0.03).

Conclusions: Inflammation and bronchial hyperresponse persist in most patients with well-controlled asthma.

Clinical relevance: The study demonstrates that eosinophilic or neutrophilic inflammation persisted in most well controlled asthma patients despite the fact that their condition was controlled and therefore, measurement of bronchial inflammation seems essential to achieve proper asthma control.
Introduction

Asthma is a chronic inflammatory disorder of the airways in which many cell and cellular elements play a role. The chronic inflammation is associated with airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing particulate at night or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment (1). The asthma definition includes four domains: symptoms and variable airway obstruction, which are readily amenable to assessment by clinicians, and airway inflammation and airway hyperresponsiveness, which characterize the underlying disease process, but are less accessible in clinical practice. The classic system for grading asthma severity was established from the first two domains, and specific treatment was recommended based on the severity of the condition (1). Nonetheless, this approach has been gradually replaced by the idea of judging severity based on the need for treatment to achieve asthma control (2); that is, the extent to which the manifestations of asthma are reduced or eliminated by treatment. Assessment of asthma control should incorporate two components: current clinical control (symptoms, use of medication to relieve symptoms, and clinical lung function) and future risk (exacerbations and rapid decline in lung function) (3).

For asthma to be considered well-controlled there should be a decrease in the number of exacerbations, and recent studies have shown that this can be more easily achieved when treatment is adjusted according to the degree of bronchial inflammation measured by eosinophil count in induced sputum than by adjusting based on clinical parameters (4-5). Although recommendations from the Cochrane Library (6) advocate this approach, there remain several uncertainties. Recent studies have suggested that there are different cellular phenotypes in bronchial asthma (7), and that the importance of eosinophils is questionable in some of them (8). Furthermore, sputum induction is not successful in all patients, the sample obtained may not be adequate, and the analysis requires immediate processing of the sample, which means that a properly equipped laboratory must be available in the center where induction is performed, a condition that is not met in all hospitals (9). Thus, several strategies for indirect evaluation of inflammation have been proposed to guide asthma treatment, such as measurement of
exhaled nitric oxide (10), study of pH in exhaled breath condensate (EBC) (11), and determination of airway hyperresponsiveness (12).

In this scenario, in which measurement of bronchial inflammation seems essential to achieve proper asthma control, only a few studies have indirectly examined the characteristics of airway inflammation in patients with well-controlled asthma (12-14), and to our knowledge, only two studies have directly focused on evaluating markers of airway inflammation in stable, well controlled asthma (15-16). The aim of this study is to determine the type and degree of bronchial inflammation present in patients with well-controlled asthma, and the relationship between the inflammation present and the degree of bronchial hyperresponsiveness. Moreover, to our knowledge, this is the first study that uses the asthma control questionnaire (ACQ) to define asthma control when studying well-controlled asthmatics.

**Material and methods**

**Patients and design**

This is a cross-sectional study, approved by the Ethics Committee of our tertiary hospital. All patients provided written informed consent for participation in the study. Patients were selected from the database of a specialized unit for bronchial asthma (Figure 1). All patients had shown reversible airway obstruction on methacholine challenge testing or bronchodilator testing, or variability >20% in the peak flow recording.
The study included 84 patients with mild (n=55) or moderate (n=29) asthma according to the Global Initiative for Asthma (GINA) classification (2), and no other lung diseases. The following inclusion criteria were applied: age 18 to 65 years, at least 1 year since the asthma diagnosis, controlled asthma established by a score lower than 0.75 on the ACQ (17) and no exacerbations defined according to GINA criteria (2) over the previous year, FEV₁ >70% the reference value, and asthma control attained by the use of inhaled corticosteroids at doses of 200-800 µg/day (equivalent dose of budesonide) with or without addition of a long-acting beta agonist (LABA) at a dose no greater than 100 µg/day. All patients had to have successfully undergone induced sputum collection.

Before their scheduled visit to the pulmonary function laboratory, patients were instructed to discontinue treatment with inhaled corticosteroids and LABA 24 hours previously and the use of short-acting beta agonists at least 6 hours previously. All patients first answered the ACQ to ensure that asthma was stable. EBC was then collected, spirometry and methacholine challenge were performed, and sputum induction was carried out.
Atopy and smoking status

Patients were considered atopic if they had at least one positive prick test to any of the common environmental allergens (18). Non-smokers were patients who had never smoked and ex-smokers were those who had not smoked for at least 6 months. The number of pack-years was calculated in all cases.

Spirometry and methacholine challenge

Spirometry was performed with a Datospir 200 (Sibel, Barcelona) instrument, according to European Respiratory Society (ERS) and American Thoracic Society (ATS) guidelines (19). The reference values used were those proposed for the Mediterranean population (20). Bronchial challenge with methacholine was undertaken following the method described by Chai et al. (21). Briefly, using a Mefar MB3 (Mefar, Ele H2O, Medicalli, Brescia, Italy) dosimeter, increasing doses of methacholine (0.03 mg/mL-16 mg/mL) were inhaled at 3-minute intervals until FEV\textsubscript{1} had fallen by 20% of the baseline value or the subject had inhaled the maximum concentration of methacholine. The methacholine concentration required to produce a 20% drop in FEV\textsubscript{1} was designated PC\textsubscript{20} and expressed in mg/mL. In keeping with the ATS guidelines (22), methacholine challenge was considered negative if the PC\textsubscript{20} FEV\textsubscript{1} was higher than 16 mg/mL. In all patients, the methacholine dose-response ratio (DRR) was calculated as the percentage fall in FEV\textsubscript{1} at the last dose, divided by the total dose administered (15).

Sputum induction and processing

Sputum induction was performed by inhaling increasing concentrations of hypertonic saline (3%, 4%, and 5%) for 5 minutes each. The nebulized solution was generated by an OMRON ultrasonic nebulizer (OMRON EUROPE BV, Hoofddorp, The Netherlands) with an output of 1 mL/min. FEV\textsubscript{1} was measured at the start and at completion of each inhalation period to ensure the patient’s safety. Patients were asked to blow their nose, rinse their mouth, and swallow water to minimize contamination with post-nasal drip and saliva. They were instructed to cough into a sterile container, and the expectorate was processed within 2 h. Sputum examination was performed as described by Pizzichini et al. (10).
In accordance with other authors (23-24), we considered that a patient had eosinophilic inflammation when induced sputum eosinophil count was >2%, neutrophilic inflammation when neutrophil count was >60% and mixed inflammation when eosinophil count was >2% and neutrophil count >60%. In cases showing an eosinophil count <2% and neutrophil count <60%, patients were considered to have paucigranulocytic inflammation.

**Statistical analysis**

Analyses of the methacholine PC$_{20}$ and DRR were carried out on log-transformed data. Results are presented as the geometric mean (95% confidence interval [CI]) for PC$_{20}$ and the DRR, and the median (range) for all other variables, unless otherwise stated. Differences between groups were analyzed using the Kruskal-Wallis test. Spearman’s rank correlation test was applied to determine correlations between the various parameters studied. SPSS 11.0 for Windows (SPSS, Inc, Chicago, IL) was used for the statistical analysis.

**Results**

Of the original 147 patients assessed for eligibility (Figure 1), 84 were able to produce sputum, yielding a success rate of 69%. The baseline characteristics of the 84 asthma patients ultimately included in the study and the 46 who could not produce sputum are shown in Table 1. There were no significant differences in the baseline characteristics between the two groups of patients. The median (range) age of the 84 asthma patients studied was 41.5 (18 – 65) years and the median (range) duration of their condition was 6 (1 – 51) years. Eighteen patients tested negative on methacholine challenge. In patients testing positive, the mean duration of their condition was longer, and they had a higher percentage of eosinophils in sputum and lower FEV$_1$ values than those testing negative (Table 2). Patients with a positive methacholine result and PC$_{20}$ $\leq$4 mg/mL showed significantly lower FEV$_1$ and FEV$_1$/FVC% findings and a higher eosinophil count than those with a PC$_{20}$ of >4 to 16 mg/mL (Table 2). A significant correlation was found between sputum eosinophil percentage and the DRR ($r=0.477$, $p<0.0001$) (Figure 2) or PC$_{20}$ ($r=-0.517$, $p<0.0001$). A total of 18 patients were smokers at the time of the
Inflammation in well-controlled asthma

study. There were no significant differences in the presence or type of inflammation observed (neutrophilic or eosinophilic) between smokers and non-smokers.

Persistent bronchial inflammation was documented in 59 patients, and was classified as eosinophilic in 28, neutrophilic in 28, and mixed in 3. The inflammation was considered paucigranulocytic in 25 patients. Results for the variables studied according to the type of inflammation (neutrophilic, eosinophilic, or paucigranulocytic) are shown in Table 3. Significantly lower PC\textsubscript{20} values were only found in patients with a positive methacholine challenge and eosinophilic inflammation relative to those with a neutrophilic or paucigranulocytic type.

![Figure 2](image_url)

**Figure 2.** Relationship between methacholine dose/response ratio and percentage of eosinophils.
<table>
<thead>
<tr>
<th></th>
<th>Patients with sputum</th>
<th>Patients without sputum</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 84</td>
<td>n = 46</td>
<td></td>
</tr>
<tr>
<td>Sex, n of subjects</td>
<td>43 / 41</td>
<td>19 / 27</td>
<td>0.323</td>
</tr>
<tr>
<td>Age, y</td>
<td>41.5 (18 - 65)</td>
<td>41.7 (20 – 65)</td>
<td>0.570</td>
</tr>
<tr>
<td>Positive atopic status,</td>
<td>51 (60%)</td>
<td>26 (57%)</td>
<td>0.225</td>
</tr>
<tr>
<td>Years of asthma</td>
<td>6 (1 - 51)</td>
<td>7 (1 – 62)</td>
<td>0.125</td>
</tr>
<tr>
<td>Smoking habit, n (%)/mean (SD) pack-years</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never smoker</td>
<td>49 (58%)</td>
<td>28 (61%)</td>
<td>0.630</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>17 (20%)/ 12.92 (9.30)</td>
<td>8 (17%)/ 12.07 (7.86)</td>
<td>0.325</td>
</tr>
<tr>
<td>Smoker</td>
<td>18 (21%)/ 24.18 (32.49)</td>
<td>10 (22%)/ 24.52 (17.37)</td>
<td>0.230</td>
</tr>
<tr>
<td>Daily IC dose (µg budesonide equivalent) mean (range)</td>
<td>392 (200-800)</td>
<td>345 (200 - 800)</td>
<td>0.430</td>
</tr>
<tr>
<td>Use of LABA, n (%) subjects</td>
<td>26 (30.5%)</td>
<td>16 (35%)</td>
<td>0.565</td>
</tr>
<tr>
<td>Baseline FVC % predicted</td>
<td>90 (67 - 141)</td>
<td>92.4 (58 - 114)</td>
<td>0.467</td>
</tr>
<tr>
<td>Baseline FEV₁ % predicted</td>
<td>89.5 (70 - 137)</td>
<td>93.3 (70 - 125)</td>
<td>0.120</td>
</tr>
<tr>
<td>Baseline FEV₁/FVC</td>
<td>78 (54 - 93)</td>
<td>79.9 (53 - 94)</td>
<td>0.265</td>
</tr>
<tr>
<td>Methacholine challenge:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive: n (%) of subjects/ PC₂₀, mg/mL</td>
<td>66 (79%) / 8.04 (6.63 - 9.45)</td>
<td>36 (78%) / 8.5 (6.06 - 9.20)</td>
<td>0.312</td>
</tr>
<tr>
<td>Negative: n (%) of subjects</td>
<td>18 (21%)</td>
<td>10 (22%)</td>
<td>0.420</td>
</tr>
<tr>
<td>Methacholine DRR</td>
<td>11.46 (5.99 - 16.93)</td>
<td>----</td>
<td>-----</td>
</tr>
<tr>
<td>Sputum eosinophils, %</td>
<td>1 (0-64)</td>
<td>----</td>
<td>-----</td>
</tr>
<tr>
<td>Sputum neutrophils, %</td>
<td>52 (1-98)</td>
<td>----</td>
<td>-----</td>
</tr>
<tr>
<td>ACQ</td>
<td>0.45 (0 - 0.75)</td>
<td>0.52 (0 - 0.75)</td>
<td>0.215</td>
</tr>
</tbody>
</table>

Abbreviations: ACQ, asthma control questionnaire; DRR, dose/response ratio; IC, inhaled corticosteroid; LABA, long-acting beta agonist
Data are presented as the geometric mean (95% confidence interval) for methacholine PC₂₀ and DRR, and the median (range) for the remaining variables, unless otherwise indicated.
Table 2. Analysis of the variables in relation to qualitative methacholine assessment

<table>
<thead>
<tr>
<th>Variable</th>
<th>Methacholine</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive PC$_{20} \leq 16$ mg/mL</td>
<td>Positive PC$_{20} &gt; 16$ mg/mL</td>
<td>P</td>
<td>Positive PC$_{20}$: 0 – 4 mg/mL</td>
</tr>
<tr>
<td></td>
<td>n = 66</td>
<td>n = 18</td>
<td></td>
<td>n = 31</td>
</tr>
<tr>
<td>Sex, M/F</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>41.5 (20 - 65)</td>
<td>42.0 (18 - 64)</td>
<td>0.600</td>
<td>42 (20 - 65)</td>
</tr>
<tr>
<td>Years of asthma</td>
<td>10.7 (1 - 51)</td>
<td>3.0 (1 - 20)</td>
<td>0.009</td>
<td>10 (1 - 51)</td>
</tr>
<tr>
<td>FVC, % predicted</td>
<td>88.5 (67 - 141)</td>
<td>97.5 (77 - 112)</td>
<td>0.069</td>
<td>84 (74 - 141)</td>
</tr>
<tr>
<td>FEV$_1$, % predicted</td>
<td>88.0 (70 - 137)</td>
<td>99.5 (70 - 125)</td>
<td>0.014</td>
<td>85 (70 - 137)</td>
</tr>
<tr>
<td>FEV$_1$/FVC, %</td>
<td>77.0 (54 - 93)</td>
<td>80.0 (72 - 91)</td>
<td>0.074</td>
<td>74 (54 - 91)</td>
</tr>
<tr>
<td>Sputum eosinophils, %</td>
<td>4.0 (0 - 64)</td>
<td>1.0 (0 - 3)</td>
<td>0.003</td>
<td>2 (0 – 64)</td>
</tr>
<tr>
<td>Sputum neutrophils, %</td>
<td>50.0 (1 - 98)</td>
<td>58.0 (15 - 97)</td>
<td>0.091</td>
<td>42 (7 - 95)</td>
</tr>
<tr>
<td>Smoking habit, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never smoker</td>
<td>38 (57%)</td>
<td>11 (61%)</td>
<td>0.715</td>
<td>13 (42%)</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>13 (20%)</td>
<td>4 (22%)</td>
<td>0.521</td>
<td>11 (35%)</td>
</tr>
<tr>
<td>Smoker</td>
<td>15 (23%)</td>
<td>3 (17%)</td>
<td>0.315</td>
<td>7 (23%)</td>
</tr>
<tr>
<td>Atopic status:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive, n (%) of subjects</td>
<td>41 (63%)</td>
<td>10 (53%)</td>
<td>0.319</td>
<td>18 (58%)</td>
</tr>
<tr>
<td>Daily IC dose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µg budesonide equivalent)</td>
<td>400 (200 - 800)</td>
<td>300 (200 - 800)</td>
<td>0.283</td>
<td>400 (20 - 800)</td>
</tr>
<tr>
<td>Use of LABA, n (%) subjects</td>
<td>22 (33.3%)</td>
<td>4 (22.2%)</td>
<td>0.263</td>
<td>9 (29.0%)</td>
</tr>
</tbody>
</table>

Abbreviations: IC, inhaled corticosteroid; LABA, long-acting beta agonist. Data are expressed as median (range) for all variables unless otherwise indicated.
Discussion

The most relevant findings in this study are the persistent bronchial inflammation seen in patients with asthma treated with inhaled corticosteroids and in a controlled phase of the disease, the high percentage of patients with neutrophilic inflammation, and the presence of a greater degree of airway hyperreactivity in patients with eosinophilic asthma. Although similar findings have been found in patients with asthma during the diagnostic process, this is the first reported data in patients with well-controlled asthma after years of disease evolution.

Perhaps one of the most important advances in the treatment of asthma occurred when the inflammatory component of the condition was demonstrated and found to be satisfactorily treated with inhaled corticosteroids. Early pathologic studies in patients with mild asthma who were not treated with corticosteroids reported high eosinophil and lymphocyte counts in the mucosa of the large airways. The number of these cells decreased significantly and overall lung function improved following high doses of inhaled corticosteroids (25-26). Later studies showed that better asthma control is obtained when treatment is adjusted to the number of eosinophils observed in samples of induced sputum, in conjunction with the general recommendations based on clinical and spirometric data (4-5). Although a recent Cochrane review has established this as a demonstrated fact (6), this approach can be difficult to apply in daily practice, and this may be explain, in part, the findings of the present study.

Treatment adjustment and asthma control in our study were carried out in accordance with the GINA strategy, which is essentially based on clinical factors (2). Therefore, it is not surprising that bronchial inflammation was detected in 66% of the cross-sectional population studied, despite the fact that their condition could be considered well controlled from the clinical viewpoint (ACQ <0.75 and no exacerbations in the previous year). Similar findings have been reported by other authors. In a population of 76 adult asthma patients with total asthma control based on GINA/NIH guidelines, Hanxiang et al. (16) observed higher levels of eosinophils, eosinophil cationic protein and IL-5 in comparison with healthy subjects, suggesting that airway inflammation persisted during total asthma control. Madhuban et al. (27), reported similar results in a pediatric
population with well-controlled asthma and presenting exercise-induced bronchoconstriction. Finally, Leuppi et al. (15) found that airway hyperresponsiveness in 31 clinically well controlled asthmatics appeared to be independent of eosinophilic airway inflammation. Although Madhuban et al. (27) establish asthma control from ACQ, to our knowledge, this is the first study investigating the relationship between asthma control according to the ACQ, and objective markers of airway inflammation in an adult population. Therefore, probably there is a need for studies investigating this relationship.

What is more uncertain is the significance of the persistent inflammation in these patients. Our study patients had been asymptomatic over the previous year and were using low or moderate doses of inhaled corticosteroids. It could be expected that if treatment had been adjusted based on the degree of inflammation, higher doses of corticosteroids would be administered and the patient would therefore be classified as having severe disease; however, the practical benefits would not have been greater than those observed, at least in terms of immediate control of the condition. Our study design does not enable future risk to be established in these patients. Thus, we cannot know whether patients who present greater bronchial inflammation despite their current disease control will have a greater risk of experiencing exacerbations or deteriorated lung function later in life (3).

