Sputum Inflammatory Patterns are Associated with Distinct Clinical Characteristics in Subjects with Occupational Asthma Independently from the Causal Agent

Short Title: Sputum inflammatory patterns in occupational asthma

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Abstract

Background: Clinical heterogeneity in sensitizer-induced occupational asthma (OA) and its relationship to airway inflammatory profiles remain poorly elucidated.

Objectives: To further characterize the interactions between induced sputum inflammatory patterns, asthma-related outcomes and the high- or low-molecular-weight category of causal agents in a large cohort of subjects with OA.

Methods: This multicenter, retrospective, cross-sectional study was conducted among 296 subjects with OA ascertained by a positive specific inhalation challenge who completed induced sputum assessment before and 24 hours after challenge exposure.

Results: Multivariate logistic regression analysis revealed that sputum eosinophilia ≥3% was significantly associated with a high dose of inhaled corticosteroid (odds ratio [95% confidence interval], 1.31 [1.11-1.55] for each 250-µg increment in daily dose), short-acting β2-agonist use less than once a day (3.54 [1.82-7.00]), and the level of baseline nonspecific bronchial hyperresponsiveness (mild: 2.48 [1.21-5.08]); moderate/severe: 3.40 [1.44-8.29]). Sputum neutrophilia ≥76% was associated with age (1.06 [1.01-1.11]), male gender (3.34 [1.29-9.99]), absence of corticosteroid use (5.47 [2.09-15.16]), short-acting β_2-agonist use once or more a day (4.09 [1.71-10.01]), ≥2 severe exacerbations during the last 12 months at work (4.22 [1.14-14.99]), and isolated early reactions during the SIC (4.45 [1.85-11.59]).

Conclusion: The findings indicate that sputum inflammatory patterns in subjects with OA are associated with distinct phenotypic characteristics and further highlight the differential effects of neutrophils and eosinophils on asthma-related outcomes. These associations between inflammatory patterns and clinical characteristics share broad similarities with what has been reported in nonoccupational asthma and are not related to the type of causal agent.

Keywords: Eosinophils. Induced sputum. Neutrophils. Occupational asthma. Phenotype.

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Resumen

Antecedentes: la heterogeneidad clínica en el asma ocupacional (AO) inducida por agentes sensibilizantes y su relación con los perfiles inflamatorios de las vías respiratorias siguen siendo muy poco conocidas.

Objetivos: Profundizar en la caracterización de las interrelaciones entre los patrones inflamatorios en esputo inducido, diversas variables relacionadas con el asma y la categoría de agentes causales de alto o bajo peso molecular, en una gran cohorte de sujetos con AO.

Métodos: Este estudio multicéntrico, retrospectivo y transversal se llevó a cabo en 296 sujetos con OA confirmada mediante una provocación bronquial específica (SIC) positiva en los que se obtuvieron muestras de esputo inducido antes y 24 horas después de la SIC. Resultados: El análisis de regresión logística multivariable reveló que la presencia de eosinofilia en esputo ≥3 % se asoció significativamente con una dosis alta de corticosteroides inhalados (odds ratio [intervalo de confianza del 95 %], 1,31 [1,11-1,55] por cada incremento de 250 µg en la dosis diaria), el uso de agonistas b2 de acción corta menos de una vez al día (3,54 [1,82-7,00]), y un nivel de hiperreactividad bronquial inespecífica inicial (leve: 2,48 [1,21-5,08]); moderado/grave: 3,40 [1,44-8,29]). La neutrofilia en esputo ≥76 %, se asoció con la edad (1,06 [1,01-1,11]), el sexo masculino (3,34 [1,29-9,99]), la ausencia de uso de corticosteroides (5,47 [2,09-15,16]), el uso de agonistas b2 de acción corta una vez o más al día (4,09 [1,71-10,01]), la presencia de ≥ 2 exacerbaciones graves en los últimos 12 meses en el trabajo (4,22 [1,14-14,99]) y reacciones inmediatas aisladas durante la SIC (4,45 [1,85-11,59]).