Another interesting finding from the present study was the fact that 33% of patients presented neutrophilic asthma. At least three pathological phenotypes of asthma have been proposed based on the predominant cell type involved: eosinophilic, neutrophilic, and paucigranulocytic (7). Several authors (24, 28, 29) have suggested that many patients with neutrophilic inflammation can have concomitant eosinophilic inflammation, but this was not the case of our series, in which only 3 of the patients included showed mixed inflammation. Although a role for neutrophilic inflammation has been established in severe asthma (30) and asthma exacerbations (31), neutrophils are not always considered important in stable asthma. Our findings are not consistent with this idea, and other studies in which neutrophils were found to be the dominant inflammatory cell in sputum of subjects with mild to moderate asthma (29, 32) support our results.
It is possible that the neutrophilia found in some of our patients could be related to their smoking habit. Smoking is surprisingly common in asthma patients, with a prevalence relatively close to that of the general population (33-34), and a recent study has shown that the induced sputum neutrophil count in asthma patients who smoke is higher than in those who do not (35). Moreover the levels of sputum eosinophil counts are reduced in smokers probably because the exogenous nitric oxide in cigarette smoke increasing the apoptosis of activated eosinophils (36). Nonetheless, only 18 patients in our population were smoking at the time of the study and there were no significant differences in this group regarding the neutrophilic or eosinophilic status of inflammation. Some authors have hypothesized that smokers with neutrophilic asthma could have a low response to corticoid treatment; thus, smoking would be a risk factor for severe asthma (37). The present study was not designed to respond to this question because our patients all had well-controlled asthma (ACQ <0.75). We can affirm, however, that it is possible to achieve disease control in at least some patients with mild or moderate asthma even though they may be smokers and present neutrophilic bronchial inflammation.

Other factors with a possible influence on neutrophilia include corticosteroid treatment exposure to air pollution, and the presence of airway infections (7). All patients were taking corticosteroids, however, at least over the previous year. This fact could have an effect on neutrophilia in two ways, by sustaining the life of neutrophils or by eliminating eosinophils in patients who initially had mixed inflammation. In this sense Cowan DC et al., assessing the inflammatory cell phenotypes in asthma after eliminating potentially confounding effects found that corticosteroid treatment was associated with increased airway neutrophils and a switch to a neutrophilic phenotype in some patients (38). Our study design did not enable assessment of the effect of pollution, and infections are unlikely because all patients were stable during the study and none had experienced exacerbations in the previous year as a requirement for inclusion. Although exacerbation is not synonymous with infection, we believe it is unlikely that infection had an influence on the neutrophilia observed.

Similar to induced sputum eosinophil count, adjusting asthma treatment according to bronchial hyperresponsiveness (BHR) measurement has been proposed as a better
method for achieving asthma control than the use of conventional guidelines (39). However, in this case, control is attained with administration of high doses of inhaled corticosteroids, without significant changes in the degree of BHR. These findings support the notion that several factors can contribute to direct BHR, and these can be broadly grouped into two categories: persistent and variable (40). The persistent contribution to BHR has been attributed to structural changes in the airway, such as subendothelial thickening, subbasement membrane thickening, smooth muscle hypertrophy, matrix deposition, and altered vascular components. The variable aspect is believed to relate to inflammatory airway events, which vary and are influenced by infection and environmental factors such as pollution.

The persistent factors are likely those that best explain the enduring BHR in the present study. BHR to methacholine was present in most patients, even though they had been under lengthy treatment with inhaled corticosteroids. The fact that all the patients had well-controlled asthma and were stable for at least one year prior to inclusion upholds the idea that therapy with inhaled corticosteroids and occasional LABA use can control the variable factors, whereas the persistent factors remain and perpetuate hyperresponsiveness. In this regard, it is important to note that patients testing positive on methacholine challenge had a longer history of asthma and a lower FEV₁, with no differences in age, compared to patients testing negative. Similar findings were obtained by McGrath KW et al. in a sample of over 300 subjects who report a history of asthma. The authors found that 27% had a negative methacholine challenge test. Compared to the methacholine positive subgroup, the clinical characteristics of the methacholine negative subgroup included an older age of asthma onset and better lung function (41).

It is also interesting that the degree of BHR was much higher in patients with eosinophilic inflammation. This finding suggests that when BHR persists despite inhaled corticosteroids, it will be more severe in patients with eosinophilic inflammation than in those with neutrophilic inflammation or normal induced sputum eosinophil and neutrophil counts. It has been reported that patients with severe corticosteroid-dependent asthma showing eosinophilic inflammation required more intubations than those with other types of inflammation (42). This could be because eosinophilic inflammation might cause greater changes in the airway architecture (e.g.
subendothelial thickening, subbasement membrane thickening, smooth muscle hypertrophy), which would contribute to perpetuating BHR, although this hypothesis remains to be demonstrated.

Some limitations of the present study should be mentioned. First, it was assumed that patients adhered to treatment. We cannot exclude the possibility that treatment compliance was not optimal in some patients because of the absence of symptoms, and that this may have been the cause of eosinophilia and BHR in some cases. Second, the inclusion of smokers could be the cause of the neutrophilia found in some of our patients and even may cause decreased levels of eosinophils. Lastly, it should be noted that the ACQ was designed for patient follow-up, whereas in this study it was used at one time point to reinforce the clinical evidence that the patient’s condition was controlled.

In conclusion, eosinophilic or neutrophilic inflammation persisted in most well-controlled asthma patients despite the fact that their condition was controlled. Direct airway hyperreactivity was documented in nearly all the patients and was higher in those with eosinophilic inflammation.

Acknowledgment: This project was supported by a research grant from Ciber Enfermedades Respiratorias (CibeRes) (Instituto de Salud Carlos III).
References


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IMPACT OF AGE ON PH, 8-ISOPROSTANE AND NITROGEN OXIDES IN EXHALED BREATH CONDENSATE

Cruz MJ, Sánchez-Vidaurre S, Romero PV, Morell F, Muñoz X

*Chest 2009;135(2):462-7. IF: 6.63*
Abstract

Background: Few studies have addressed the effects of aging on levels of inflammatory markers in exhaled breath condensate (EBC). The aim of this study was to determine whether there are significant age-associated differences in pH, 8-isoprostane, and nitrogen oxide values in EBC from a population of healthy adults.

Material and methods: EBC samples were obtained from 75 healthy volunteers aged 18 to 80 years and stratified into five age groups (n=15): 18-29, 30-39, 40-49, 50-59 and 60-80. The following were measured in the samples collected: pH before and after deaeration, nitrite, nitrate, and 8-isoprostane. Differences between the groups were assessed by the Kruskal-Wallis test.

Results: Significant differences in deaerated pH ($P<0.0001$) were found in the group of individuals 60 to 80 years of age as compared to the remaining groups. Significant differences were also found in 8-isoprostane levels between the younger and older groups (18 to 29 years and 30 to 39 years of age; $P=0.006$ and $P=0.034$, respectively). There were no significant differences in nitrite or nitrate values between younger and older individuals.

Conclusion: The results of this study indicate that pH and 8-isoprostane levels in EBC show a relationship with age. Thus, values obtained in studies with control groups may require adjustment for these factors.
Introduction

Airway inflammation has a central role in the development and progression of many lung diseases (1, 2). Exhaled breath contains aerosols and vapors that can be condensed by breathing through a cooling system and analyzed to non-invasively elucidate physiologic and pathologic processes in the lung (3). Although exhaled breath condensate (EBC) is predominantly derived from water vapor, several non-volatile and volatile compounds are dissolved in it, such as ions, proteins, oxidation products and other substances that can reflect lower respiratory tract changes (4). Measurement of markers in EBC has proven to be a useful, non-invasive method for assessing and monitoring airway inflammation (3).

Several mediators implicated in the inflammatory cascade have been detected in EBC, including prostaglandins (5, 6), nitrogen oxides (7, 8), 8-isoprostane (9), and cytokines (10-11). Endogenous airway acidification, as assessed by pH (predominantly controlled by volatile components), and oxidative stress, defined as increased exposure to oxidants or decreased antioxidant capacity, are also implicated in inflammatory airway disease (12-13). The biomarkers of oxidative stress are elevated in EBC of patients with many lung diseases, including asthma and COPD (14). But analysis of EBC is not limited to monitoring patients and elucidating the mechanisms of lung disease. It may also become a useful tool for screening healthy individuals for possible early pulmonary tissue damage (3).

While EBC shows promise as a source of biomarkers in lung disease, substantial variability has been reported in the concentration of solutes in EBC samples, with considerable overlap between normal subjects and disease groups. Nevertheless, to our knowledge, only two studies have analyzed the influence of age on levels of exhaled markers (15-16), and the normal age-related range has only been established for pH values. The aim of the present study was to determine whether there are significant age-associated differences in pH values, 8-isoprostanone, and nitrogen oxides in EBC from a population of healthy adults in different age groups.
Material and methods

Study population

Seventy-five healthy, non-smoking subjects participated in the study. The age range was 18 to 80 years (n=15), stratified into five age groups: 18 to 29 years (group 1), 30 to 39 years (group 2), 40 to 49 years (group 3), 50 to 59 years (group 4), and 60 to 80 years (group 5). The 18- to 65-year-olds were recruited from the staff of our department. The population from 65 to 80 years old was a group of retired individuals. Subjects were excluded from the study if they had a history of asthma, allergy, COPD or other lung diseases, and if they had experienced a respiratory tract infection during the last month prior to the study, physician-diagnosed gastroesophageal reflux disease, or any acute or chronic systemic illness. None of the participants were taking any medication.

In all subjects, the clinical examination, spirometry, and x-ray study were normal. All signed informed consent documents for participation. The local Ethics Committee approved the study.

Exhaled breath concentrate collection

EBC was collected during tidal breathing with a commercially available condenser (EcoScreen; Jaeger, Würzburg, Germany), as described (3). Exhaled air entered and left the chamber through one-way valves at the inlet and outlet, thus maintaining the chamber closed. Subjects breathed tidally through a mouthpiece connected to the condenser while wearing nose clips. The low temperature within the condensing chamber throughout the collection time cooled the sample. Samples were collected in sampling tubes, which, prior to use, had been disinfected for 30 minutes using sodium dichloroisocyanurate (Inibsa Lab, Barcelona, Spain), and rinsed for 24 h with distilled water and 2 h with ultra pure water (Fresenius Kabi; Barcelona, Spain). To determine the ventilatory pattern, a spirometer (EcoVent; Jaeger, Würzburg, Germany) was connected to the equipment at the expiratory valve. The spirometer recorded the total exhaled breath, time of collection, tidal volume (Vt), minute ventilation (MV), and breathing frequency (BF). Subjects were instructed to refrain from food intake during the 2 h prior to sample collection. A fixed volume of 150 L of exhaled breath was collected per subject. Each EBC sample was divided into 500 µL aliquots in two to four
plastic tubes. The aliquots, used for measuring nitrite, nitrate, and 8-isoprostane were immediately stored at −70°C, and analyzed within 1 month of collection. Other aliquots were used to measure the pH before and after deaeration.

**pH measurement**

pH was measured in one of the aliquots immediately after EBC collection and after deaerating with helium (350 mL/min for 10 min), using a calibrated pH meter (Model GLP 21; Crison Instruments SA; Barcelona, Spain) with an accuracy of ±0.01 pH, and a probe for small volumes (Crison 50 28; Crison Instruments SA). The probe was calibrated daily with standard pH 7.02 and 4.00 buffers (12).

**EBC nitrate, nitrite, and 8-isoprostane**

Nitrate ($\text{NO}_2^-$) and nitrite ($\text{NO}_3^-$) concentrations were determined with a colorimetric assay based on the Griess reaction in which sample duplicates were reacted with Griess reagent (Cayman Chemical; Ann Arbor, MI) and measured at 540 nm absorbance with a microplate reader. Assay sensitivity was 1 µmol/L for nitrite and 2.5 µmol/L for nitrate. Within-run were 6%, and 4%, and between-run coefficients of variation were 9% and 5% for nitrate and nitrite, respectively. The 8-isoprostane concentration was determined by a competitive enzyme immunoassay using a commercially available kit (Cayman Chemical). Assay sensitivity was 4 pg/mL (3). Within-run and between-run coefficients of variation were 11% and 15%, respectively.

**Statistical analysis**

Data are presented as the median and range. A one-sample Kolmogorov-Smirnov test was calculated to assess normality. The Kolmogorov-Smirnov test showed non-normal distribution of the parameters studied; hence, differences between groups were analyzed by the Kruskal-Wallis test. Spearman’s rank correlation test was applied to determine correlations between the various parameters studied. Non-detectable 8-isoprostane concentrations were assigned the value of the detection limit of the method (4 pg/mL). Statistical software (SPSS release 11.0 for Windows; SPSS; Chicago, IL) was used for the statistical analyses.
Results

Demographic data and parameters recorded during EBC collection

Demographic data and parameters recorded during EBC collection are summarized in Table 1. EBC was collected without discomfort in all cases, and no adverse effects were documented in any subject over the course of the study. The mean volume of EBC collected from the 75 participants was 2.4 mL (range, 1 to 3.2 mL), and the time required to collect this volume was 5.38 to 20.15 min. Vt values were significantly lower in the oldest age group as compared to the remaining groups (Table 1). No differences were found in the volume, collection time, peak expiratory flow (PEF), MV, or BF between the various age groups in the study population.

pH measurement

pH results are summarized in Table 2 and Figure 1. No significant differences were found in the pH values before deaeration among the study groups. Nevertheless, after deaeration, the 60- to 80-year age group had significantly lower pH values as compared to the remaining groups (P<0.001). There were no significant differences in pH values between the other groups (Figure 1).

EBC nitrate, nitrite, and 8-isoprostane

Nitrate and nitrite were detectable in exhaled breath condensate of all subjects and no significant differences were seen between any of the age groups. 8-isoprostane was detectable in EBC of all subjects in the oldest group (group 5). In the remaining groups, 8-isoprostane was undetectable in nine subjects (60%) in group 1, five subjects (33%) in group 2, four subjects (27%) in group 3, and two subjects (13%) in group 4. Significant differences were found in 8-isoprostane levels between the youngest groups (groups 1 and 2) and the oldest group (group 5) (P=0.006 and P=0.034, respectively). Groups 3 and 4 also showed differences relative to group 5, although they did not reach statistical significance (P=0.051 and P=0.062, respectively). There were no significant differences according to sex in pH before and after deaeration, nitrite, nitrate, or 8-isoprostane values.
### Table 1. Characteristics of study population

<table>
<thead>
<tr>
<th>Age range (yr)</th>
<th>18-29</th>
<th>30-39</th>
<th>40-49</th>
<th>50-59</th>
<th>60-80</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (Female/male)</td>
<td>15 (13 / 2)</td>
<td>15 (9 / 6)</td>
<td>15 (5 / 10)</td>
<td>15 (9 / 6)</td>
<td>15 (13 / 2)</td>
</tr>
<tr>
<td>Mean age ± SD</td>
<td>25.13 ± 2.61</td>
<td>34.13 ± 2.99</td>
<td>43.46 ± 2.97</td>
<td>54.93 ± 3.15</td>
<td>71.20 ± 5.60</td>
</tr>
<tr>
<td>EBC Vol (mL)</td>
<td>2.30 (1.60 – 2.98)</td>
<td>2.48 (1.70 – 3.20)</td>
<td>2.17 (1.00 – 3.15)</td>
<td>2.37 (1.71 – 2.80)</td>
<td>2.53 (1.50 – 3.19)</td>
</tr>
<tr>
<td>Vt (L)</td>
<td>0.64 (0.36 – 1.93)</td>
<td>0.84 (0.44 – 1.43)</td>
<td>0.95 (0.51 – 2.00)</td>
<td>0.65 (0.33 – 2.00)</td>
<td>0.51* (0.30 – 1.08)</td>
</tr>
<tr>
<td>MV (L/min)</td>
<td>12.99 (6.10 – 21.90)</td>
<td>10.20 (4.80 – 22.20)</td>
<td>15.9 (5.90 – 29.70)</td>
<td>11.10 (7.20 – 43.00)</td>
<td>11.10 (7.00 – 16.20)</td>
</tr>
</tbody>
</table>

Ventilatory pattern: values are expressed as median (range) unless otherwise indicated. Vt, tidal volume; MV, minute ventilation; BF, breathing frequency; EBC Vol, Exhaled breath condensate volume.
Table 2. Values of the parameters studied in the different age groups

<table>
<thead>
<tr>
<th>Age range (yr)</th>
<th>18-29</th>
<th>30-39</th>
<th>40-49</th>
<th>50-59</th>
<th>60-80</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pH</strong></td>
<td>6.67</td>
<td>6.75</td>
<td>7.08</td>
<td>7.13</td>
<td>6.86</td>
</tr>
<tr>
<td></td>
<td>(6.05 – 7.54)</td>
<td>(5.54 – 7.85)</td>
<td>(6.22 – 7.77)</td>
<td>(6.18 – 7.67)</td>
<td>(5.28 – 7.81)</td>
</tr>
<tr>
<td><strong>Deaerated pH</strong></td>
<td>8.12</td>
<td>8.21</td>
<td>8.20</td>
<td>8.25</td>
<td>7.74</td>
</tr>
<tr>
<td></td>
<td>(7.23 – 8.84)</td>
<td>(6.74 – 8.64)</td>
<td>(7.75 – 8.68)</td>
<td>(7.94 – 8.60)</td>
<td>(5.52 – 8.23)</td>
</tr>
<tr>
<td><strong>8-isoprostane, pg/mL</strong></td>
<td>4.00</td>
<td>6.41</td>
<td>7.85</td>
<td>9.11</td>
<td>9.90</td>
</tr>
<tr>
<td></td>
<td>(4.00 – 12.94)</td>
<td>(4.00 – 14.34)</td>
<td>(4.00 – 13.27)</td>
<td>(4.00 – 12.33)</td>
<td>(6.76 – 16.71)</td>
</tr>
<tr>
<td><strong>Nitrite, µmol/L</strong></td>
<td>5.77</td>
<td>4.28</td>
<td>5.18</td>
<td>4.85</td>
<td>3.21</td>
</tr>
<tr>
<td></td>
<td>(2.42 – 15.32)</td>
<td>(1.04 – 25.34)</td>
<td>(1.88 – 22.77)</td>
<td>(1.03 – 19.54)</td>
<td>(1.70 – 26.36)</td>
</tr>
<tr>
<td><strong>Nitrate, µmol/L</strong></td>
<td>10.56</td>
<td>13.52</td>
<td>10.04</td>
<td>12.39</td>
<td>10.82</td>
</tr>
<tr>
<td></td>
<td>(1.90 – 67.80)</td>
<td>(3.29 – 39.00)</td>
<td>(2.26 – 34.30)</td>
<td>(2.57 – 47.47)</td>
<td>(3.05 – 35.79)</td>
</tr>
</tbody>
</table>

Values expressed as median (range).