Conclusión: los resultados del estudio indican que los patrones inflamatorios del esputo en sujetos con OA están asociados con características fenotípicas distintas y resaltan aún más los efectos diferenciales de la inflamación bronquial neutrofílica o eosinofílica en las distintas variables relacionadas con el asma. Estas asociaciones entre patrones inflamatorios y características clínicas comparten amplias similitudes con lo que se ha descrito en el asma de origen no ocupacional y no están relacionadas con el tipo de agente causal.

Palabras clave: Eosinófilos. Esputo inducido. Neutrófilos. Asma ocupacional. Fenotipo.

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INTRODUCTION

Sensitizer-induced occupational asthma (OA), a distinguishable subset of adult asthma, is characterized by the *de novo* inception of asthma or the recurrence of previously quiescent asthma induced by immunologically mediated sensitization to specific agents at the workplace [1, 2]. These workplace sensitizing agents are conventionally categorized into high-molecular-weight (HMW) (glycol)proteins from animal, vegetal or microbiological origin and low-molecular-weight (LMW) agents that include reactive chemicals, metals and wood dusts [1, 2]. OA caused by HMW agents is associated with demonstrable specific IgE antibodies while LMW agents act as haptens, binding to endogenous proteins to initiate a specific immunologic response through mechanisms that remain largely uncertain [3].

The noninvasive induced sputum technique allowed the identification of eosinophilic and noneosinophilic inflammatory patterns of asthma that are associated with different clinical phenotypes and are likely related to differences in underlying pathobiological pathways [4-13]. However, clinical heterogeneity in patients with OA and its relationship to sputum inflammatory profiles remain poorly elucidated. The available studies provided sparse and often discordant information pertaining to the relationships between eosinophilic inflammation and asthma outcomes [14-21]. In addition, most of these studies failed to specifically investigate the clinical and functional characteristics associated with sputum neutrophilia, although it has been suggested that OA induced by LMW agents may be associated with higher sputum neutrophilia compared to HMW agents [14, 16, 22].

This study aimed at further characterizing the relationships between sputum inflammatory patterns, asthma-related outcomes at the time of the diagnostic evaluation, and the type of causal agent in a large cohort of subjects with OA ascertained by a positive specific inhalation challenge (SIC).

METHODS

Study Design and Population

This retrospective cross-sectional study was conducted among subjects with OA ascertained by a positive SIC completed between 2006 and 2018 in tertiary centers participating to the European network for the PHenotyping of OCcupational Asthma (E-PHOCAS) [23-27]. Eligible subjects for this analysis were those with complete information on key asthma outcomes (i.e. detailed medication and number of severe exacerbations) and induced sputum samples collected both before and 24 hours after the SIC procedure. Cohort recruitment is further

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detailed in the online Supplementary Materials. This report conformed to the Strengthening of the Reporting of Observational Studies in Epidemiology statement for cross-sectional studies (*www.strobe-statement.org*).

Ethics

This retrospective E-PHOCAS study was approved by the local Institutional Review Board of all participating sites as well as the "*Comité Consultatif sur le Traitement de l'Information en Matière de Recherche dans le Domaine de la Santé*" and the "*Commission Nationale de l'Informatique et des Libertés*".

Asthma Outcomes

Details of data collection and interpretation are given in the online Supplementary Materials [23, 24]. Information on asthma medication, including the frequency of short-acting β_2-agonist (SABA) use, and severe exacerbations while the subjects were exposed at work (last 12 months at work for exacerbations) was used for this analysis. "Poor symptom control" was defined by the need for an inhaled short-acting β2-agonist (SABA) once or more a day [28]. Severe asthma exacerbations and severe asthma were defined according to the European Respiratory Society/American Thoracic Society consensus guidelines [29].

Lung Function Assessments

Prechallenge spirometric values as well as the level of nonspecific bronchial hyperresponsiveness (NSBH) measured at baseline of the SIC procedure and 24 hours after challenge exposure were collected. NSBH was graded as "absent", "mild", or "moderate-to-severe" [23] according to the bronchoprovocation method used in each center (see online Supplementary Materials and Table S1). The methodology and interpretation of SIC is further described in the online Supplementary Materials and conformed with international recommendations [30].

Induced Sputum Assessment

Induced sputum was collected at baseline and 24 hours after the SIC. Detailed information on the methods used for the induction and analysis of sputum samples in participating centers is included in the online Supplementary Materials. "Sputum eosinophilia" was defined by a sputum eosinophil count ≥3% while a sputum neutrophil count ≥76% was regarded as reflecting "sputum neutrophilia" [11]. Accordingly, the sputum inflammatory patterns were classified as "eosinophilic" (i.e., ≥3% eosinophils and <76% neutrophils); "neutrophilic" (i.e.,

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neutrophils ≥76% and <3% eosinophils); “paucigranulocytic” (i.e., <3% eosinophils and <76% neutrophils); or “mixed granulocytic” (i.e., ≥76% neutrophils and ≥3% eosinophils).

In this study, we used sputum cell counts obtained 24 hours after the SIC as the primary outcome since a significant decrease in sputum eosinophil counts has been reported within two weeks after removal from exposure [31] and 173 of 296 (58.4%) subjects in this cohort were already removed from the causal exposure for more than one week at the time of the SIC procedure.

Data Analysis

Data are presented as the median and interquartile range for continuous variables and percentages for categorical variables. The comparison between groups of subjects was made using the Fisher’s exact or chi-squared test for categorical variables and non-parametric tests for numerical variables. Pairwise comparisons between the four sputum inflammatory patterns in order to identify which groups differed from each other were corrected according to the Benjamini-Hochberg method for multiple comparisons.

Multivariable logistic regression analyses were conducted in order to identify the clinical and physiological characteristics that were associated with a ≥3% sputum eosinophil count or a ≥76% neutrophil count in samples collected 24 hours after challenge exposure. These multivariable analyses were carried out using a binomial generalized linear model with the best parsimonious models selected using a stepwise procedure based on the Akaike information criterion. The independent variables incorporated into these regression models included both sociodemographic characteristics (age, gender, smoking status, and body mass index ≥30 kg/m^2) and the variables with a *P*-value ≤0.1 in univariate comparisons. Missing values were not imputed. Peripheral bood eosinophil counts and fractional exhaled nitric oxide (FeNO) were not included in the multivariate models because these biomarkers and sputum eosinophils are known to be interrelated [32] and reflect T2-high airway inflammation, and these data were missing in a substantial proportion of the subjects.

Sensitivity analyses were made by restricting the regressions to the pre-challenge (baseline) sputum eosinophil or neutrophil counts in the subjects who were still exposed to the offending agent within one week before the SIC procedure (n=123). Statistical analysis was performed using the R software version 3.4.1 (www.r-project.org, Vienna, Austria). A *P* value <0.05 was considered significant.

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RESULTS

Population

The study included 296 subjects with available sputum samples collected both before and 24 hours after the SIC procedure. The clinical and physiological characteristics of the subjects grouped according to their post-challenge sputum inflammatory pattern are presented in Tables 1 and 2. The majority (67.9%) of the subjects demonstrated a post-challenge eosinophilic pattern, whereas a paucigranulocytic, neutrophilic, or mixed granulocytic pattern was observed in 18.2%, 7.8%, and 6.1% of the cohort, respectively. The clinical and functional characteristics associated with the four sputum inflammatory patterns are compared in the online Supplementary Materials. The workplace agents causing OA in this cohort are detailed in Table S2 of the Supplementary Materials.

Determinants of Sputum Eosinophilia

The clinical and physiological characteristics of the subjects with (n=219) and without (n=77) a post-challenge sputum eosinophil count ≥3% and the univariate associations with post-challenge sputum eosinophilia are detailed in Table 3. The multivariate logistic regression analysis (Table 4; multivariate model 1) revealed that a high dose of ICS while the subjects were exposed at work (odds ratio, 1.31; 95% confidence interval [CI], 1.11-1.55 for each 250-µg increment in daily dose of ICS; *P*=0.002), SABA use less than once a day at work (odds ratio, 3.54; 95% CI, 1.82-7.00; *P*<0.001), as well as mild (odds ratio, 2.48; 95% CI, 1.21-5.08; *P*=0.012) and moderate-to-severe NSBH (odds ratio, 3.40; 95% CI, 1.44-8.29; *P*=0.006) were significant predictors of a post-challenge sputum eosinophilia.