Figure 1. EBC pH values, 8-isoprostane and nitrogen oxides in the groups studied. Significant differences were found in the de-aerated pH values between the oldest group as compared to the remaining groups ($P<0.001$); and in 8-isoprostane levels between the two youngest groups (groups 1 and 2) and the oldest group (group 5) ($P=0.006$ and $P=0.034$, respectively).
Discussion

The present study suggests that aging has an effect on EBC pH and 8-isoprostane values. The EBC pH is the result of a balance among several buffer systems, and the production and release of acids and bases in the airways (17, 18). NH$_4^+$ and HCO$_3^-$ have an important effect on the pH of these solutions in normal EBC (19). Some authors (20) have suggested that the main source of NH$_3$ in EBC is the mouth, and that contamination with oral ammonia would have an important alkalinizing effect in EBC. Others (21) have reported that the effect of oral ammonia on the EBC pH is uncertain. The equipment used in this study is constructed in a way that the sampling tube initiates within the mouthpiece so that saliva cannot reach the tube but, instead, is trapped in a cylinder covering the tube, thereby obviating an effect of salivary contamination on the measurements (22). However, pH is controlled by volatiles and these volatiles may arise from the mouth in part. Although a saliva trap will not avoid oral volatiles, the system used can minimize their effect and any impact on the results by oral contamination.

In this aqueous environment, exhaled CO$_2$ from H$^+$ and HCO$_3^-$ profoundly affects the pH of EBC. Deaeration with an inert gas causes a significant decrease in CO$_2$ content (23, 24). After deaeration, the pH reflects the status of the fluid lining the airway (12). In fact, in the present study, significantly lower pH values were observed after deaeration in the 60- to 80-year age group as compared to the other groups, whereas these differences were not observed before deaeration.

The absence of an increase in pH values after deaeration in the older group may be due to the fact that changes occur in the cellular pattern of the airways with age. According to some authors (12), neutrophils and eosinophils have the greatest influence on EBC pH values. Along this line, Thomas et al. (25) found that the induced sputum differential neutrophil count increased significantly with age in a healthy volunteer population. This increase may be related to the decrease in pH values and could be the result of longstanding environmental exposure. There may also be age-related changes in the immune response of the lung that could lead to an amplification of the response to environmental and other triggers (25). Moreover, oxidative stress is greater with age and
there is an accumulation of metabolic substances that produce a larger amount of non-volatile acids, which do not disappear after deaeration.

To our knowledge, only two studies (15-16) have focused on age-related pH in EBC. In the study of Brooks et al. (15), no relationship was found between pH and age. The differences between the results of that study and ours may be attributable to the lower pH values the authors found in their youngest group, which are outside the reported range (7.4 to 8.8) described by the ATS/ERS Task Force (3). The pH range obtained in the present study was within the reported limits except in four subjects in group 5 and one in group 2. In the study by Paget-Brown et al. (16), median pH after deaeration was 8.0, with a range of 7.8 to 8.1, but 6.4% of the EBC samples had a pH <7.4. This percentage of outlying values is comparable to that obtained in the present study (6.6%). Nevertheless, these authors also concluded that EBC pH is not systematically affected by patient age.

The findings obtained for 8-isoprostane in the present study are similar to those seen for pH. Levels of 8-isoprostane are increased in EBC of patients with several respiratory diseases such as asthma (26), COPD (27), and cystic fibrosis (28). In our study population, significant differences were found in 8-isoprostane levels between the younger groups (groups 1 and 2) and the oldest group (group 5) despite the fact that the values obtained in all the groups studied were within the normal limits described by other authors in the healthy population (3). Moreover, in the present study, the number of samples with detectable values increased with age. According to some authors (3, 29), 8-isoprostane is present in detectable amounts in all normal tissues and biological fluids and has been detected in EBC in healthy subjects, thus allowing definition of the normal range (30). The findings from the present study should be taken into account when establishing the 8-isoprostane normal values.

It is possible that subtle, subclinical disease was present in some of the population of this study, but it is also possible that the differences recorded were produced by “normal” age-related respiratory system changes (31). The lung parenchyma loses its supporting structure, causing senile emphysema (31). This would produce greater
oxidative stress and might be related to changes in the cellular pattern of the airways occurring with aging (12) and to the absence of increased pH values.

No relationships between age and nitrate or nitrite levels were found in this study. Nitrite and nitrate generated by nitric oxide in aqueous media are often considered markers of inflammation (7, 32). It has been shown that nitrite can be consumed by neutrophil myeloperoxidase as a substrate for enzymatic nitration, and when it is protonated, nitrite releases nitric oxide (33, 34). The increase in neutrophil count with aging would lead to higher metabolism of these nitrogen redox forms and increase the levels of exhaled nitric oxide. Because of differences in the techniques used for EBC collection and analysis, comparison of reported nitrite and nitrate concentrations between studies is difficult. Nonetheless, the levels observed in our study are in agreement with those reported by Chladkova et al. (35) and Balint et al. (36) using the EcoScreen condenser, although with different assay methods.

In conclusion, according to the results of this study, pH values and 8-isoprostane levels in EBC appear to be associated with age. These effects would not be disease-specific but they could have important implications for interpreting acidic and oxidative stress, as well as for understanding nitrogen oxide chemistry in the lung of older subjects. Thus, the values obtained in studies with control groups should be adjusted for this factor.
References


SPUTUM INFLAMMATORY PROFILE BEFORE AND AFTER SPECIFIC INHALATION CHALLENGE IN INDIVIDUALS WITH SUSPECTED OCCUPATIONAL ASTHMA

Sánchez-Vidaurre S, Cruz MJ, Gómez-Ollès S, Morell F, Muñoz X

Journal of Allergy and Clinical Immunology (Submitted)
Abstract

**Background:** The aim of this study was to establish the sputum inflammatory profile and changes in levels of leukotriene B$_4$ (LTB$_4$) and a panel of Th1/Th2 cytokines in subjects with suspected occupational asthma (OA) following specific inhalation challenge (SIC) to high-molecular-weight (HMW) and low-molecular-weight (LMW) agents.

**Material and methods:** Fifty-one consecutive subjects undergoing SIC for suspected OA were enrolled. Sputum induction was performed the day before and 24 h after exposure to the offending agent. In all samples, total and differential cell counts were assessed. LTB$_4$ and a 10 Th1/Th2 cytokines were measured in sputum supernatant.

**Results:** Thirty-four patients tested positive to SIC and were diagnosed with OA (in 10 due to HMW agents and in 24 to LMW agents). SIC was negative in 17 subjects (1 had been exposed to a HMW agent and 16 to LMW agents). As compared to baseline an increase was found in the percentage of sputum eosinophils and neutrophils, and in IL-10 concentration after SIC (p=0.0078, p=0.0195, and p=0.046, respectively), and a decrease was seen in LTB$_4$ level (p=0.0078) in patients with OA due to HMW agents. No changes were observed in patients with OA caused by LMW agents. An increase in the percentage of sputum neutrophils after SIC (p=0.0040) was observed in subjects without OA exposed to LMW agents. IL-8 levels after SIC were higher in patients without OA compared with patients with OA (p=0.0146)

**Conclusion:** When conducting airway inflammation studies in OA, patients should be divided according to the causal agent (HMW or LMW) due to the fact that probably more than one mechanism may be involved in the genesis of the disease.
Introduction

As its name indicates, work-related asthma (WRA) is a type of asthma in which the symptoms occur in relation to work (1). The term encompasses both occupational asthma (OA) and work-exacerbated asthma (WEA). OA refers to de novo asthma caused by exposure to an agent specific to a workplace and not to stimuli outside the work environment (2), and WEA is defined as a worsening of pre-existing or concomitant asthma that is exacerbated by working conditions (3).

Traditionally, OA is differentiated into two types according to whether the condition is immunologically mediated or non-immunologically mediated (4). In immunologically mediated OA, also known as ‘OA with latency’, sensitization against a workplace agent occurs after a latency period of months to years. Depending on the molecular weight of the offending agents, immunologically mediated OA can be further divided into two types according to whether the cause is high-molecular-weight (HMW) agents, most of which induce OA via immunoglobulin-E (Ig-E)-dependent mechanisms, or low-molecular-weight (LMW) agents, many of which (though not all) appear to induce OA via unknown pathways that do not involve IgE-dependent mechanisms (5, 6).

Specific inhalation challenge (SIC) may be an effective test for establishing an accurate diagnosis of the different types of WRA (7). Moreover, in the case of OA, SIC may be useful for determining the causal mechanism (6), in particular if analysis of sputum cell counts is performed before and after SIC (8-9). Few studies have evaluated the performance of induced sputum in conjunction with SIC: some of them are case reports (10-13), others are clinical series with small numbers of participants (8, 14-17), and in only two clinical series (9, 18) is OA divided into HMW- and LMW-induced asthma.

The aim of this study was to establish the sputum inflammatory cell profile in subjects with suspected OA following SIC to HMW and LMW agents and to determine possible changes in the levels of leukotriene B₄ (LTB₄) and a panel of Th1/Th2 cytokines. Information in this line can help to clarify the pathophysiological mechanisms involved in the genesis of OA, in particular OA caused by LMW agents.
Material and methods

Subjects
The study included all individuals older than 18 years of age referred to our center for SIC to investigate possible OA in the period of 2005 to 2010, and from whom adequate sputum samples could be obtained before and after SIC. All subjects had a medical history consistent with OA and a workplace agent was considered the probable cause of their respiratory symptoms. Concurrent treatment with anti-inflammatory drugs was maintained at the same level during the study. None of the patients were receiving leukotriene modifiers or non-steroidal anti-inflammatory drugs. Long- and short-acting β₂-agonists were stopped at least 24 h and 6 h before SIC, respectively.

Subjects testing positive to SIC were diagnosed with OA and were advised to avoid exposure to the offending agent. Subjects negative on SIC underwent various tests based on clinical suspicion to establish a definite diagnosis. Those who presented symptoms and abnormal findings on pulmonary function study in relation to work received a diagnosis of WEA (3). The local Ethics Committee approved the study, and all subjects signed informed consent documents for participation.

Inhalation Challenges
In each subject, SIC was performed with the suspected offending agent, provided there was no contraindication for this test (19). SIC was carried out according to the guidelines proposed by Pepys et al. (20) and our group (21-22). Briefly, subjects were examined on 5 consecutive days. On the first day (control day), full medical and occupational histories were collected, and skin-prick tests with a battery of common allergens, radiography study, pulmonary function testing, methacholine challenge, and sputum induction were performed. On day 2, a first placebo inhalation challenge was performed. On days 3 and 4, subjects underwent SIC with the suspected workplace agent. On day 5, pulmonary function testing, methacholine challenge and sputum induction were carried out again. Changes in lung function were monitored in each patient by measuring FEV₁ every 10 minutes during the first hour after exposure and then every hour to complete 15 hours after inhalation. Response was considered positive
when FEV₁ fell more than 20% of the baseline value in the absence of any change to placebo.

**Induced sputum collection and processing**

Sputum induction was performed using the method described by Pizzichini *et al.* (23) by inhaling an aerosol of hypertonic saline at increasing concentrations (3%, 4%, and 5%) for 7 minutes per concentration, generated by an OMRON ultrasonic nebulizer (Peróxidos Farmacéuticos S.A., Barcelona, Spain) through a mouthpiece with a nose clip in place. At the end of each 7-min inhalation period, subjects were asked to blow their noses, rinse their mouths with water, and swallow the water before expectorating to minimize contamination with postnasal drip and saliva and decrease the number of squamous cells. Then they were instructed to cough and expectorate sputum into a sterile plastic container. During the procedure, lung function was measured before and after every period of inhalation to ensure the patient’s safety. Sputum induction was stopped if the FEV₁ value fell by at least 20% of baseline, or if troublesome symptoms occurred.

Sputum samples were examined and processed within 2 h, as described by Pizzichini *et al.* (23). Briefly, all opaque and/or dense portions of the expectorate that appeared different from saliva or were free of squamous cell contamination under the inverted microscope were selected, placed in a 15-mL polystyrene tube and weighed. The sample was treated with 0.1% dithiothreitol (DTT) (Sigma-Aldrich, Saint Louis, MO, USA) for 10 minutes and then diluted with phosphate-buffered saline (PBS). The resulting suspension was filtered through a 48-µm nylon mesh and centrifuged at 2500 rpm for 10 minutes. The supernatant was removed and stored in aliquots at -80°C for later measurements. The cell pellet was resuspended in PBS. Cytospin slides were prepared for staining with May Grünwald Giemsa and a differential cell count of 500 non-squamous cells was performed. The percentage of salivary squamous cells was noted and a cut-off of 20% squamous cells was used to define adequate samples. Total cell count, cell viability, and percentage of squamous cells were determined using trypan blue exclusion staining in a Neubauer hematocytometer.
Differential cell count of macrophages, neutrophils, lymphocytes, and eosinophils was carried out by optic microscopy and expressed as a percentage of the total of non-squamous cells. The specimen was considered adequate if total and differential cell counts could be obtained; this required as little as 50 mg of selected material. Results are expressed as the absolute number of cells in millions per mL of sputum sample.

**LTB₄ and cytokine measurements**

LTB₄ concentration in sputum supernatant samples was determined by a commercially available LTB₄ enzyme immunoassay (EIA) kit (Cayman Chemical Company, Ann Arbor, MI). The LTB₄ assay has a detection limit of 13 pg/mL, specificity of 100%, intra-assay coefficient of variation (CV) of 14.87%, and inter-assay CV of 11.84%.

A panel of 10 cytokines, including interferon-gamma (IFN-γ), interleukin (IL)-1beta (β), IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, and tumor necrosis factor-alfa (TNF-α) and -beta (TNF-β), were analyzed by flow cytometry using a commercially available kit (Bender MedSystems GmbH, Vienna, Austria). Assay sensitivity was 1.6, 4.2, 16.4, 20.8, 1.6, 1.2, 0.5, 1.9, 1.5, 3.2, 2.4 pg/mL for each analyte, respectively.

**Statistical Analysis**

The characteristics of the subjects are expressed as the median and range. A one-sample Kolmogorov-Smirnov test, calculated to assess normality, showed non-normal distribution of the parameters studied. Between-group differences were analyzed by the Mann-Whitney test and within-group differences by the Wilcoxon signed rank test. Differences were considered significant at a \( p \) value of ≤0.05. The variable "difference" (VD) for differential cell count, cytokines, and LTB₄ was calculated by subtracting the value before SIC from the value after SIC. SPSS release 17.0 for Windows (SPSS; Chicago, IL) and GraphPad InStat4 (GraphPad Software Inc; San Diego, CA) were used for the statistical analyses.
Results

Study Population
Of the original 175 individuals who were referred for suspected OA, adequate sputum samples before and after SIC were obtained in 51, yielding a success rate of 49% in positive SICs (n=70) and 16% in negative SICs (n=105) (Figure 1). In the 51 subjects assessed in the study, SIC was positive to the workplace agents tested in 34, who were then diagnosed with OA (OA Group), and negative in 17 (NoOA Group). In the negative group, 4 patients received a diagnosis of WEA, 2 patients had asthma unrelated to work, 2 patients had chronic obstructive pulmonary disease (COPD), 2 had bronchiectasis and 7 subjects were considered not to have conclusive pulmonary disease.

Figure 1. Study population and agents tested.
Demographic data and clinical characteristics of the study subjects are summarized in Table 1. Methacholine challenge 24 hours after exposure could not be performed in 11 subjects in the OA group and in 3 in the NoOA Group because FEV$_1$ tested \(<70\%\) the theoretical value following SIC. The percentages of methacholine-positive tests before exposure and 24 h after exposure were higher in the OA Group than NoOA (\(p=0.030\) and \(p=0.043\), respectively). PC$_{20}$ values before exposure and 24 h after exposure differed significantly between the OA group and NoOA group, with lower values in OA (\(p=0.0037\) and \(p=0.0150\), respectively). A PC$_{20}$ decrease of at least two-fold at 24 h occurred in 43.5\% of patients in the OA group and no patients in the NoOA Group (\(p=0.007\)). No significant differences between groups were found for any of the remaining variables analyzed.
Table 1. Characteristics of Subjects Studied

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>OA Group (n = 34)</th>
<th>NoOA Group (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, M/F</td>
<td>28/6</td>
<td>8/9</td>
</tr>
<tr>
<td>Age, yr</td>
<td>40.50 (21 - 59)</td>
<td>41 (18 - 64)</td>
</tr>
<tr>
<td>Smoking habits, NS/exS/CS</td>
<td>11/8/12</td>
<td>4/7/6</td>
</tr>
<tr>
<td>Atopy, n (%)</td>
<td>10 (38.5)</td>
<td>5 (35.7)</td>
</tr>
<tr>
<td>Duration of exposure, m</td>
<td>113 (13 - 533)</td>
<td>121 (5 - 366)</td>
</tr>
<tr>
<td>Duration of symptoms, m</td>
<td>43 (5 - 365)</td>
<td>34 (3 - 266)</td>
</tr>
<tr>
<td>Interval from last exposure, m</td>
<td>1 (0 - 15)</td>
<td>0 (0 - 26)</td>
</tr>
<tr>
<td>Treatment, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhaled corticosteroid</td>
<td>17 (50)</td>
<td>9 (52.9)</td>
</tr>
<tr>
<td>Long-acting β₂-agonist</td>
<td>17 (50)</td>
<td>7 (41.2)</td>
</tr>
<tr>
<td>FEV₁, % predictive before exposure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24h after exposure</td>
<td>85 (70 - 124)</td>
<td>91.3 (71 - 125)</td>
</tr>
<tr>
<td>Methacholine test before exposure, n positive tests, n (%)</td>
<td>34</td>
<td>17</td>
</tr>
<tr>
<td>PC₂₀, mg/mL ≠</td>
<td>27 (79.4)*</td>
<td>8 (47.1)*</td>
</tr>
<tr>
<td>FEV₁, % predictive before exposure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24h after exposure</td>
<td>82.5 (39 - 125)</td>
<td>87 (61 - 125)</td>
</tr>
<tr>
<td>Methacholine test 24h after exposure, n positive tests, n (%)</td>
<td>23</td>
<td>14</td>
</tr>
<tr>
<td>PC₂₀, mg/mL ≠</td>
<td>18 (78.3)*</td>
<td>6 (42.9)*</td>
</tr>
<tr>
<td>PC₂₀ decrease ≥ 2 fold, n (%)</td>
<td>10 (43.5)§</td>
<td>0§</td>
</tr>
<tr>
<td>Occupational agents, HMW/LMW</td>
<td>10/24</td>
<td>1/16</td>
</tr>
<tr>
<td>Type of asthmatic reactions,</td>
<td>10/17/6/1</td>
<td>10/17/6/1</td>
</tr>
<tr>
<td>immediate/late/dual/others</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as No. (%) or median (range), unless otherwise stated. CS, current smoker; F, female; FEV₁, forced expiratory volume in one-second; HMW, high-molecular-weight; LMW, low-molecular-weight; M, male; NoOA, No occupational asthma; NRD, non-respiratory diseases; NS, never smoker; OA, occupational asthma; ORD, other respiratory diseases; PC₂₀, concentration of methacholine inducing a 20% fall in FEV₁; SIC, specific inhalation challenge; exS, ex-smoker.