The multivariate analysis of the pre-challenge sputum eosinophil count ≥3% restricted to subjects still exposed at work within one week of the SIC procedure (n=123) (Table 4) also identified a high dose of ICS, SABA use less than once a day and the level of NSBH as the main factors associated with sputum eosinophilia. Univariate associations with pre-challenge sputum eosinophilia are detailed in Table S3.

Determinants of Sputum Neutrophilia

Table 5 provides the characteristics of subjects with (n=41) and without (n=255) a post-challenge sputum neutrophil count ≥76% and the univariate associations with post-challenge sputum neutrophilia. The multivariate analysis (Table 6) showed that sputum neutrophilia was significantly associated with older age (odds ratio for a 1-yr increase, 1.06; 95% CI, 1.01-1.11; *P*=0.014), male gender (odds ratio, 3.34; 95% CI, 1.29-9.99; *P*=0.019), absence of ICS use

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while at work (odds ratio, 5.47; 95% CI, 2.09-15.16; *P*<0.001), poor asthma control at the workplace (odds ratio, 4.09; 95% CI, 1.71-10.0; *P*=0.024), a history of two or more severe exacerbations during the last 12 months while exposed at work (odds ratio, 4.22; 95%CI, 1.14-14.9; *P*=0.025), and the development of isolated immediate reactions during the SIC (odds ratio, 4.45; 95% CI, 1.85-11.59; *P*=0.001).

Table S3 in the online Supplementary Materials provides the univariate associations for pre-challenge sputum neutrophili count ≥76% among the 123 subjects still exposed at work. The multivariate analysis of pre-challenge sputum neutrophilia retained only the absence of ICS use (odds ratio, 5.09; 95% CI, 1.82-15.22; *P*=0.002) as a significant factor associated with sputum neutrophilia (Table 5).

Associations between Causal Agents and Sputum Inflammatory Patterns

Although Table 3 indicates that HMW agents were more frequently involved in subjects who showed an eosinophilic pattern, exposure to a HMW agent was not retained as a significant determinant for sputum eosinophilia in the multivariate analysis (Table 4; multivariate model 1). Interestingly, however, the presence of work-related rhinitis in subjects exposed to a HMW agent (Table 4; model 2) was significantly associated with a post-challenge sputum eosinophil count ≥3% (odds ratio, 2.78; 95% CI, 1.55-5.0; *P*=0.001), while the dose of ICS, SABA use less than once a day, and NSBH remained significant. In contrast, the multivariate analysis conducted among subjects still exposed at work at the time of the SIC procedure (n=123) failed to show an association between pre-challenge sputum eosinophilia and exposure to HMW agents, even when associated with work-related rhinitis (Table 4; models 1 and 2).

Table S4 in the online Supplementary Materials provides detailed information on the HMW and LMW agents that elicited asthmatic reactions associated with a neutrophilic (n=23) or a mixed granulocytic (n=18) pattern. Notably, HMW agents were involved in 24 of 41 (58.5%) subjects with a post-challenge sputum neutrophilia ≥76%. Flour was the predominant agent, accounting for 19 of 24 cases related to HMW agents. Interestingly, IgE-mediated sensitization to flour was documented by skin-prick testing and/or the determination of specific IgE antibodies in 20 of these 24 subjects with HMW-induced sputum neutrophilia.

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DISCUSSION

This large cohort study is, to our knowledge, the first that comprehensively characterizes the clinical and functional characteristics associated with neutrophilic and eosinophilic airway inflammation in OA.

The multivariate analysis revealed that post-challenge sputum neutrophilia ≥76% was significantly and independently associated with older age, poorer asthma control, more frequent severe asthma exacerbations, and low ICS use while exposed at work. These findings are consistent with studies conducted in general adult asthma populations that documented associations between neutrophilic airway inflammation and age [6-8], more severe disease and poorer asthma control [4, 5, 12], although sputum neutrophilia was not associated with lower lung function in our cohort of subjects with OA [6, 8].

Although this cohort indicated that neutrophilic OA showed phenotypic similarities with nonoccupational asthma, there were also slight differences compared with the findings of studies conducted in general adult asthma populations. Male gender was associated with an increase likelihood of sputum neutrophilia, while a female preponderance has been reported in some studies on general asthma [7]. Another intriguing observation was the association between post-challenge sputum neutrophilia and isolated immediate asthmatic reactions that has to our knowledge not been described after inhalation challenges with common inhalant allergens in subjects with allergic asthma [33].

The mechanisms underlying neutrophilic airway inflammation and its role in asthma remain an area of intense research [34, 35]. It is currently acknowledged that neutrophilic inflammation may reflect innate immune responses to environmental triggers, such as ozone, diesel exhaust particulates and bacterial endotoxin (lipopolysaccharide) which have been documented as inducing an increase in sputum neutrophils. Remarkably, a substantial proportion (58.5%) of the subjects with sputum neutrophilia in our cohort were challenged with HMW protein agents, mainly flour, while IgE-mediated sensitization to these HMW agents was documented in the vast majority (83.3%) of these subjects (Table S4). This obervation is consistent with an earlier study that demonstrated neutrophilic airway inflammation in subjects with OA who developed predominantly immediate asthmatic reaction after challenge exposure to cereal grain dust extracts [36]. Sputum neutrophilia, either isolated or combined with sputum eosinophilia, has also been documented in 11% of subjects with allergic asthma after an inhalation challenge with nonoccupational inhalant allergens [37]. Endotoxins that likely "contaminate" allergen extracts and HMW workplace agents may promote neutrophilic inflammation via Toll-like

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receptor-4 signalling [38]. Collectively, these data further indicate that a HMW agent is capable of initiating either type 2 (T2), non-T2, or mixed immune responses. Whether the elicitation of T2 vs. non-T2 immune responses is affected by host-related (e.g., airway microbiome) and environmental factors (e.g., endotoxin) that interact with HMW occupational agents warrants further prospective investigations.

The majority (67.9%) of patients with OA demonstrated a post-challenge sputum eosinophilic pattern which is consistent with what has been described in subjects exposed to common inhalant allergens [37]. However, there is limited information about the determinants of eosinophilic airway inflammation and its relationship with the clinical and functional phenotypes in subjects with OA. Previous studies in OA related sputum eosinophilia to more severe disease at the time of the diagnostic evaluation, being associated with worse quality of life [16] and more frequent use of ICS [16] compared to noneosinophilic OA in subjects sensitized to LMW agents. In this OA cohort, the eosinophilic pattern was associated with higher dose of ICS and mild disease activity in terms of symptoms, exacerbations, and airway obstruction. Therefore, eosinophilic OA shared common features with the "eosinophilic inflammation-predominant" cluster described by Haldar et al. [9] in a secondary care cohort of adult asthmatics. These findings further highlight the possible dissociation between eosinophilic inflammation and asthma symptoms [9].

Previous studies conducted in limited series of subjects with OA predominantly exposed to LMW agents, such as isocyanates or Western red cedar, found that sputum eosinophil counts correlated positively with the degree of baseline airflow obstruction [15, 16] and a higher level of NSBH [16], although sputum eosinophilia failed to correlate with NSBH in some of these studies [14, 15]. This large cohort study provides definitive evidence that sputum eosinophilia is strongly associated with a higher level of baseline NSBH, which is consistent with studies in general asthma populations [6, 11, 39]. In contrast, we failed to document a relationship between sputum eosinophilia and the baseline FEV_1/FVC ratio or the FEV_1 [15, 16]. Such relationships might have been blunted in this OA cohort because spirometry was measured at baseline of the SIC while 58.4% of the subjects were no longer exposed to the offending workplace. Nevertheless, analyzing pre-challenge sputum eosinophils among subjects still exposed at work within one week of the SIC procedure further confirmed that sputum eosinophilia was not associated with the level of airflow obstruction (Table 4 and Table S3).