≠ Only patients with PC₂₀ ≤16 mg/mL
* p = 0.03; ** p = 0.0037, ¥ p = 0.0037, ± p = 0.015, § p = 0.007
**Differential sputum cell counts**

a) Comparison OA group vs NoOA group

Total and differential cell counts (percentage of sputum eosinophils and neutrophils) in the two groups studied (OA/NoOA) are summarized in Table 2. There were no significant within-group differences in sputum differential cell counts depending on smoking status or treatment. Total cell count and sputum neutrophil count after SIC were higher in NoOA compared to OA (p=0.016 and p=0.0170, respectively). In the NoOA group we also found a significant increase in percentage of sputum neutrophils after SIC (p=0.0024). In OA group, there was a non-significant trend towards higher percentages of sputum eosinophils after SIC (p=0.0826). Analysis of the VD of sputum eosinophils and neutrophils between OA and NoOA groups is shown in Figure 2. The sputum eosinophil VD was more elevated in the OA Group than in NoOA (p=0.0287). The sputum neutrophil VD was higher in NoOA compared to OA, although it did not reach statistical significance (p=0.0526).

![Figure 2](image-url)

**Figure 2.** Differential cell count, A) Sputum eosinophils percentages; B) Sputum neutrophils percentages. A: Before SIC; B: After SIC. HMW, High-molecular-weight; LMW, Low-molecular-weight; NoOA, Non-occupational asthma; OA, occupational asthma; SIC, Specific inhalation challenge.
Table 2. Total and differential cell counts in induced sputum

<table>
<thead>
<tr>
<th></th>
<th>Global</th>
<th></th>
<th>HMW Agents* (n = 11)</th>
<th>LMW Agents (n = 40)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before SIC</td>
<td>After SIC</td>
<td>Before SIC</td>
<td>After SIC</td>
</tr>
<tr>
<td>OA Group (n = 34)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCC, x10^6/mL</td>
<td>0.36 (0.03 - 7.74)</td>
<td>0.47** (0.01 - 5.47)</td>
<td>0.38 (0.15 - 7.74)</td>
<td>0.61 (0.12 - 5.47)</td>
</tr>
<tr>
<td>Eosinophils, %</td>
<td>1.00 (0.0 - 52.0)</td>
<td>2.00 (0.0 - 75.0)</td>
<td>0.50‡ (0.0 - 2.0)</td>
<td>2.50‡ (0.0 - 8.0)</td>
</tr>
<tr>
<td>Neutrophils, %</td>
<td>61.00 (1.0 - 96.0)</td>
<td>63.00¥ (2.0 - 98.0)</td>
<td>71.00§ (23.0 - 93.0)</td>
<td>75.00§ ± (24.0 - 97.0)</td>
</tr>
<tr>
<td>NoOA Group (n = 17)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCC, x10^6/mL</td>
<td>0.36 (0.01 - 6.58)</td>
<td>0.71** (0.07 - 44.96)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eosinophils, %</td>
<td>1.00 (0.0 - 26.0)</td>
<td>0.00 (0.0 - 23.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophils, %</td>
<td>70.00† (15.0 - 91.0)</td>
<td>77.00¥† (52.0 - 98.0)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as median (range). HMW, High-molecular-weight; LMW, Low-molecular-weight; OA, occupational asthma; SIC, Specific inhalation challenge; TTC, total cell count. NoOA, No occupational asthma.

* Absence of NoOA HMW Agents Group data because n = 1

** p = 0.016; *** p = 0.0454; ¥ p = 0.0170; † p = 0.0024; ‡ p = 0.0078; § p = 0.0195; # p = 0.0040, ± p = 0.0413
b) Analysis of OA group

Patients with OA due to HMW agents showed a significant increase in percentage of sputum eosinophils and neutrophils after SIC (p=0.0078 and p=0.0195, respectively). This increase was not observed in OA patients exposed to LMW agents (Table 2 and Figure 3). No significant differences in pre-SIC sputum eosinophils or neutrophils were found between patients with OA due to HMW or LMW agents (Table 2) although the highest eosinophil percentages, were observed in OA due to LMW agents, particularly in 4 patients with OA due to isocyanates and in one caused by cyanoacrylate (Figure 3). A higher percentage of sputum neutrophils was found after SIC in patients with HMW-induced asthma compared to LMW-induced asthma (p=0.0413) (Table 2). This difference was not observed in the eosinophil percentages between the two groups. Nevertheless, analysis of the VD of sputum eosinophils showed higher values in patients with OA exposed to HMW agents than those exposed to LMW agents (p=0.0424) (Figure 2).

**Figure 3.** % Sputum cell type after SIC minus % Sputum cell type before SIC, A) Eosinophils Difference; B) Neutrophils Difference. HMW, High-molecular-weight; LMW, Low-molecular-weight; NoOA, Non-occupational asthma; OA, occupational asthma; SIC, Specific inhalation challenge.
c) Analysis of NoOA group
The single subject in this group exposed to a HMW agent was not included in the analysis (Figure 1). In subjects exposed to LMW agents (n=16) we observed a significant increase in the percentage of sputum neutrophils after SIC (p=0.0040) (Table 2 and Figure 3). In this group, 9 individuals were diagnosed with other respiratory diseases, as described above. The median percentage (range) of sputum neutrophils in these 9 subjects was 50.0 (15.0-91.0) before SIC and 86.0 (53.0-95.0) after SIC (p=0.0273). No differences were found in the 7 subjects without conclusive pulmonary disease.

**Measurements in induced sputum supernatant**
Table 3 shows LTB₄ and cytokine levels in the study population. Following SIC, IL-2 and IL-10 concentrations were higher in patients with OA compared to those without (p=0.0040 and p=0.0064, respectively) (Figure 4), whereas IL-8 levels were higher in NoOA patients compared to OA (p=0.0146) (Figure 4).

In the OA group, cytokine levels before SIC did not differ between patients exposed to HMW agents and those exposed to LMW agents. In patients exposed to HMW agents, we observed a decrease in LTB₄ and an increase in IL-10 levels after SIC compared to baseline values (p=0.0313 and p=0.046, respectively) (Table 3 and Figure 5). There was no correlation between LTB₄ or IL-10 levels and eosinophil or neutrophil percentages before and after SIC. In subjects exposed to LMW agents, there were no significant differences in any of the cytokines determined before and after SIC.
Table 3. Markers in sputum supernatant

<table>
<thead>
<tr>
<th>LTB₄ and Citokines (pg/mL)</th>
<th>HMW Agents*</th>
<th>LMW Agents</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive SIC (n = 10)</td>
<td>Positive SIC (n = 24)</td>
</tr>
<tr>
<td></td>
<td>Before SIC</td>
<td>After SIC</td>
</tr>
<tr>
<td>LTB₄</td>
<td>1260.0†</td>
<td>(408.0 - 5449.0)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>14.51</td>
<td>(1.6 – 633.2)</td>
</tr>
<tr>
<td>IL-2</td>
<td>80.82</td>
<td>(17.5 - 616.0)</td>
</tr>
<tr>
<td>IL-10</td>
<td>32.47‡</td>
<td>(1.9 - 61.0)</td>
</tr>
<tr>
<td>IL-8</td>
<td>3283.90</td>
<td>(80.1 - 14812.9)</td>
</tr>
<tr>
<td>IL-6</td>
<td>61.02</td>
<td>(8.7 - 844.9)</td>
</tr>
<tr>
<td>IL-4</td>
<td>28.90</td>
<td>(20.8 - 654.1)</td>
</tr>
<tr>
<td>IL-5</td>
<td>227.82</td>
<td>(28.3 - 678.6)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>106.19</td>
<td>(35.2 - 5014.4)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>70.80</td>
<td>(3.2 - 337.2)</td>
</tr>
<tr>
<td>TNF-β</td>
<td>35.60</td>
<td>(3.2 - 68.0)</td>
</tr>
</tbody>
</table>

Data are presented as median (range). HMW, High-molecular-weight; IFN-γ, Interferon-gamma; IL, Interleukin; LTB₄, Leukotriene B₄; LMW, Low-molecular-weight; SIC, Specific inhalation challenge; TNF-α, Tumor necrosis factor-alpha; TNF-β, Tumor necrosis factor-beta.

* Absence of data on HMW agents in the NoOA (no occupational asthma) group because n=1. p = 0.0313; † p = 0.046
Figure 4. Cytokines in sputum supernatant. NoOA, non-occupational asthma; OA, occupational asthma; SIC, specific inhalation challenge. *p = 0.0040; **p = 0.0064; ***p = 0.0146.

Figure 5. Sputum inflammatory markers in patients with OA exposed to HMW agents. HMW, high-molecular-weight; OA, occupational asthma; SIC, specific inhalation challenge.
Discussion

To our knowledge, this is the first study comparing inflammatory cell percentages and inflammatory markers in sputum samples of subjects with and without OA, distinguishing between HMW-induced and LMW-induced asthma. Increases in sputum eosinophils, neutrophils, and IL-10 concentration and decrease in LTB4 levels were observed in patients with OA due to exposure to HMW agents, but not in those exposed to LMW agents. An increase in sputum neutrophils and IL-8 was also observed in subjects exposed to LMW agents who were ultimately diagnosed as having other respiratory diseases.

Only three studies have evaluated the differences in cell counts in induced sputum before and after SIC in patients with and without OA (8, 14, 17). In all these studies, OA patients exposed to HMW or LMW agents were analyzed together and a significant increase in sputum eosinophils was observed after SIC. This fact has not been observed in the present study and this discrepancy is probably due to their different study populations. In the previously published studies, about 50% of patients were exposed to HMW agents, whereas in the present study they were only 29%. This may be the cause of these differences and conditions that probably the best approach to this analysis is to differentiate between individuals exposed to HMW and LMW. In fact, in the present study, classifying patients according to the type of agent they were exposed, we observed, as already described by other authors (9, 24), an increasing percentage of sputum eosinophils after SIC in OA patients exposed to HMW, a fact that was not found in patients with OA exposed to LMW. These results are in agreement with the known fact that HMW agents cause OA through an IgE-dependent mechanism (6). We also observed an increased percentage of sputum neutrophils in patients with OA caused by HMW agents, in keeping with the results documented by Prince et al. (18), also in the context of SIC, and with the findings of Di Franco et al. (25), who investigated the inflammatory cell pattern on and off work. The mechanism causing this increase in sputum neutrophils is unknown; nevertheless, it has been shown that neutrophilic inflammation can occur during asthma exacerbations in parallel to the increase of eosinophils (26-28). Therefore, it is possible that, during SIC, an inflammatory response is produced similar to that occurring in an exacerbation of asthma. Recent investigations
have shown that airway exposure to allergens in sensitized individuals causes release of IL-17, which orchestrates allergic airway inflammation by inducing the expression of various pro-inflammatory mediators such as cytokines, chemokines, and adhesion molecules, in turn leading to recruitment and activation of neutrophils and Th2-mediated eosinophils (29).

In the present study, no significant changes in sputum eosinophils or neutrophils were found after SIC in patients with OA due to exposure to LMW agents. In this sense, there are discrepancies in the related literature. Some authors have reported an increase in sputum eosinophils after SIC in these patients (9, 11, 12, 30), others have documented increased sputum neutrophils only in patients with OA induced by isocyanates (15-16, 31) or exposed to welding fumes (13), and one study found increases in both eosinophils and neutrophils (18). This is the first study in which no significant differences in cell types in induced sputum after SIC were observed in patients with OA due to LMW agents. These differences could be due to the fact that the population included in our study is superior in number to that of previous studies, except the work of Prince et al. (18), and because it includes patients exposed to very different agents. Moreover, we found higher baseline percentages of sputum neutrophils in OA induced by LMW agents than those reported by Prince et al. (18), which suggests that the higher the levels of these cells at baseline, the lower will be the increase after SIC. It is interesting to note that the higher levels of sputum eosinophils before SIC we observed were mainly found in patients exposed to isocyanates. It is known that certain LMW agents induce OA through IgE-mediated mechanisms (6). However, most LMW agents induce OA without production of specific IgE antibodies, and therefore induce non-immunological OA, suggesting involvement of a different mechanism (32).

Another interesting finding of the present study is that subjects testing negative on SIC and diagnosed with other respiratory diseases experienced an important increase in sputum neutrophils after SIC. This finding has been reported by other authors, who have suggested that these patients may have had false-negative responses to SIC (14, 24). In this sense, Vandenplas et al. (24) recently postulated that the observed increase in sputum inflammatory cells in patients testing negative to SIC was the most accurate parameter to predict the development of an asthmatic response on subsequent
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challenges with a sensitivity of 67% and a specificity of 97%. However, this conclusion particularly referred to cases showing sputum eosinophils. Another possible explanation could be the effect of exposure to LMW agents in subjects with baseline pulmonary disease. In this sense, Girard et al. (33) found that subjects testing negative to SIC had more sputum neutrophils when at work. The mechanisms causing this neutrophilic inflammation are unclear, but we believe it may be due to an irritant effect. In our previous study (34) analyzing exhaled breath condensate (EBC) in conjunction with SIC, we observed that EBC pH decreased significantly in patients diagnosed with WEA, the majority of whom were exposed to LMW agents. Thus, we hypothesize that exposure to LMW agents in patients with baseline pulmonary disease may enhance neutrophil recruitment in the airways. This would correlate with the observed increase in IL-8 levels after SIC in this group of patients compared to those with OA.

In the present study, a panel of TH1-TH2 cytokines was analyzed. LTB₄ levels were measured because previous studies have reported that it could be an important marker of disease in patients with OA (15, 35). In these studies, which were focused on OA due to isocyanates, the authors found increased LTB₄ levels after SIC in induced sputum (15) or bronchoalveolar lavage samples (35), associated with sputum neutrophilia and increased IL-8 concentrations. There are no studies investigating the role of LTB₄ in OA induced by HMW agents. Interestingly, in the present study a decrease in LTB₄ levels after SIC was found in all patients except one with OA due to HMW agents. It has been demonstrated in multiple studies that in IgE-mediated asthmatic reactions caused by allergens there is an increase in cysteiny1 leukotriene (Cys LT) (36). Because production of LTB₄ and Cys LT occurs through alternative pathways (35), overproduction of Cys LT could imply a decrease in LTB₄ production. Although this hypothesis has not been demonstrated in relation to SIC, it has been observed in asthma exacerbation (37).

We also found increased levels of IL-10 after SIC in patients with OA caused by HMW agents. It is known that individuals with asthma and rhinitis have lower IL-10 levels than the healthy population. IL-10 is a cytokine with broad anti-inflammatory properties that plays an important role in regulating Th2 cell responses (38). To our knowledge, only two studies have evaluated IL-10 in patients with OA (8, 39). Mapp et al. (39) reported that occupational exposure to isocyanates is associated with high baseline
levels of IL-10 secreted by peripheral blood mononuclear cells. In contrast, Fernández-Nieto et al. (8) documented lower IL-10 levels in OA patients with either negative or positive SIC compared to the healthy population. Nevertheless, in both studies, IL-10 levels before and after SIC were similar in patients testing positive (8) and after stimulation of peripheral blood mononuclear cells (39). The increase in IL-10 levels observed in the present study after SIC associated only with HMW agents could be related to an immune response directed to regulation of the Th2-mediated allergic response. When individuals with allergic asthma are exposed to immunotherapy treatment, an increase in IL-10 occurs during the first months of treatment (40-42). Furthermore, in an experimental study of allergen exposure in sensitized asthmatic patients Bettiol et al. (43) reported an increase in the amount of IL-10 spontaneously generated by ex vivo sputum cells. The fact that Fernandez-Nieto et al. (8) did not find these differences may be because the study population consisted of individuals exposed to both HMW and LMW agents. IL-2 showed a (non-significant) trend to increase after SIC in OA patients, whereas in patients without SIC-proven OA, the trend was toward a decrease. Moreover, IL-2 levels were higher after SIC in patients with OA compared to those without. This difference is difficult to interpret and casts doubts on the role of IL-2 in OA since no differences were found after segmenting patients according to the type of causal agent.

Finally, it is interesting to note that there were no changes in post-SIC cytokine levels in LMW-exposed individuals who had OA and those who did not. A possible explanation for this could be the heterogeneity of the mechanisms involved in the genesis of this type of asthma, which would support the idea that in future studies it would be appropriate to distinguish not only between HMW and LMW agents, but also between agents that cause asthma by an IgE-mediated mechanism and those in which this mechanism is not demonstrated.

The study has some limitations. Although no significant differences were found in the differential cell count of the groups studied, it cannot be excluded that there may have been some influence due to smoking habit. In any case, this effect would be observed before SIC, but would likely be independent of the response after SIC, considering that during the test the patients did not smoke. Another aspect that may have influenced the
observed results is the fact that approximately 50% of patients in both groups received treatment with inhaled corticosteroids. Although treatment with B-2 agonists was withdrawn in all patients prior to initiation of the SIC, inhaled corticosteroids treatment was not changed to avoid destabilization of asthma, which would invalidate the test. Lastly, the low yield in obtaining sputum samples from patients in the NoAO group might seem to be a limitation, but we believe the study illustrates the true situation of our laboratory and does not show selection bias.

In conclusion, the results of this study show that it is necessary to differentiate between patients with OA due to HMW agents and LMW agents when investigating airway inflammation. In OA patients exposed to HMW agents, an increase in the number of neutrophils can be found in parallel to the increase of eosinophils, although this does not contradict an IgE-mediated mechanism. Exposure to LMW agents can result in increased neutrophilic inflammation in patients with airway diseases unrelated to OA, suggesting different mechanisms of action according to whether the LMW agent is the cause of OA or provokes aggravation of a pre-existing respiratory disease. The variability in the responses observed in patients with OA exposed to LMW agents suggests that more than one mechanism may be involved in the genesis of the disease. Future studies should examine this possibility.