Remarkably, this cohort study demonstrates that both sputum eosinophilia and neutrophilia develop independently from the molecular-weight category of the causal agents, which is in

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line with the study by Prince et al. [17] who compared the changes in sputum cells during SICs with HMW and LMW agents. Nevertheless, in our cohort HMW agents were significantly associated with post-challenge sputum eosinophilia only when there was coexisting work-related rhinitis. This finding is consistent with the observation that nasal exposure to inhalant allergens enhances eosinophil recruitment into the lower airways in subjects with allergic rhinitis [40, 41] and may account for the discordant information on the patterns of airway inflammation induced by HMW and LMW agents reported by previous studies that did not take into account the coexistence of occupational rhinitis [14, 16, 19]. Of note, the effect of HMW agents on sputum eosinophilia was not confirmed when analysing pre-challenge sputum cells in subjects still at work at the time of the SIC procedure, even when exposure to HMW agents was associated with work-related rhinitis, suggesting that the "synergic" effect of rhinitis on lower airways eosinophilia is predominantly apparent during "acute" exposures to occupational agents, as demonstrated for common allergens [40, 41].

The findings of this study challenge the traditional concept of categorizing the agents causing OA into HMW and LMW agents, presuming implicitly that they act through different underlying pathophysiological mechanisms. Our data provide convincing evidence that the molecular weight category of the causal agent does not determine the pattern of airway inflammation, although HMW and LMW agents are associated with distinct clinical characteristics, especially those pertaining to "IgE-related" clinical features. Indeed, a previous analysis conducted in a larger sample of the E-PHOCAS cohort that did not take into account sputum inflammatory data demonstrated that OA caused by HMW agents was characterized by a higher rate of work-related rhinitis, atopy, isolated early asthmatic reactions, and a greater post-challenge increase in FeNO compared with OA induced by LMW agents [23]. In addition, recent data from this E-PHOCAS cohort revealed pathophysiological heterogeneity among LMW agents. Compared to other LMW agents, acrylate-induced OA [25] showed some characteristics (i.e., concomitant work-related rhinitis and a greater post-challenge increase in fractional exhaled nitric oxide) that are similar to OA caused by HMW agents while OA caused by quaternary ammonium compounds was associated with a greater eosinophilic response compared to other LMW agents [27]. However, the number of subjects with OA caused by most of the LMW agents was too limited in this cohort to allow further comparison of the clinical and inflammatory characteristics among the various types of LMW agents.

Limitations of the Study

The major strengths of this study were the homogeneous diagnostic criteria used for identifying OA and the multicenter design that allowed for the recruitment of a large cohort of patients

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evaluated by SIC and induced sputum. Nevertheless, several limitations deserve further consideration. The major potential limitation of the study resulted from the use of sputum cell counts obtained 24 hours after challenge exposure to the causal agents as a surrogate for the airway inflammatory profile of subjects while exposed at work. However, the multivariate analysis of baseline sputum eosinophilia restricted to the subjects who were still exposed at work at the time of the diagnosis yielded similar results as the analysis of post-challenge sputum eosinophilia among the whole cohort (Table 4). In contrast, low ICS use was the only determinant of baseline neutrophilia among subjects still at work, while post-challenge neutrophilia was significantly associated with demographic and clinical characteristics (Table 6), suggesting that neutrophil counts after "acute" exposure to occupational agents may be more clinically relevant than those recorded during "chronic" exposure at work. Although the multicenter design of this study enabled the recruitment of a large cohort of subjects with induced sputum assessment, the number of subjects with sputum neutrophilia was still low and might not have allowed for fully capturing the influence of potential environmental and host factors and distinguishing accurately between neutrophilia alone (n=23) or in combination with sputum eosinophilia (i.e. mixed granulocytic pattern, n=18) (see Table 1 and online Supplementary Materials).