**Acknowledgements:** This project was supported by the FIS PI05100 (Instituto de Salud Carlos III), the Sociedad Española de Patología Respiratoria (SEPAR), the Fundació Catalana de Pneumologia (FUCAP), and the Societat Catalana de Pneumologia (SOCAP).
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DIAGNOSTIC UTILITY OF EXHALED BREATH CONDENSATE ANALYSIS IN CONJUNCTION WITH SPECIFIC INHALATION CHALLENGE IN INDIVIDUALS WITH SUSPECTED WORK-RELATED ASTHMA

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Annals of Allergy and Asthma Immunolgy 2012;108 (3): 151-6. IF: 2.801
Abstract

Background: Establishing the role of exhaled breath condensate (EBC) analysis in work-related asthma (WRA), and more specifically, in conjunction with specific inhalation challenge (SIC), is difficult.

Objective: To measure EBC pH, and nitrite/nitrate concentrations before and after SIC in individuals with suspected WRA exposed either high-molecular-weight (HMW) or low-molecular-weight (LMW) agents and evaluate whether these changes are useful to distinguish between occupational asthma (OA) and work exacerbated asthma (WEA).

Material and methods: One hundred twenty-five consecutive workers undergoing SIC were enrolled. EBC was collected at the end of the baseline day and 24h after exposure to the offending agent. In all EBC samples, pH was measured, and nitrite and nitrate concentrations were determined.

Results: SIC was positive in 66 individuals, who were then diagnosed with OA. WEA was diagnosed in 14, and in 45 patients it was not possible to establish a direct relationship between the symptoms and work exposure. In patients with WEA, EBC pH values after SIC were significantly lower than those before SIC ($P=0.0047$). Using the ROC curve, we found that an EBC pH decrease greater than 0.4 units after SIC achieved the most satisfactory sensitivity 79% (CI: 49 - 94) and specificity of 100% (CI: 68 - 100), considering only patients with asthma and without OA. A decrease in EBC pH ≥0.4 was more common in those exposed to HMW agents (8/19, 42%) than in those exposed to LMW agents (7/47, 15%).

Conclusions: EBC pH in conjunction with SIC may be useful for diagnosing WEA.
Introduction

An estimated 10% to 25% of all asthma cases developing in adulthood are caused by occupational exposure (1, 2). Work-related asthma, which includes occupational asthma (OA) and work-exacerbated asthma (WEA), presents a major health challenge with significant potential for acute morbidity, long-term disability, and an adverse social and economic impact (3). OA refers to de novo asthma caused by exposure to an agent specific to a workplace and not to stimuli outside the work environment, and WEA refers to a worsening of pre-existing or concomitant asthma due to working conditions (4). Specific inhalation challenge (SIC) is considered the reference standard method for the diagnosis of OA, which is often difficult to confirm objectively. SIC is intended to demonstrate a direct relationship between exposure to a test substance and an asthmatic response (5). However, false-positive and false-negative responses to SIC can occur (6-8).

Like bronchial asthma, work-related asthma is a heterogeneous chronic inflammatory disorder of the airways. Study of airway inflammation could be of great value for establishing a precise diagnosis, being a direct reflection of the disease. In this sense, induced sputum analysis preceding and following SIC has been used to support the diagnosis of OA in the laboratory (9) or at the workplace (10, 11), although it is currently a tool with limited availability. Another noninvasive, comprehensive view of airway inflammation can be obtained by analyzing exhaled breath condensate (EBC) (12, 13). EBC collection is completely noninvasive, repeatable, and does not disturb the underlying disease process, making it a useful approach for cross-day variation studies and longitudinal studies that monitor inflammation (14-16).

However, in contrast to the situation with induced sputum, few studies have evaluated the role of EBC in work-related asthma and only two have done so in conjunction with SIC (17, 18). Klusackova et al. (17), monitored leukotrienes and 8-isoprostane before and after SIC, but the results cannot be considered conclusive, because SIC was positive in only 6 of the 47 patients studied. Furthermore, the authors did not specify the workplace agent. The other study conducted by Ferrazzoni et al. (18), performed only in 15 patients with OA due to isocyanates, found no association between asthmatic
reactions and EBC acidification after SIC. From these studies, it is difficult to establish the role of EBC analysis in work-related asthma, and more specifically, in conjunction with SIC. Thus, the aims of this study were to measure EBC pH and nitrite/nitrate concentrations before and after SIC in individuals with suspected work-related asthma exposed either high-molecular-weight (HMW) or low-molecular-weight (LMW) agents and evaluate whether these changes are useful to distinguish between OA and WEA.

Material and methods

Study design and subjects

This is a cross-sectional observational study in order to know the utility of EBC analysis in conjunction with SIC in individuals with suspected work-related asthma. The study included all individuals older than 18 years of age referred to our center to investigate possible OA by SIC testing between 2004 and 2010. All patients had a medical history consistent with OA, and a workplace agent was considered the probable cause of their respiratory symptoms. Patients with a positive SIC result received a diagnosis of OA, and those with a negative SIC were divided into 2 groups, according to clinical criteria (19). Patients who presented symptoms and abnormal findings on pulmonary function study in relation to work received a diagnosis of WEA, whereas those who did not fulfill the criteria for asthma or who had asthma that was unrelated to work were grouped as no work-related asthma (NWRA). All patients signed informed consent documents for participation. The local Ethics Committee approved the study.

Inhalation Challenge

SIC was performed with the substance suspected to be the causal agent in each patient, only when there was no contraindication to this testing (20). The test was mainly carried out according to the method proposed by Pepys et al. (21) and by our group (22, 23). Briefly, patients were examined on 5 consecutive days. On day 1 (baseline), clinical and occupational histories were collected, skin-prick tests with common allergens, radiography study, pulmonary function tests, and methacholine challenge were performed, and EBC was collected. On day 2, a first placebo inhalation challenge was performed. On days 3 and 4, patients underwent SIC with the appropriate workplace agent. On day 5 following SIC, pulmonary function tests, and methacholine challenge
were performed, and EBC was collected again. Pulmonary function changes were monitored in each patient by measuring the FEV\(_1\) every 10 minutes during the first hour after exposure and every hour thereafter up to 15 h after inhalation. The response was considered positive when FEV\(_1\) decreased by more than 20% the baseline value in the absence of any change with the placebo test.

**EBC Collection**

EBC was collected during tidal breathing with a commercially available condenser (EcoScreen; Jaeger, Würzburg, Germany), as described (16). Exhaled air entered and left the chamber through one-way valves at the inlet and outlet, thus maintaining the chamber closed. Patients breathed tidally through a mouthpiece connected to the condenser while wearing nose clips. The low temperature within the condensing chamber cooled the sample throughout the collection time. Samples were collected in sampling tubes, which, prior to use, had been disinfected for 30 minutes using sodium dichloroisocyanurate (Inibsa Lab, Barcelona, Spain), and rinsed for 24 h with distilled water and 2 h with ultra pure water (Fresenius Kabi, Barcelona, Spain). To determine the ventilatory pattern, a spirometer (EcoVent; Jaeger, Würzburg, Germany) was connected to the equipment at the expiratory valve. The spirometer recorded the total exhaled breath, time of collection, tidal volume (Vt), minute ventilation (MV), and breathing frequency (BF). Patients were instructed to refrain from food intake during the 2 hours before sample collection. A fixed volume of 150 L of exhaled breath was collected per patient. Each EBC sample was divided into 500 µL aliquots in two to four plastic tubes. The aliquots, used for measuring nitrite and nitrate, were immediately stored at –70°C, and analyzed within 1 month of collection. Other aliquots were used to measure the pH after deaeration.

**EBC pH measurement**

pH was measured in one of the aliquots after EBC deaeration with helium (350 mL/min for 10 min), using a calibrated pH meter (Model GLP 21; Crison Instruments SA; Barcelona, Spain) with an accuracy of ± 0.01 pH, and a probe for small volumes (Crison 50 28; Crison Instruments SA; Barcelona, Spain). The probe was calibrated daily with standard pH 7.02 and 4.00 buffers (24). Evaluation of the reproducibility of EBC pH measurements from five consecutive measurements revealed a mean coefficient of
variation of 2% (range 0.5 to 14%). The mean intraday coefficient of variation was 4% (range 1 to 20%).

**EBC Nitrite and Nitrate**

Nitrite (NO$_2^-$) and nitrate (NO$_3^-$) concentrations were determined with a colorimetric assay based on the Griess reaction. Briefly, NO$_3^-$ was measured as NO$_2^-$ after enzymatic conversion by nitrate reductase, and the total NO$_2^-$/ NO$_3^-$ (converted NO$_3^-$ plus NO$_2^-$) was measured in sample duplicates using Griess reagent (Cayman Chemical Company, Ann Arbor, Michigan). Concentrations were measured with a microplate reader at 540 nm absorbance. Assay sensitivity was 1 µmol/L for nitrite and 2.5 µmol/L for nitrate. Within-run coefficients of variation were 6%, and 4%, and between-run coefficients of variation were 9% and 5% for nitrate and nitrite, respectively. The authors decided to measure the end products of NO metabolism instead of exhaled NO in order to compare compounds generated from the same specimen.

**Data Analysis**

A one-sample Kolmogorov-Smirnov test was calculated to assess whether the assumption that variables analyzed had normal distributions was tenable. The results of the test refuted the assumption; hence, the Kruskal-Wallis test and the adjusted Mann-Whitney $U$ test were used to compare the age, duration of exposure and symptoms, interval from last exposure, lung function data, EBC pH and nitrite/nitrate concentrations between OA, WEA and NWRA group. Wilcoxon signed rank test was used to compare lung function data, EBC pH and nitrite/nitrate concentrations within groups. Spearman’s rank correlation test was applied to determine correlations between EBC, pH, and nitrite or nitrate concentrations within groups. A $p$ value of < 0.05 was considered significant. Non-detectable nitrite and nitrate concentrations were assigned the value of the detection limit of the method (1µmol/L and 2.5 µmol/L, respectively). Receiver-operating characteristic (ROC) curves were constructed to determine the most discriminating cut-off value of changes in EBC pH before and after SIC for predicting WEA. SPSS 17.0 for Windows (SPSS Inc, Chicago, Illinois) was used for the statistical analyses.
Results

Study Population

One hundred and twenty-five consecutive patients referred for possible OA were assessed. Sixty-six patients had a positive SIC and were diagnosed with OA (31 patients had an immediate asthmatic reaction, 23 a late asthmatic reaction, 10 a dual asthmatic reaction, and 2 patients had other types of asthmatic reactions), and 59 patients had a negative SIC to the workplace agents tested (Figure 1). Among the 59 SIC-negative patients, 14 were diagnosed with WEA according to clinical criteria and 45 were considered NWRA, either because they did not meet the criteria for asthma (n=34) or because they had asthma in which the symptoms were unrelated to the workplace (n=11). Patients in the NWRA group who did not have asthma were ultimately diagnosed with eosinophilic bronchitis (n=2), chronic obstructive pulmonary disease (COPD) (n=2), chronic bronchitis (n=2), bronchiolitis (n=1), smoker’s bronchiolitis (n=1), pulmonary tuberculosis sequelae (n=1), and bronchiectasis (n=1); in 24 patients, the respiratory disease could not be identified.

Demographic data and clinical characteristics of the patients included are presented in Table 1. The FEV$_1$ values (% predicted) before exposure and 24 hours after exposure were lower in the WEA group compared to the NWRA group ($P=0.034$ and 0.039, respectively). Methacholine challenge 24 hours after exposure could not be performed in 22 patients in the OA group because their FEV$_1$ was less than 60% the theoretical value following SIC. Two patients in the WEA group and 2 in the NWRA group did not agree to repeat methacholine challenge testing at 24 hours after SIC. The PC$_{20}$ was significantly lower in the OA group compared to NWRA before exposure ($P=0.028$) and at 24 hours after exposure ($P=0.004$). A PC$_{20}$ decrease of at least two-fold at 24 hours occurred in 36% of patients in the OA group and none of the patients in the WEA or NWRA groups. Symptoms duration was also greater in OA than in NWRA ($P=0.009$). There were no significant differences between the groups in any of the remaining variables analyzed.
Figure 1. Study population and agents tested.
Table 1. Characteristics of the subjects studied

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>WRA Group</th>
<th>NWRA Group</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>OA Group (n=66)</td>
<td>WEA Group (n=14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Sex, M/F</td>
<td>50/16</td>
<td>6/8</td>
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<tr>
<td>Age, years</td>
<td>40 (22 - 57)</td>
<td>43.5 (24 - 62)</td>
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<tr>
<td>Smoking habits, NS/exS/CS</td>
<td>30/17/19</td>
<td>5/6/3</td>
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<tr>
<td>Atopy, %</td>
<td>48</td>
<td>62</td>
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<tr>
<td>Duration of exposure, months</td>
<td>115 (3 - 533)</td>
<td>153.5 (23 - 365)</td>
</tr>
<tr>
<td>Duration of symptoms, months</td>
<td>48 (1 - 365)*</td>
<td>36 (5 - 197)</td>
</tr>
<tr>
<td>Interval from last exposure, months</td>
<td>1.25 (0 - 65)</td>
<td>3 (0 - 21)</td>
</tr>
<tr>
<td>FEV\textsubscript{1} % pred. before exposure</td>
<td>88.5 (53 - 124)</td>
<td>82 (69 - 99)†</td>
</tr>
<tr>
<td>24h after exposure</td>
<td>86 (39 - 125)</td>
<td>82 (48 - 95)‡</td>
</tr>
<tr>
<td>Methacholine before exposure, n % positive</td>
<td>66</td>
<td>14</td>
</tr>
<tr>
<td>PC\textsubscript{20}, mg/mL ≠</td>
<td>72</td>
<td>75</td>
</tr>
<tr>
<td>≥2-fold % PC\textsubscript{20} decrease</td>
<td>2 (0.19 - 16)**</td>
<td>4 (0.22 - 13)</td>
</tr>
<tr>
<td>Methacholine 24h after exposure, n % positive</td>
<td>44</td>
<td>12</td>
</tr>
<tr>
<td>PC\textsubscript{20}, mg/mL ≥</td>
<td>75</td>
<td>67</td>
</tr>
<tr>
<td>≥2-fold % PC\textsubscript{20} decrease</td>
<td>2.4 (0.05 - 16)***</td>
<td>8 (0.85 - 12)</td>
</tr>
<tr>
<td>Occupational agents, HMW/LMW</td>
<td>19/47</td>
<td>2/12</td>
</tr>
<tr>
<td>Type of asthmatic reactions, immediate/late/dual/others</td>
<td>31/23/10/2</td>
<td>……</td>
</tr>
</tbody>
</table>

Data are given as the number or median (range), unless otherwise noted. CS, current smoker; exS, ex-smoker; F, female; HMW, high-molecular-weight agent; LMW, low-molecular-weight agent; M, male; NS, never smoker; NWRA, no work-related asthma; OA, occupational asthma; PC\textsubscript{20}, methacholine concentration inducing a 20% fall in FEV\textsubscript{1}; SIC, specific inhalation challenge; WEA, work-exacerbated asthma; WRA, work-related asthma.

≠ Only positive tests; * $P=0.009$; ** $P=0.028$; *** $P=0.004$; † $P=0.034$; ‡ $P=0.039$
**EBC pH measurement**

The EBC pH values before and after exposure are shown in Figure 2. Following SIC, a significant decrease in EBC pH was seen in the WEA group ($P=0.0047$). Most patients with WEA (12/14) had been exposed to low-molecular-weight (LMW) agents.

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**Figure 2.** EBC pH in the 3 groups studied. A: Before SIC; B: After SIC. NWRA, Non-work related asthma; OA, Occupational asthma; SIC, specific inhalation challenge; WEA, Work exacerbated asthma; WRA, Work related asthma.

The ROC curves showed the most relevant differences in EBC pH before and after SIC for predicting WEA. Considering only patients with negative SIC and reliable diagnosis of asthma (all 14 patients with WEA group and 11 patients of the NWRA group), we found that an EBC pH decrease greater than 0.4 units after SIC achieved the most satisfactory sensitivity: 79% (49 - 94) and specificity of 100% (68 - 100) (Figure 3 A). Considering all the patients included in the study, and the same decrease of 0.4 units in the EBC pH, we obtained a sensitivity of 78% (CI: 49 - 94) and specificity: 82% (CI: 73 - 88) for the diagnosis of WEA (Figure 3 B).
Figure 3. Receiver operating characteristic curves constructed to determine the most relevant difference in EBC pH before and after SIC for predicting WEA in: (A) patients with negative SIC and reliable diagnosis of asthma and (B) all the study population. The dots represent the optimal cut-off points.

In the group of patients with OA, a decrease in EBC pH ≥0.4 was more common in those exposed to high-molecular-weight (HMW) agents (8/19, 42%) than in those exposed to LMW agents (7/47, 15%). In patients with WEA, this significant pH decrease was observed in 2 patients in contact with HMW agents and in 9/12 (75%) exposed to LMW agents. In the NWRA group, a significant pH decrease occurred in only 5 (11%) patients, all of whom had been in contact with LMW agents. The final diagnoses in these patients were eosinophilic bronchitis in 2 and bronchiectasis in 1; a definite diagnosis could not be established in the remaining 2 patients.

Nitrite and nitrate measurement
In the group of OA patients exposed to HMW agents, a significant increase in NO$_3^-$ values occurred following SIC ($P=0.0002$) (Figure 4A). In these same patients, the pH decrease after SIC was greater in those with more elevated baseline NO$_2^-$ levels ($r=-0.685$, $P=0.001$) (Figure 4B). There were no significant differences in NO$_2^-$ or NO$_3^-$ concentrations before and after SIC in the remaining groups analyzed.
Figure 4. OA group exposed to HMW agents, A) EBC NO₂⁻ and NO₃⁻ concentrations; B) correlation between EBC pH after SIC and EBC NO₂⁻ concentration before SIC. EBC, exhaled breath condensate; HMW, high-molecular-weight; OA, occupational asthma; SIC, specific inhalation challenge.

Discussion

The most important result of this study is that a decrease higher than 0.4 units in the EBC pH after SIC, in patients with asthma in which we exclude the diagnosis of OA, has a sensitivity of 79% and a specificity of 100% for the diagnosis of WEA. Most of patients with WEA had been exposed to LMW agents. In OA patients, a decrease higher than 0.4 units was only found in 15% of those exposed to LMW agents, and in 42% of those exposed to HMW agents. In addition to the potential value of these findings for diagnostic purposes, they suggest that the mechanism of action of LMW agents may differ according to whether they provoke OA or WEA.