The retrospective cross-sectional design did not allow determining whether the persistence of sputum eosinophilia despite ICS treatment might result from suboptimal doses of ICS, poor treatment adherence, or the mishandling of inhaler devices. Nevertheless, functional stability of the subjects was established by monitoring FEV_1 during the control day before challenge exposure to the causal agents. Likewise, we were not able to evaluate whether the higher rates of poor asthma control and severe exacerbations in subjects with sputum neutrophilia were related to the neutrophilic inflammation *per se* or to the lower use of ICS.

Another limitation of this retrospective multicenter study resulted from the use of slightly different methods for inducing and processing sputum samples and the lack of quality control. There is conflicting information as to whether variations in the methods may have impacted the differential sputum cell counts [42], although using different nebulizers and saline concentrations does not affect/influence sputum cell counts [43, 44]. There were also between-center differences in the bronchoprovocation methods used for assessing the level of NSBH, but the interpretation of results was standardized for the whole cohort (Table S1).

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Conclusion

This large cohort study indicates that sputum inflammatory patterns are associated with distinct clinical phenotypes of OA. However, the associations between sputum inflammatory patterns and phenotypic charactristics in OA share broad similarities with what has been decribed in nonoccupational asthma. These findings may improve our understanding of the pathophysiological mechanisms involed in OA and enhance precision medicine. Nevertheless, determining whether inflammatory patterns have an impact on the long-term outcome of the disease needs further prospective investigation. In addition, the data provide definitive evidence that a sensitizing occupational agent may induce OA through different inflammatory pathways, independently from its HMW or LMW category.

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Table 1. Demographic and clinical characteristics of the subjects according to their post-challenge sputum inflammatory pattern

Characteristic	Missing values	Eosinophilic pattern (n=201)	Mixed granulocytic pattern (n=18)	Neutrophilic pattern (n=23)	Paucigranulocytic pattern (n=54)	*P* value
Age, yr *	0	42 (34-51)	47 (40-54)	45 (37-54)	46 (34-51)	0.566
Sex, male	0	133 (66.2)	14 (77.8)	17 (73.9)	30 (55.6)	0.247
Body mass index, kg/m^2 *	0	27 (24-30)	26 (24-31)	27 (22-30)	28 (25-32)	0.413
Smoking status	0					0.711
Current-smoker	0	46 (22.9)	3 (16.7)	8 (34.8)	14 (25.9)	
Ex-smoker		54 (26.9)	6 (33.3)	7 (30.4)	17 (31.5)	
Never-smoker		101 (50.2)	9 (50.0)	8 (34.8)	23 (42.6)	
Atopy†	1/0/0/1	95 (47.5)	11 (61.1)	13 (56.5)	25 (47.2)	0.616
Chronic rhinosinusitis	1/0/0/1	13 (6.5)	3 (16.7)	3 (13.0)	7 (13.2)	0.119
Asthma pre-existing to the causal exposure	0	12 (6.0)	3 (16.7)	4 (17.4)	6 (11.1)	0.061
Duration of exposure before symptom onset, mo*	1/0/0/1	120 (60-216)	72 (28-240)	132 (50-228)	84 (25-204)	0.362
Duration of asthma symptoms at work, mo*	1/1/0/1	36 (18-86)	33 (13-72)	36 (16-48)	24 (15-60)	0.246
Interval since last work exposure, mo*	1/0/0/0	3.0 (0.03-11.2)	6.5 (0.1-16.8)	5.0 (0.1-14.5)	2.8 (0.1-10.8)	0.388
Type of causal agent, HMW	0	139 (69.2)	8 (44.4)	16 (69.6)	25 (46.3)	**0.005**
Work-related rhinitis	0	164 (81.6)	14 (77.8)	14 (60.9)	37 (68.5)	**0.042**
Asthma treatment at work						
ICS use	0	160 (79.6)	10 (55.6)	11 (47.8)	36 (66.7)	**0.001**
Daily dose of ICS, µg*‡	0	500 (400-1000)	325 (0-1000)	0 (0-1000)	500 (0-605)	0.052
Long-acting β2-agonist	0	155 (77.1)	10 (55.6)	10 (43.5)	32 (59.3)	**0.001**
Leukotriene receptor antagonist	1/1/1/0	50 (25.0)	3 (17.6)	2 (9.1)	9 (16.7)	0.262
Poor asthma control (SABA ≥ once a day) at work	0	45 (22.4)	5 (27.8)	16 (69.6)	13 (24.1)	**<0.001**
≥2 severe exacerbations last 12 mo at work	0	16 (8.0)	4 (22.2)	2 (8.7)	1 (1.9)	**0.045**
Severe asthma at work§	0	35 (17.4)	5 (27.8)	5 (21.7)	8 (14.8)	0.549