In studies on work-related asthma, the percentage of patients with WEA ranges from 13% to 58% (4). Studies investigating socioeconomic outcomes in work-related asthma have generally shown that OA and WEA patients report similar income loss, rates of unemployment, and partial work disability (25, 26). Nevertheless, some authors suggest that patients with WEA, unlike those with OA, can usually return to their workplace with adjustments to reduce exposure to likely airway irritants and/or with optimized asthma medication (27). As compared to OA, studies on WEA are limited and this leads to relative uncertainty regarding the definition, prevalence, diagnosis, and management of these conditions. The diagnosis of WEA is usually based on the existence of asthma
prior to occupational exposure and to changes occurring in the symptoms, medication use, and/or lung function temporally related to work, whereas the diagnosis of OA is based on the fact that asthma onset occurs after starting a job, with similar changes in symptoms, medication use, and/or lung function temporally related to work (3-4). Nonetheless, according to the American College Of Chest Physicians Consensus Statement (5), the possibility that an individual with previous asthma will present with OA cannot be excluded, nor can it be ruled out that a patient will develop non-occupational asthma during his/her working life and that this will be exacerbated by work. In fact, only 10% to 25% of asthma cases initiating in adults are of occupational origin (1). In this context, a simple differentiation between OA and WEA based on a temporal relationship between the onset of asthma and the start of an occupation can be erroneous. Thus, it would be useful to have diagnostic methods that would enable differentiation between these two conditions. Peak expiratory flow recording cannot reliably distinguish OA from WEA, as both may be associated with work-related changes in airway caliber (5, 19). Some authors have suggested that SIC could be useful because a positive result confirms the diagnosis of OA. Nonetheless, when SIC is negative, the situation is more complex. In a study population comprised of work-related asthma patients, one can conclude that individuals with a negative SIC have WEA (28). However, in clinical practice, SIC is often performed in patients with asthma unrelated to the workplace and in patients who do not have asthma (29). In these situations, it would be of use to perform other tests in conjunction with SIC to help establish the definite diagnosis.

In the present study, in the WEA group, 79% (11/14) experienced an EBC pH decrease $\geq$0.4 units after SIC. Moreover, most patients who experienced this significant pH decrease had been exposed to LMW agents (9/11). WEA can result from a variety of occupational triggers, including physical factors (eg, extreme temperatures, exercise), behavioral states (eg, strong emotions, stress), odors (eg, perfume), general irritants and dust, and second-hand cigarette smoke (4). Because of this large variety of potential triggering factors, we cannot exclude a nonspecific mechanism that would lead to a decrease in EBC pH. In fact, LMW substances may more easily cause non-specific airway irritation with symptoms in patients with WEA or other respiratory diseases. This could be the case, for example, of 3 patients in our NWRA group, who also
showed a pH decrease following SIC and were affected with eosinophilic bronchitis or bronchiectasis. It is important to highlight that there was no decrease in EBC pH after SIC in 22 of the 24 patients in whom no disease was documented or in any of the patients with asthma unrelated to work.

In patients with OA, there was no significant decrease in EBC pH following SIC regardless of whether they had been exposed to HMW or LMW agents. Nonetheless, 42% of OA patients exposed to HMW agents and only 15% of those exposed to LMW agents presented an EBC pH decrease of ≥0.4 units after SIC. In the case of HMW agents, the decrease was related to the fact that baseline EBC NO$_2^-$ levels were more elevated. Furthermore, an increase in NO$_3^-$ levels after SIC was found in OA patients in contact with HMW agents, but not in those exposed to LMW agents. There have been no other studies in this area, and only a few studies have focussed on NO determination in OA. In general, in these studies the authors observed a increase in the levels of NO in response to HMW agents as this increase was not observed in response to LMW agents (18, 30-32). These findings might be due to differences in the pathophysiology of asthma induced by these two types of agents. The airway changes produced by LMW and HMW agents differ, and these differences could be reflected in the EBC pH. Nevertheless, there is no clear evidence that LMW agent-induced asthma is associated with acidification of EBC pH, as the available studies report conflicting findings. The EBC pH results in the present study are consistent with those of Ferrazzoni et al. (18), who found no evidence that isocyanate-induced asthma is associated with EBC acidification. However, Boyce et al. (33) reported increased pH values in workers exposed to welding fumes, and Fireman et al. (34) found significantly lower levels in welders exposed to cadmium/chromium/iron/lead/nickel, and no significant changes in those exposed to aluminium/iron. The discrepancies existing between studies suggest a different mechanism of action in the two types of work-related asthma and would explain the differences found in the present study regarding EBC pH and NO$_2^-$ y NO$_3^-$ levels in patients with OA exposed to HMW and LMW agents.

This study has some limitations. First, it is an observational study with no follow-up data. Thus, we do not know if the diagnoses established by SIC have been maintained over time. Another limitation is derived from the diagnosis of WEA. In the present
study, we chose to use a combination of clinical data and SIC findings, such that WEA was diagnosed when the patient had asthma, the asthma was exacerbated by work, and SIC was negative. Despite these limitations, the present study is of value, being the first in a large number of patients in which the role of EBC pH in conjunction with SIC is analyzed. Moreover, the results demonstrate that EBC pH in conjunction with SIC may be useful for diagnosing WEA and suggest that mechanism of action of LMW agents seems to differ according to whether they cause OA or induced WEA. We can conclude that these EBC inflammatory markers should be carefully evaluated to investigate their potential capability to establish differences between OA caused by LMW or HMW agents and to distinguish between patients with OA, WEA, and asthma unrelated to work.

Acknowledgements: This project was supported by Fis PI07/90086 (Instituto de Salud Carlos III), Societat Catalana de Peumologia (SOCAP) and Fundació Catalana de Pneumologia (FUCAP).
References


GENERAL DISCUSSION
The assessment and monitoring of airway inflammation has established itself as a useful approach in the management and treatment of asthma. In this thesis, we assess airway inflammation using two non-invasive methods, induced sputum (IS) and exhaled breath condensate (EBC), in the context of asthma and, especially, in work-related asthma (WRA). Our main results using IS are the persistent airway inflammation observed in asthmatic patients treated with inhaled corticosteroids when they are evaluated during a controlled phase of the disease, as well as, an airway hyper-reactivity (AHR) documented in the majority of them (Chapter 1). Analysing IS in the context of WRA, we noted different inflammatory profiles depending on the causative agent: high-molecular-weight (HMW) agents seem to induce more airway inflammation in subjects with occupational asthma (OA), while low-molecular-weight (LMW) agents induce more inflammation in subjects without OA but diagnosed with other respiratory diseases (Chapter 3). In the case of EBC, after establishing normal values related to age for several biomarkers in healthy adults (Chapter 2), we found significant changes in EBC pH values after exposure to the causative agent in subjects with asthma in those the diagnosis of OA were excluded, suggesting that this biomarker may be useful for diagnosing work-exacerbated asthma (WEA) (Chapter 4).

To confirm the working hypothesis of this thesis and of studies specifically designed to evaluate the usefulness of non-invasive methods to study airway inflammation in subjects with suspected WRA, data from control populations are required. Given the scarcity of information available in the literature, it seems appropriate to study airway inflammation in stable asthmatic patients regardless of the treatment they are receiving. This point is significant because most of the subjects evaluated for suspected WRA are under treatment at the time of the evaluation, and so patients with well-controlled asthma appear to be a suitable reference population in these studies. The second control population comprised healthy adults and served to establish normal values in our laboratory for several EBC biomarkers for which little information is available at present, and also to study the effect of age on these EBC biomarkers. The age range among workers is wide, and since airway cellularity varies with age, it is reasonable to suppose that the physical-chemical characteristics and different biomarkers of the airways may also vary with age. Certainly, establishing this point is important to the correct interpretation of the results obtained from this sample.
1. Airway inflammation in well-controlled asthma

Asthma is increasingly recognized to be a multifactorial, chronic inflammatory disease of the airways. This airway inflammation has implications for the diagnosis, management, and potential prevention of the disease (1). There is now good evidence that the clinical manifestations of asthma (symptoms, sleep disturbances, limitations of daily activity, impairment of lung function, and use of rescue medications) can be controlled with appropriate treatment. When asthma is controlled, there should be no more than occasional recurrence of symptoms and severe exacerbations should be rare (2). Current asthma guidelines emphasize that asthma control is a key therapeutic goal and its assessment is recommended to guide step therapy. In this context, the assessment of asthma control should include not only the current clinical control based on the clinical manifestations (symptoms, use of medication to relieve symptoms, and clinical lung function), but also control of the expected future risk to the patient such as exacerbations, accelerated decline in lung function, and side-effects of treatment (2-4).

Bearing in mind that in general clinical practice focuses on current clinical control, we wanted to evaluate the possibility of airway inflammation in asthmatic patients who are in principle well-controlled.

For this purpose, we applied the widely used IS technique to provide insights into the airway inflammation characteristics of patients with stable, well-controlled asthma and to assess its relationship with AHR. We obtained a success rate in sputum induction among our stable asthmatics of 69%, similar to the rate reported in a previous study also analysing patients who were stable and well-controlled upon treatment with inhaled corticosteroids (5), as well as in two other studies reporting characteristics of patients with asthma symptoms in whom sputum induction was successful or unsuccessful (6, 7). As in these three earlier studies, we did not find any differences in the baseline characteristics between patients with successful and unsuccessful induction. One interesting observation extracted from one of these studies is that successful sputum induction may be more likely in patients with better asthma control and better quality of life; this finding argues in favour of IS use in clinical assessment of these asthmatics in order to prevent undertreatment with inhaled corticosteroids and disease exacerbation (7).
Underlying airway inflammation was detected in 70% of stable asthmatics studied, thus reinforcing the idea that airway inflammation persists in spite of the absence of symptoms. Treatment adjustment and asthma control in our study were carried out in accordance with the Global Initiative for Asthma (GINA) strategy, which is essentially based on clinical factors (2). Therefore, this higher percentage of patients with airway inflammation was not unexpected. Furthermore, our results are consistent with other studies in the literature reporting persistent airway inflammation in well-controlled asthma or even during total asthma control (5, 8). This persistent airway inflammation could explain future asthma exacerbations and worse asthma outcomes; indeed, previous studies adjusting asthma treatment tailored in accordance with control of sputum inflammation have reported decreased asthma exacerbations (9-11). The first of these studies was by Green et al. (9) who evaluated 74 patients with moderate to severe asthma, randomly allocated to management either according to standard asthma guidelines or by normalization of the IS eosinophil count and reduction of symptoms. The authors reported that a treatment strategy directed at normalization of the IS eosinophil count reduces asthma exacerbations and admissions without the need for additional anti-inflammatory treatment. These findings have led to a recent Cochrane review (12), which established that adjusting asthma treatment according to sputum eosinophilia is useful for asthma control, although this approach can be difficult to apply in daily practice.

In accordance with other authors, we classified the type of airway inflammation as eosinophilic when the IS eosinophil count was >2%, neutrophilic when the neutrophil count was >60%, mixed when the eosinophil count was >2% and the neutrophil count was >60%, and paucigranulocytic when the eosinophil count was <2% and the neutrophil count was <60% (13, 14). We observed three predominant inflammatory profiles: eosinophilic, neutrophilic and paucigranulocytic inflammation, in accordance with the asthma phenotypes proposed and based on the predominant cell types involved (15). Mixed inflammation was only noted in three patients, in contrast to other reports suggesting that many asthmatics with neutrophilic inflammation have concomitant eosinophilic inflammation (14, 16).
As mentioned in the introduction, sputum eosinophilia is a typical feature of asthma, but non-eosinophilic asthma is also common, accounting for 25% to 55% of corticosteroid-naïve asthmatics (17, 18). Although the role of eosinophils in airway inflammation in chronic asthma has been extensively studied, a role for neutrophils has not been well characterized. It is now known that their role in the inflammatory process is not only restricted to phagocytosis and the release of enzymes and other cytotoxic agents, but that these cells can release a variety of mediators that have profound effects on the airways of asthmatic individuals (19). Sputum neutrophilia has been reported in the airways of patients with severe asthma and also in asthma exacerbations (20, 21). Other studies have found that neutrophils are the dominant inflammatory cell type in sputum of subjects with mild to moderate asthma (16, 18), in agreement with our finding of a higher rate of patients with neutrophilic inflammation in this study. One possible explanation for this neutrophilic inflammation may be smoking habit; a close link has been reported between asthma and smoking, and one previous study showed that sputum neutrophilia in asthmatic smokers is higher than in non-smokers (22). Furthermore, neutrophilic inflammation is probably enhanced by smoking because the exogenous nitric oxide in cigarette smoke increases the apoptosis of activated eosinophils; thus, eosinophilic inflammation is reduced in smokers (23). However, only 21% of asthmatics with successful IS in our population were smokers at the time of the study and we did not find any difference regarding the type of inflammation in this group of patients. Another factor that may influence sputum neutrophilia is corticosteroid treatment. All patients in our study had been treated with inhaled corticosteroids, at least over the previous year. This may have affected sputum neutrophilia in two ways: by prolonging the survival of neutrophils through inhibition of the apoptosis of these cells or by eliminating eosinophils in patients who initially presented mixed inflammation (19). In this context, a recent study assessing the inflammatory cell phenotypes in asthma after eliminating potentially confounding effects found that corticosteroid treatment was associated with increased airway neutrophils and with a switch to a neutrophilic phenotype in patients with moderate stable asthma (24). Because sputum neutrophilia was not decreased in subjects using inhaled corticosteroids, other anti-inflammatory therapies directed specifically at controlling neutrophilic inflammation might be useful in improving airway calibre in patients with chronic asthma (25).
Sputum eosinophilia was observed in almost half of our asthmatic patients (28 out of 59) with documented persistent or chronic airway inflammation. Eosinophilic airway inflammation has been shown to predict worse asthma outcomes (26) and a broad correlation between clinical asthma severity and the degree of airway eosinophilia has been recorded, especially when eosinophils appear to be activated (27). Furthermore, inverse relationships have been observed between sputum eosinophil counts and lung function measures, such as forced expiratory volume in one second (FEV$_1$) and methacholine responsiveness (25).

In this study, 79% of asthmatic patients presented AHR, measured by methacholine challenge, despite treatment with inhaled corticosteroids. This finding supports the hypothesis that several factors can contribute to AHR, and that they can be divided into two categories: persistent and variable. The persistent contribution to AHR has been attributed to structural changes in the airways, such as subendothelial thickening, subbasement membrane thickening, smooth muscle hypertrophy, matrix deposition, and altered vascular components. The variable contribution is believed to relate to inflammatory airway events, which vary and are influenced by infection and environmental factors (28). These latter factors are probably not responsible for the persistent AHR in our study because all the patients had well-controlled asthma and had been stable for at least one year prior to inclusion. In contrast, the persistent factors are probably the ones that best explain the persistence of AHR in our study, despite treatment with inhaled corticosteroids.

Just as control of sputum eosinophil counts has been shown to prevent worse asthma outcomes (26), monitoring AHR may have an important role in the long-term management of asthma, and adjusting asthma treatment according to AHR measurement has been proposed as a better method for achieving asthma control than the use of conventional guidelines (29). Sont et al. (30) reported that adults with mild to moderate asthma in whom the medication with inhaled corticosteroids was adjusted according to the severity of their AHR had lower rates of mild exacerbations than patients in whom the treatment was adjusted following recommendations in the existing guidelines. The authors therefore concluded that reducing AHR in conjunction with optimizing
symptoms and lung function leads to more effective control of asthma, while alleviating chronic airway inflammation.

One last interesting finding in this study was the fact that the degree of AHR was much higher in patients presenting eosinophilic airway inflammation, in agreement with previous studies (25). This finding suggests that when AHR persists despite treatment, its severity will be linked to an eosinophilic airway inflammation and not to a neutrophilic or paucigranulocytic airway inflammation. In this regard, it has been reported that patients with severe corticosteroid-dependent asthma and eosinophilic airway inflammation require more intubations than those with other types of airway inflammation (31). One possible explanation could be that eosinophilic airway inflammation might cause greater structural changes in the airways which would contribute to perpetuating AHR, although further investigation is needed to identify the mechanisms responsible.

2. Exhaled breath condensate biomarkers in healthy population

Before evaluating the utility of EBC analysis in conjunction with SIC in WRA, we wanted to standardize EBC collection and establish reference values in a healthy adult population for certain EBC biomarkers of airway inflammation, in order to define normal ranges for these biomarkers in different age groups. Thomas et al. (32) investigated the effects of age on sputum differential cell counts in a healthy adult population and reported a trend for sputum neutrophils counts to increase with age, indicating that this effect was not disease-specific and suggesting the possible presence of subclinical disease in older individuals as a result of longstanding environmental exposure. Following the same lines, we decided to investigate whether the age-related changes in sputum inflammatory profile mentioned above might be reflected in EBC biomarkers. We obtained EBC samples from seventy-five volunteers aged 18 to 80 years, stratified into five groups according to age (n = 15): 18 to 29 years, 30 to 39 years, 40 to 49 years, 50 to 59 years, and 60 to 80 years. In all EBC samples we measured EBC pH, 8-isoprostane concentration and nitrogen oxide values: NO$_2^-$ and NO$_3^-$. 
Like Thomas et al. (32), we observed lower EBC pH values in the older group of subjects after de-aeration than in the other studied groups. EBC pH is known to be the result of a balance between several buffer systems, and the production and release of acids and bases in the airways (33, 34). In this regard, some authors have suggested that the main source of NH$_3$ in EBC is the mouth, and that contamination with oral ammonia may have an important alkalinizing effect in EBC (35). Others authors have reported that the effect of oral ammonia on EBC pH is uncertain (36). The equipment used in our study, ECoScreen™, is constructed in a way that the sampling tube initiates within the mouthpiece so that saliva cannot reach the tube but, instead, is trapped in a cylinder covering the tube, thereby obviating an effect of salivary contamination on the measurements (37). However, pH is controlled by volatiles and these volatiles may arise in part from the mouth. Although a saliva trap will not avoid oral volatiles, our system can minimize their effect and any impact on the results of oral contamination. In this aqueous environment, exhaled CO$_2$ from H$^+$ and HCO$_3^-$ profoundly affects the EBC pH. Thus, de-aeration with an inert gas has been proposed to enhance the stability of the EBC pH readings because it causes a significant decrease in CO$_2$ content (38, 39). The most accepted data for EBC pH values are those after de-aeration, when the EBC pH reflects the status of the fluid lining the airways (40). In fact, we observed lower EBC pH values after de-aeration in the 60- to 80-year age group as compared to the others groups, while no difference was observed in the EBC pH values before de-aeration. To the best of our knowledge, only two studies have focused on age-related pH in EBC (41, 42). In the first study (41), the authors found no relationship between EBC pH and age. The differences between the results of that study and ours may be attributable to the lower EBC pH values found by Brooks et al. in their youngest group, which were outside the reported range (7.4 to 8.8) described by the American Thoracic Society/European Respiratory Society Task Force (43). In the second study (42), 6.4% of the EBC samples had a pH < 7.4; this percentage of outlying values was comparable to that obtained in our study (6.6%). Nevertheless, these authors also concluded that EBC pH is not systematically affected by age.

The decrease in EBC pH values after de-aeration in the older group of subjects may be due to the fact that changes occur in the cellular pattern of the airways with age. According to some authors (40), neutrophils and eosinophils have the greatest influence
on EBC pH values. This would corroborate the findings reported by Thomas et al. (32) mentioned above, where the induced-sputum differential neutrophil count increased significantly with age in a healthy volunteer population. This increase may be related to the decrease in EBC pH values and may be the result of longstanding environmental exposure. There may also be age-related changes in the immune response of the lung that could lead to an amplification of the immune response to environmental and other triggers (32). Furthermore, oxidative stress is likely to be greater with age, and there is an accumulation of metabolic substances as a consequence of longstanding environmental exposure thus increasing the amount of non-volatile acids, which do not disappear after de-aeration and which may affect the EBC pH values.

The findings we obtained for EBC pH are similar to those observed for 8-isoprostane. This compound, which is derived from free radical-catalyzed peroxidation of arachidonic acid, is one of the most reliable biomarkers of oxidative stress. It has been shown that levels of 8-isoprostane are increased in EBC of patients with several respiratory diseases, such as asthma (44), COPD (45), cystic fibrosis (46), pulmonary fibrosis (47), and sarcoidosis (48). We observed increased 8-isoprostane levels with age, and significant differences in 8-isoprostane levels between the two younger groups and the oldest group despite the fact that the values obtained in all the groups were within the normal limits previously described by other authors in the healthy population (43). Moreover, we noted that the number of EBC samples with detectable values increased with age. According to some authors (43, 49), 8-isoprostane is present in detectable amounts in all normal tissues and biological fluids and has been detected in EBC in healthy subjects, thus allowing the definition of a normal range (50). Nevertheless, the findings from our study should be taken into account when establishing the 8-isoprostane normal values.

In view of our results, we believe that a subtle, subclinical disease could be present in some of the population of our study, but it is also possible that the differences recorded were produced by “normal” aging-related changes in the respiratory system (51). It has been reported that the respiratory system undergoes various anatomical, physiological and immunological changes with age. For example, the lung parenchyma loses its supporting structure, causing “senile emphysema” (51); this would produce greater
oxidative stress with higher levels of 8-isoprostane and might be related to changes in the cellular pattern of the airways occurring with aging (40) and to the decrease in EBC pH values.

Regarding NO\textsubscript{2\textsuperscript{-}} and NO\textsubscript{3\textsuperscript{-}} measurements, we did not find any relationship between the levels of these biomarkers and age. NO\textsubscript{2\textsuperscript{-}} and NO\textsubscript{3\textsuperscript{-}} generated by NO in aqueous media are considered biomarkers of airway inflammation (52). It has been shown that NO\textsubscript{2\textsuperscript{-}} can be consumed by neutrophil myeloperoxidase as a substrate for enzymatic nitration, and when it is protonated, NO\textsubscript{2\textsuperscript{-}} releases NO (53, 54). Thus, the increase in sputum neutrophil counts with aging may lead to higher metabolism of these nitrogen redox forms, increasing the levels of exhaled NO. Comparison of reported NO\textsubscript{2\textsuperscript{-}} and NO\textsubscript{3\textsuperscript{-}} concentrations between studies in the literature is difficult because of differences in the techniques used for EBC collection and analysis of its biomarkers. Nonetheless, the levels observed in our study are in agreement with those reported by other authors (55, 56) who also used the ECoScreen\textsuperscript{TM} condenser, although with different assay methods.

3. Airway inflammation in work-related asthma

As in the case of patients whose asthma was stable and well-controlled, we established the inflammatory profile in subjects reporting respiratory symptoms related to occupational exposures. To do so we used IS and EBC analysis in the context of specific inhalation challenge (SIC), and assessed whether these non-invasive techniques might help to improve the diagnostic yield of SIC.

3. 1. Sputum inflammatory profile

As mentioned in the introduction, SIC is currently considered the reference standard method for the correct diagnosis of OA (57). We studied 51 subjects with medical history compatible with OA undergoing SIC to a workplace agent considered as a probable cause of their respiratory symptoms. We used IS to assess whether the exposure following SIC in the laboratory induces changes in airway inflammation, distinguishing between types of occupational agent: HMW and LMW.
Sputum induction was performed the day before and 24 h after exposure to the offending agent. It has been reported that the best timing for the collection of IS with respect to the exposure to occupational agents is probably 7-24 h after exposure (58). Indeed, an increase in sputum eosinophils has been shown to occur 7 h after exposure to occupational agents and persisting 24 h after exposure (59). After processing sputum samples, total and differential sputum cell counts (with eosinophils and neutrophils as main inflammatory cell types) were assessed. We also measured leukotriene B\textsubscript{4} (LTB\textsubscript{4}) and a panel of 10 Th1/Th2 cytokines in sputum supernatants.

In the 51 subjects assessed in the study, SIC was positive to the workplace agents tested in 34 (67%), who were then diagnosed with OA, and negative in 17 (33%), who were then diagnosed with non-OA (NoOA). Very few studies have evaluated changes in sputum cell counts before and after SIC in subjects with suspected OA; all these studies analysed OA patients together, regardless of whether they had been exposed to HMW or to LMW agents, and a significant increase in sputum eosinophil counts was observed after SIC responses compared with baseline values (60-62). We did not find this increase in our OA patients as a whole; one possible explanation could be the differences in the characteristics of the study populations. In the studies mentioned, about 50% of patients were exposed to HMW agents, whereas in our study this rate was only 29%. Indeed, it is well known that a large number of HMW agents induce an increase in sputum eosinophils after SIC (58), corroborating the theory that the majority of HMW agents cause OA through an immunoglobulin (Ig)E-dependent mechanism (63). Considering this mechanism of action and the sputum eosinophilia associated with a great number of HMW agents, we believe that the best approach in the assessment of airway inflammation in the context of SIC might be to distinguish between patients according to the type of occupational agent. In fact, one previous study reported differences in patterns of response, expiratory flows and AHR in a large group of patients with OA due to HMW and to LMW agents (64).

Regarding sputum cell counts, we observed significant increases in sputum eosinophil and neutrophil percentages after SIC compared to baseline in patients with HMW-induced OA, but not in those with LMW-induced OA. These results are in agreement with recently reported findings also in the context of a SIC (65) and with findings
investigating the inflammatory pattern in and out of work (66), suggesting that not only is an eosinophilic inflammation characteristic of exposure to HMW agents, but a neutrophilic inflammation is also present. The mechanism by which this increase in sputum neutrophils occurs is still unknown; nevertheless, it is possible that, in the context of a SIC, an inflammatory response is produced similar to that occurring in an exacerbation of asthma, in which there may be early activation of both the neutrophilic and eosinophilic responses (67). In fact, recent investigations revealed that airway exposure to allergen in sensitized individuals causes the release of adenosine triphosphate (ATP) and uric acid, activating the inflammasome complex and leading to the production of mature interleukin (IL)-1β, which creates a pro-inflammatory milieu with the production of IL-6 and chemokines which mobilize neutrophils and enhance Th17 cell differentiation in the lung. IL-17 orchestrates allergic airway inflammation and plays a critical role in neutrophil and Th2-mediated eosinophil recruitment to the lung by regulating the expression of various pro-inflammatory mediators such as cytokines, chemokines, and adhesion molecules (68).

In patients with OA due to exposure to LMW agents we did not find any significant change in sputum eosinophils or neutrophils after SIC compared to baseline, and to the best of our knowledge this is the first study to report no significant differences in IS cell types after SIC. There are discrepancies in this regard in the literature. Some authors have reported an increase in sputum eosinophils after SIC in patients exposed to LMW agents (69), whereas others have documented increased sputum neutrophils only in patients with OA exposed to isocyanates (70) or to welding fumes (71), and one study found the same findings reporting in OA due to HMW agents, that is, increases in both eosinophils and neutrophils after SIC (65). Our study population is larger than those of previous studies, with the exception of (65), and it includes patients exposed to very different agents, which may explain the discrepancies between the previous results and our findings. Moreover, we found higher baseline percentages of sputum neutrophils in OA induced by LMW agents than those previously reported (65), suggesting that the higher the levels of these cells at baseline, the lower the increase after SIC. Interestingly, we mainly observed higher levels of sputum eosinophils before SIC in patients exposed to isocyanates. This finding corroborates a previous report that certain LMW agents induce OA through IgE-mediated mechanisms (63). However, most LMW
agents seem to induce OA without production of specific IgE antibodies, suggesting the involvement of a different mechanism (72).

Regarding sputum inflammatory biomarkers, we found increases in IL-10 concentrations and reductions in LTB₄ levels after SIC in patients with OA due to exposure to HMW agents, but not in those exposed to LMW agents. We measured levels of LTB₄ because it has been previously demonstrated that this leukotriene could be an important biomarker in OA (70, 73). In these two studies, evaluating the role of LTB₄ in OA due to isocyanates, the authors reported increased LTB₄ levels after SIC in IS (70) or bronchoalveolar lavage (BAL) (73) associated with sputum neutrophilia and increased IL-8 concentrations. To our knowledge, our study is the first to investigate the role of LTB₄ in OA induced by HMW agents, and we observed a decrease in LTB₄ levels after SIC in all patients with OA due to HMW agents except one. In IgE-mediated asthmatic reactions caused by allergens an increase in cysteinyl leukotriene (CysLT) has been demonstrated (74). Furthermore, it has been reported that overproduction of CysLT could cause a decrease in LTB₄ production probably due to the involvement of alternative pathways in the synthesis of both types of leukotrienes (73). In fact, although this hypothesis has not yet been tested in the context of a SIC, it has been demonstrated in asthma exacerbations (75). Regarding cytokine measurements, we found increased levels of IL-10 after SIC in sputum samples of patients with OA caused by HMW agents. IL-10 is a cytokine that plays a key role in regulating Th2 cell responses, inhibiting the expression of many pro-inflammatory cytokines, chemokines, and chemokine receptors (76). The increase in IL-10 levels that we observed after SIC associated only with HMW agents may be related to an immune response directed to the regulation of the Th2-mediated allergic response. When allergic asthmatic individuals are exposed to immunotherapy, IL-10 increases during the first months of treatment (77, 78). Furthermore, spontaneous production of IL-10 has been reported in sputum cell cultures of sensitized asthmatic patients who underwent a bronchial allergenic challenge (79). To the best of our knowledge, only two studies have evaluated IL-10 in patients with OA (60, 79), and the results are inconclusive. One found lower IL-10 sputum levels in patients with suspected OA (with either negative or positive SIC responses) compared to the healthy population (61) and the second reported that occupational exposure to isocyanates is associated with high baseline
levels of IL-10 secreted by peripheral blood mononuclear cells (80). Nevertheless, neither of these studies evaluated IL-10 distinguishing between patients with HMW-induced and LMW-induced OA.

In subjects with OA exposed to LMW agents, we found no significant differences in LTB$_4$ levels or in any of the cytokines determined before and after SIC. Moreover, when comparing LMW-exposed individuals with and without OA, there were no changes in post-SIC cytokine levels. This finding may again be due to the heterogeneous mechanisms involved in the genesis of LMW-induced asthma, which suggests that future studies should distinguish not only between HMW and LMW agents, as mentioned above, but also between pathophysiological mechanisms, i.e., between IgE- and non IgE-dependent mechanisms.

Finally, among subjects with a negative SIC response, all but one had been exposed to LMW agents. They presented an increase in the percentage of sputum neutrophils after SIC, which was more significant in those in whom OA diagnosis was excluded but who were finally diagnosed with other respiratory diseases, such as WEA, asthma unrelated to work, chronic obstructive pulmonary disease (COPD), and bronchiectasis. Our hypothesis is that this finding could be related to the effect of exposure to LMW agents in subjects reporting any previous pulmonary disease (baseline pulmonary disease), which may enhance neutrophil recruitment in the airways. This would correlate with the observed increase in IL-8 levels after SIC in the group of patients without OA compared to those with OA, as IL-8 is a potent chemokine whose major effector function is the recruitment of neutrophils to the site of infection or injury (76). Girard et al. (81) reported that subjects testing negative to SIC had increased percentage of sputum neutrophils after periods at work compared with periods away from work. However, the mechanisms causing this neutrophilic inflammation in patients with other respiratory diseases are still unclear, but it may be due to an irritant effect of certain LMW agents. Indeed, a neutrophilic inflammatory response has been found in the IS of healthy subjects after short-term exposure to irritant agents such as ozone, diesel exhaust and endotoxins (82-84).
3.2 EBC inflammatory profile

Following the same approach as in the previous study, we wanted to investigate the usefulness of EBC analysis in the context of SIC for assessing changes in airway inflammation due to exposure to causal occupational agents. For this purpose, we evaluated 125 subjects with suspected WRA who underwent SIC to HMW and LMW agents. In 66 subjects (53%) SIC was positive to the workplace agents tested and these patients were then diagnosed with OA. The 59 subjects with a negative SIC response were subdivided into two groups according to clinical criteria. Thus, individuals who presented symptoms and abnormal findings on pulmonary function study in relation to work (n=14) received a diagnosis of WEA, whereas those who did not fulfil the criteria for asthma (n=34) or who had asthma in which the symptoms were unrelated to work (n=11) were grouped as non-work-related asthma (NWRA). As in the case of IS, EBC collection was performed the day before and 24 h after exposure to the offending agent. In the EBC samples, we measured the pH and nitrite (NO$_2^-$) and nitrate (NO$_3^-$) concentrations as biomarkers of airway inflammation.

Our findings showed that among all the subjects assessed with suspected OA, 11% had WEA. In studies in general populations or general health care settings providing overall estimates of WEA prevalence, the percentage of patients with WEA ranges from 13% to 58%; most of these studies define WEA based on self-reports of a relationship between work and asthma symptoms (85). Our percentage of patients with WEA is lower than those reported in the previous studies, because of differences in the study populations: our study was conducted in subjects with suspected OA who had a consistent medical history, without including other types of WRA, which probably affected the prevalence.

Although WEA appears to be common, it has received much less systematic study than asthma caused by work. Most of the published literature has addressed OA rather than WEA. Studies on WEA are limited, and this leads to relative uncertainty regarding the definition, prevalence, diagnosis, and management of these conditions (57). Studies investigating socioeconomic outcomes in WRA have generally shown that OA and WEA patients report similar income loss, rates of unemployment, job changes due to asthma, and work disability (86, 87). Nevertheless, some authors suggest that patients with WEA, unlike those with OA, can usually return to their workplace with
adjustments in order to reduce work exposures to likely airway irritants or with optimized asthma medication (88). The diagnosis of WEA is usually based on the existence of asthma before occupational exposure and depends on demonstrating a relationship between work exposures and asthma exacerbations, most commonly documented by changes in symptoms (e.g., frequency, severity), medication use, or lung function temporally related to work, whereas the diagnosis of OA is based on the fact that asthma onset occurs after starting a job, with similar changes in symptoms, medication use, or lung function temporally related to work (85, 89). Nonetheless, according to the American College of Chest Physicians Consensus Statement (57), the possibility that an individual with previous asthma will present with OA cannot be excluded; it may also be the case that a subject will develop non-occupational asthma during his or her working life and that this will be exacerbated by work. In this context, a simple differentiation between OA and WEA based on a temporal relationship between the onset of asthma and the start of an occupation can be erroneous. Thus, it would be useful to have diagnostic methods that make it possible to differentiate between these two conditions. In this sense, currently available diagnostic methods are limited. Peak expiratory flow (PEF) monitoring during periods at and away from work cannot reliably distinguish OA from WEA, because both may be associated with work-related changes in airway calibre (57, 90). Some authors have suggested that SIC could be useful because a positive result confirms the diagnosis of OA; however, when SIC is negative, the situation is more complex. In a study population comprising patients with WRA, one can conclude that individuals with a negative SIC response can be diagnosed with WEA (91). However, in clinical practice, SIC is often performed in patients reporting symptoms but whose asthma is unrelated to work and also in patients who do not have asthma (92). In this context, we wanted to assess the utility of EBC in conjunction with SIC to help establish the definitive diagnosis, trying to differentiate between OA and WEA.

Regarding EBC pH results, we found a significant decrease in EBC pH after SIC in patients with WEA, but no decrease was observed after SIC in the other groups. Furthermore, we used receiver-operating characteristic (ROC) curve analysis to determine the most discriminating cut-off value for changes in EBC pH before and after SIC for predicting WEA. To do so, we used data from a previous study by our group.
(93) in which subjects with suspected OA were monitored for two weeks at work and two weeks off work. EBC pH was measured at the end of each period, and by using ROC curve analysis to predict a diagnosis of OA, we observed that a decrease in EBC pH greater than 0.4 units during periods at work compared to off work achieved the most satisfactory sensitivity (40%) and specificity (90%). Therefore, since a decrease in EBC pH greater than 0.4 units at work had high specificity for diagnosing OA, we used the same cut-off for predicting WEA in conjunction with SIC. Considering all the subjects included in the study, we noted that an EBC pH decrease greater than 0.4 units after SIC achieved a sensitivity of 78% and a specificity of 82% for the diagnosis of WEA. Considering only patients with negative SIC responses and a reliable diagnosis of asthma (14 patients with WEA and 11 patients in the NWRA group who had asthma in which the symptoms were unrelated to the workplace), we found that the same decrease of 0.4 units in the EBC pH after SIC achieved the most satisfactory sensitivity (79%) and specificity of 100%. These results suggest that measurement of EBC pH in the context of SIC may be useful for diagnosing WEA.

Most of the patients diagnosed with WEA (79%) experienced a decrease in EBC pH of 0.4 units or more after SIC. Taking into account the type of occupational agent to which they had been exposed, we observed that 82% of patients who experienced this significant EBC pH decrease had been exposed to LMW agents, and only 18% had been exposed to HMW agents. WEA can result from a variety of occupational triggers and conditions at work, including physical factors (e.g., extreme worksite temperatures, exercise), behavioural states (e.g., strong emotions, stress), odours (e.g., perfumes), general irritants, accidental exposures to high levels of chemicals, common aeroallergens not specific to the work environment (e.g., dust), and second-hand cigarette smoke (85). Because of this large variety of potential triggering factors that can exacerbate asthma, we cannot rule out a non-specific mechanism that would lead to a decrease in EBC pH after exposure to the offending agent. In fact, LMW agents are substances that may easily cause non-specific airway irritation with symptoms in patients with WEA or other respiratory diseases; this may have been the case of three patients testing negative in SIC in the NWRA group, who also showed an EBC pH decrease after SIC and had eosinophilic bronchitis or bronchiectasis. These results are consistent with the findings of our previous study of sputum inflammatory profiles,
where we found that exposure to LMW agents in patients reporting any previous pulmonary disease resulted in an increased airway inflammation, especially a neutrophilic airway inflammation. Thus, these findings in both IS and EBC studies suggest that exposure to LMW agents induces higher airway inflammation in subjects with baseline pulmonary disease. In the case of patients with OA, we did not find any significant decrease in EBC pH after SIC regardless of whether they had been exposed to HMW or to LMW agents. However, taking into account the type of occupational agent, a decrease in EBC pH of 0.4 units or greater after SIC was more common in OA patients exposed to HMW agents (42%) than in those exposed to LMW agents (15%).

Regarding NO\textsubscript{2} and NO\textsubscript{3} measurements, we observed an increase in NO\textsubscript{3} levels after SIC in OA patients exposed to HMW agents, but not in those exposed to LMW agents. Furthermore, in these same patients with OA due to HMW agents, the pH decrease after SIC was related to the fact that baseline EBC NO\textsubscript{2} levels were more elevated. NO\textsubscript{2} and NO\textsubscript{3} are considered stable oxidative end products of nitric oxide (NO) metabolism which may be associated with respiratory diseases. In the American Thoracic Society/European Respiratory Society statement on EBC (43), NO\textsubscript{2} and NO\textsubscript{2}/NO\textsubscript{3} levels are described to be elevated in asthma, cystic fibrosis, and bronchiectasis and it is attributed to an increased NO metabolism. Furthermore, it has been reported that NO\textsubscript{3} varies in patients with inflammatory airway diseases, being higher in asthmatics and lower in COPD patients than in healthy subjects (94). However, in a recent study increased NO\textsubscript{2}/NO\textsubscript{3} levels were observed in COPD and cystic fibrosis patients as well as in lung transplant recipients compared to healthy subjects, but not in mild asthmatics (95). The source of NO\textsubscript{2}/NO\textsubscript{3} is currently under debate because it is known that changes in nitrogen oxide concentrations not only reflect NO formation, but also depend on a variety of other physiological or pathophysiological conditions in the airways (96).

To our knowledge, only a few studies have focused on NO determination in OA, and in general, in these studies, an increase in NO levels is reported in response to HMW agents but not in response to LMW agents (97-100). These findings might be again attributable to differences in the pathophysiology of asthma induced by these two types of occupational agents. Thus, the airway changes produced by exposure to HMW and LMW agents differ, and these differences may be reflected in EBC biomarkers like...
EBC pH. Nevertheless, no clear evidence exists that LMW agent-induced asthma is associated with acidification of EBC pH, because the studies currently available report conflicting findings. Our results regarding EBC pH are consistent with those reported by Ferrazzoni et al. (97), who found no evidence that isocyanate-induced asthma is associated with EBC acidification. However, Boyce et al. (101) reported increased pH values in workers exposed to welding fumes, and Fireman et al. (102) found significantly lower levels in welders exposed to cadmium/chromium/iron/lead/nickel, and no significant changes in those exposed to aluminium/iron. The discrepancies between these studies suggest the presence of different mechanisms of action in the two types of WRA and would explain the differences that we observed regarding EBC pH and NO\textsubscript{2} and NO\textsubscript{3} concentrations in patients with OA exposed to HMW and LMW agents. Furthermore, our results suggest that the mechanism of action of LMW agents seems to differ according to whether they cause OA or induced WEA.
References


CONCLUSIONS
Chapter 1: Inflammation in well-controlled asthma

- Airway inflammation and airway hyper-reactivity (AHR) persist in most well-controlled asthma patients despite the fact that their condition is controlled. When AHR persists despite treatment, it is more severe in patients with eosinophilic inflammation than in those with neutrophilic inflammation or with normal induced sputum cell counts.

- Measurement of airway inflammation in patients with well-controlled asthma seems to be essential to achieve proper asthma control.

Chapter 2: Exhaled breath condensate biomarkers

- pH values and 8-isoprostane levels in exhaled breath condensate (EBC) show a relationship with age. The interpretation of acidic and oxidative stress, as well as understanding nitrogen oxide chemistry in the lung of older subjects, should take into account that changes occur in the cellular pattern of the airways with age.

- The values obtained for EBC biomarkers in studies with control groups should be adjusted for age.

Chapter 3: Inflammation in occupational asthma

- In patients with occupational asthma (OA) exposed to high-molecular-weight (HMW) agents, in parallel with the increase in sputum eosinophils there may also be an increase in the number of sputum neutrophils, although this does not rule out an IgE-mediated mechanism.

- Exposure to low-molecular-weight (LMW) agents in subjects with a baseline pulmonary pathology unrelated to OA seems to enhance neutrophil recruitment in the airways, suggesting the presence of different mechanisms of action depending on whether LMW agents induce OA or cause an aggravation of a pre-existing respiratory disease.

- The variability in the responses observed in patients with OA exposed to LMW agents suggests that more than one mechanism may be involved in the genesis of the disease and future studies should aim to examine this possibility.
Conclusions

Chapter 4: Exhaled breath condensate biomarkers in work-related asthma

- EBC pH, in conjunction with specific inhalation challenge, is useful for diagnosing work-exacerbated asthma (WEA).

- EBC inflammatory biomarkers should be carefully evaluated to investigate their potential capacity to establish differences between OA caused by HMW or LMW agents and to distinguish between patients with OA, WEA and asthma unrelated to work.
Airway inflammation plays a central role in the development and progression of many respiratory diseases. Asthma is a chronic disorder of the airways characterized by a reversible airway obstruction, airway inflammation and non-specific airway hyperreactivity (AHR). Currently, asthma is considered to be a heterogeneous, multifactorial disease that includes distinct phenotypes, each one with its own natural history and its own response to treatment. It is estimated that up to 25% of all asthma cases developing in adulthood are caused by occupational exposure. This condition is known as work-related asthma (WRA); it includes both occupational asthma (OA) and work-exacerbated asthma (WEA), and it presents a major health challenge with significant potential for acute morbidity, long-term disability, and adverse social and economic impact. OA refers to \textit{de novo} asthma caused by exposure to an agent specific to a workplace and not to stimuli outside the work environment, and WEA is defined as a worsening of pre-existing or concomitant asthma which is exacerbated by working conditions. Occupational agents inducing OA or causing WEA can be divided into two categories: high-molecular-weight (HMW) agents and low-molecular-weight (LMW) agents.

Like bronchial asthma, WRA is a heterogeneous chronic inflammatory disorder of the airways. The study of airway inflammation is of great value for establishing a precise diagnosis, as it is a direct reflection of the disease. Assessment of airway inflammation in a non-invasive manner does not disturb the underlying disease process and allows the monitoring of airway inflammation, and in recent years this practice has aroused growing interest in the attempts to understand the pathophysiological mechanisms of inflammatory airway diseases. This thesis aimed to establish the usefulness of two non-invasive methods: induced sputum (IS) and exhaled breath condensate (EBC) for the assessment of airway inflammation in subjects with suspected WRA.

Two studies were carried out in asthmatic patients with stable, well-controlled asthma and in a healthy adult sample as control groups in order to provide reference data for the respective studies performed later with IS and EBC samples in subjects with suspected WRA. Evaluating the type and degree of airway inflammation present in these control patients with well-controlled asthma we found that airway inflammation and AHR persist in most patients in spite of treatment, and that when AHR persists, it is more
severe in patients with eosinophilic inflammation than in those with neutrophilic inflammation or with normal IS cell counts. The second study was carried out in healthy adults stratified into groups according to age, in order to establish reference values for certain biomarkers of airway inflammation and to determine whether there are age-associated differences. pH values and 8-isoprostane levels in EBC showed a relationship with age. Therefore, our data suggest that the values obtained in studies with control groups should be adjusted for this factor.

Assessment of airway inflammation in WRA improved our understanding of the pathophysiological mechanisms implicated in the genesis of the different types of WRA. In this context, it seems necessary to distinguish between the different types of occupational agents when conducting airway inflammation studies. We investigated the inflammatory profile by evaluating sputum differential cell counts and several inflammatory biomarkers in sputum supernatants of subjects with suspected WRA preceding and following a specific inhalation challenge (SIC). Increases in sputum eosinophils and neutrophils and in interleukin (IL)-10 concentration and a decrease in leukotriene B₄ (LTB₄) after exposure to HMW agents have been reported. These findings support the notion that most HMW agents induce OA via an IgE-mediated mechanism inducing a Th2-mediated allergic response. No significant changes in sputum differential cell counts or inflammatory biomarkers were found after SIC in patients with OA due to exposure to LMW agents. However, exposure to LMW agents can result in increased neutrophilic inflammation in patients with airway diseases unrelated to OA, suggesting different mechanisms of action according to whether the LMW agent is the cause of OA or provokes aggravation of a pre-existing respiratory disease. Investigating the inflammatory profile by analysing EBC in subjects with suspected WRA, EBC pH after exposure to the offending agent had a sensitivity of 79% and specificity of 100% for the diagnosis of WEA, demonstrating that in conjunction with SIC this biomarker may be useful for diagnosing WEA, and suggesting again that the mechanism of action of LMW agents seems to differ according to whether they cause OA or induce WEA.
RESUMEN
La inflamación bronquial desempeña un papel central en el desarrollo y la progresión de muchas enfermedades respiratorias. El asma es un trastorno crónico de las vías respiratorias caracterizado por una inflamación bronquial que condiciona una obstrucción reversible y/o una hiperrespuesta no específica de la vía aérea. En la actualidad, el asma es considerado una enfermedad heterogénea y multifactorial que incluye distintos fenotipos, cada uno con su propia historia natural y su propia respuesta al tratamiento. Se estima que hasta un 25% de los casos de asma que se desarrollan en la edad adulta están relacionados con una exposición laboral. Esta entidad, que se conoce con el nombre de asma relacionado con el trabajo (ART), incluye el asma ocupacional (AO) y el asma exacerbado por el trabajo (AET), y representa un problema de salud importante con un significativo potencial sobre la morbilidad aguda, discapacidad a largo plazo, así como un negativo impacto socio-económico. El AO se refiere al asma causado de novo por la exposición a un agente específico en un lugar de trabajo y no debido a estímulos externos al entorno laboral, y el AET se define como un empeoramiento de un asma preexistente o concomitante que se ve agravanado por las condiciones de trabajo. Los agentes ocupacionales que inducen AO o causan AET se pueden dividir en dos categorías: agentes de alto peso molecular (APM) y agentes de bajo peso molecular (BPM).

Al igual que el asma bronquial, el ART es una enfermedad inflamatoria crónica heterogénea de las vías respiratorias. El estudio de la inflamación bronquial es de gran utilidad para establecer un diagnóstico preciso, ya que ésta es un reflejo directo de la enfermedad. La evaluación de esta inflamación bronquial de forma no invasiva no interfiere en el proceso subyacente de la enfermedad, permitiendo así su monitorización. Además, en los últimos años, existe un interés creciente en su utilización en un intento por comprender los mecanismos fisiopatológicos implicados en las enfermedades inflamatorias respiratorias. El objetivo de esta tesis fue establecer la utilidad de dos métodos no invasivos: el esputo inducido (EI) y el condensado de aire exhalado (CAE) en la evaluación de la inflamación de las vías respiratorias en pacientes con sospecha de ART.

Inicialmente se realizaron dos estudios, uno en pacientes asmáticos con asma estable, bien controlado y otro en una población adulta sana, que sirvieron como grupos
controles con el fin de proporcionar datos de referencia para los respectivos estudios realizados posteriormente con muestras de EI y de CAE en sujetos con sospecha de ART. Al analizar el tipo y el grado de inflamación bronquial en los pacientes controles con asma estable, se observó que la inflamación y la hiperrespuesta bronquial persisten en la mayoría de estos pacientes a pesar del tratamiento, y que cuando la hiperrespuesta persiste, ésta es más grave en los pacientes con inflamación eosinofílica que en aquellos que presentan inflamación neutrofílica o recuentos celulares normales en EI. El segundo estudio se llevó a cabo en adultos sanos estratificados por grupos según su edad, a fin de establecer valores de referencia para ciertos marcadores de inflamación bronquial y para determinar si existían diferencias asociadas con la edad. Los valores de pH y los niveles de 8-isoprostaneno en el CAE presentaron una relación con la edad. Por lo tanto, nuestros datos sugieren que los valores obtenidos en estudios con grupos controles deberían ser ajustados por este factor.

La evaluación de la inflamación bronquial en el ART ha permitido mejorar nuestros conocimientos a cerca de los mecanismos fisiopatológicos implicados en la génesis de los diferentes tipos de ART. En este contexto, cuando se realizan estudios de inflamación bronquial, parece ser necesario distinguir entre los diferentes tipos de agentes ocupacionales. Se investigó el perfil inflamatorio de sujetos con sospecha de ART mediante la evaluación de contajes celulares diferenciales en muestras de EI y de varios biomarcadores inflamatorios en el sobrenadante de estas muestras antes y después de la realización de una prueba de provocación bronquial específica (PPBE). Se encontró un incremento de eosinófilos y neutrófilos en esputo y de la concentración de interleuquina (IL) -10, así como una disminución de los niveles de leucotrieno B4 (LTB4) tras la exposición a agentes de APM. Estos resultados son concordantes con el hecho de que la mayoría de los agentes de APM inducen AO a través de un mecanismo mediado por IgE, dando lugar a una respuesta alérgica mediada por células Th2. No se observaron diferencias significativas en los recuentos celulares diferenciales ni en los niveles de biomarcadores inflamatorios en las muestras de EI de los pacientes con AO causada por exposición a agentes de BPM. Sin embargo, se observó que la exposición a agentes de BPM puede ocasionar un aumento de la inflamación neutrofílica en pacientes con enfermedades respiratorias no relacionadas con AO, hecho que sugiere la existencia de diferentes mecanismos de acción en función de si el agente de BPM es la causa del
AO o bien si éste provoca un empeoramiento de una enfermedad respiratoria preexistente. Al investigar el perfil inflamatorio de sujetos con sospecha de ART mediante el análisis del CAE, se observó que tras la exposición al agente causal durante la PPBE el pH del CAE mostró una sensibilidad del 79% y una especificidad del 100% para el diagnóstico del AET, lo que demuestra que este biomarcador conjuntamente con la PPBE puede ser útil para el diagnóstico del AET, y sugiere de nuevo que el mecanismo de acción de los agentes de BPM parece variar en función de si éstos causan AO o inducen AET.
Esta tesis doctoral no hubiera sido posible sin la ayuda y el apoyo de todas aquellas personas que, de una manera u otra, han contribuido a hacer de ella una realidad, a todas muchísimas gracias.

En primer lugar, quisiera empezar dando las gracias a mis directores de tesis. A la Dra. Mª Jesús Cruz, por permitirme formar parte del equipo de investigación en Neumología, por la confianza depositada en mí y ofrecerme la posibilidad de realizar este proyecto, por su dedicación, y por ser más que una jefa, muchas gracias Chus. Al Dr. Xavier Muñoz, por aportar la vertiente más clínica, por su entusiasmo, motivación y rigurosidad, y por hacernos partícipes de sus dos grandes pasiones. A los dos, muchas gracias por esos “momentos agenda” y los ratos tan agradables en el laboratorio y fuera de él. Al Dr. Ferran Morell, por su inestimable dedicación a la investigación, por su amabilidad y profesionalidad, y por el interés y el apoyo recibido durante la realización de esta tesis.

Muchísimas gracias también a mis compañeros de laboratorio, por todos los momentos vividos durante estos años. A Lola, fiel compañera de despacho, por su dedicación y por ser nuestra proveedora oficial de chocolate. A Susana, por su amabilidad, por estar siempre ahí, por tantas conversaciones varias, y por enseñarme Londres. A Marta y Dani, los últimos fichajes, muchas gracias también por vuestro apoyo en esta última etapa de la tesis, sobre todo Marta, que lo ha vivido muy de cerca. A los que ya no están, Laura y Maribel, gracias por empezar conjuntamente esta larga travesía, espero que a vosotras también os llegue este momento. En especial a Laura, mi “Pili”, por tantísimas cosas y momentos compartidos, te echo de menos. Y a Oriol, fue un placer compartir dos años de “recerca” contigo.

Quisiera agradecer al Servicio de Citometría de la UCTS del Institut de Recerca Vall d’Hebron, en especial a Álex, por su disposición y ayuda.

Gracias a Ana y a Mónica, las “chicas de funcionales”, que pese a la distancia de la segunda planta también habéis vivido muy de cerca esta tesis, gracias por vuestros ánimos y apoyo.
A todas las personas del Servicio de Neumología, por el trabajo en equipo y por crear un ambiente tan agradable. A Rosa y a Montse, por estar siempre dispuestas a ayudar y junto con Marian, por tantas conversaciones durante las comidas. A las enfermeras del Gabinete de Pruebas Funcionales Respiratorias, Teresa, Montse y Nieves, muchas gracias por vuestra colaboración semanal en la recogida de muestras. De la misma manera, me gustaría hacer llegar un agradecimiento especial a todos los pacientes y voluntarios que consintieron participar en los diferentes estudios, sin su colaboración no habría sido posible esta tesis.

También me gustaría agradecer al Dr. Romero del Hospital de Bellvitge, con quien inicié esta apasionante aventura que es la investigación, por su entrega y perseverancia, siempre recibiendo con una agradable sonrisa, y por ser el vínculo con el Hospital Vall d’Hebron.

Finalmente, dar las gracias a los míos. Muchas gracias a mi madre, por serlo todo para mí, por su paciencia y apoyo incondicional. A mi padre, a quien se la dedico especialmente, porque allá donde esté estoy segura que se sentirá muy orgulloso. Muchísimas gracias a los dos, esta tesis también es vuestra. A mi familia, mis dos yayas, tíos y primos, por animarme y acompañarme durante estos años. Y gracias también a los amigos que han estado siempre ahí, siguiendo de cerca este viaje, y a todos los salseros, por ser una gran familia. Y para terminar, gracias a Néstor, por todo, por estar ahí, por ponerle “salsa” a mi vida en todos los sentidos.

Muchísimas gracias a todos.
SCIENTIFIC PRODUCTION & FINANCIAL SUPPORT
List of publications


List of national and international presentations


**Financial support related to this doctoral thesis**

1. Grant FIS PI050100 of the Instituto de Salud Carlos III.
2. Grant FIS/Evaluación de tecnologías PI07/90086 of the Instituto de Salud Carlos III.
3. Sociedad Española de Neumología y Cirugía Torácica (SEPAR).
4. Societat Catalana de Pneumologia (SOCAP).
5. Fundació Catalana de Pneumologia (FUCAP).
6. Ciber Enfermedades Respiratorias (CibeRes), Insituto de Salud Carlos III.