Legend: HMW, high-molecular-weight; *ICS,* inhaled corticosteroid; *SABA,* short-acting β2-agonist. The sputum inflammatory patterns were characterized as "eosinophilic" (i.e. ≥3% eosinophils and <76% neutrophils); "neutrophilic" (i.e. ≥76% and <3% eosinophils); "paucigranulocytic" (i.e. <3% eosinophils and <76% neutrophils); and "mixed granulocytic" (i.e. ≥76% neutrophils and ≥3% eosinophils. Data are presented as n (% of available data) unless otherwise specified. Bold indicates statistical significance (*P*<0.05).

* Median value with interquartile range (IQR) within parentheses.

† Atopy defined by the presence of at least one positive skin prick test result to common allergens.

‡ Daily dose of inhaled corticosteroid expressed as beclomethasone dipropionate equivalent.

§ Multidimensional definition of severe asthma adapted from the European Respiratory Society/American Thoracic Society guidelines [29].

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Table 2. Functional characteristics and markers of airway inflammation according to the post-challenge sputum inflammatory pattern

Characteristic	Missing values	Eosinophilic pattern (n=201)	Mixed granulocytic pattern (n=18)	Neutrophilic pattern (n=23)	Paucigranulocytic pattern (n=54)	*P* value
Baseline spirometry						
FVC, % pred*	0	102 (91-110)	100 (91-104)	105 (91-112)	102 (91-109)	0.928
FEV_1, % pred*	0	90 (78-97)	88 (72-92)	85 (73-96)	93 (84-101)	0.251
FEV_1/FVC, %*	0	74 (66-80)	71 (66-77)	73 (64-78)	78 (72-81)	**0.024**
Baseline level of NSBH†	14/2/0/4					
Absent		30 (16.0)	2 (12.5)	4 (17.4)	18 (36.0)	**0.020**
Mild		99 (52.9)	10 (62.5)	12 (52.2)	23 (46.0)	0.703
Moderate-to-severe		58 (31.0)	4 (25.0)	7 (30.4)	9 (18.0)	0.324
Post-SIC change in NSBH	71/6/2/11					
Pre/post-SIC NSBH ratio*		2.33 (1.40-4.64)	2.40 (1.31-4.05)	1.48 (1.00-2.67)	2.58 (1.00-6.70)	0.293
Maximum fall in FEV_1, % baseline*	0	24 (19-33)	29 (24-37)	22 (18-27)	22 (19-27)	0.193
Pattern of bronchial response to SIC	12/4/4/4					**0.008**
Isolated immediate reaction		67 (35.4)	9 (64.3)	13 (68.4)	17 (34.0)	
Late reaction‡		122 (64.6)	5 (35.7)	6 (31.6)	33 (66.0)	
Blood eosinophils	33/3/4/14					
Cells/µl*		300 (200-428)	203 (150-300)	211 (144-305)	200 (100-291)	**<0.001**
>300/µl		93 (55.4)	5 (33.3)	6 (31.6)	9 (22.5)	**<0.001**
Baseline FeNO, ppb*	136/6/5/25	26 (14-39)	24 (16-61)	12 (7-26)	18 (9-28)	0.123
Post-SIC change in FeNO	139/6/5/31					
ppb*§		20 (6-46)	11 (4-29)	2 (0-12)	2 (0-12)	**0.001**
>17.5 ppb§		35 (56.5)	3 (25.0)	3 (16.7)	4 (17.4)	**0.001**
Baseline sputum inflammatory pattern	0					ND
Eosinophilic		95 (47.3)	0	1 (4.3)	7 (13.0)	
Neutrophilic		18 (9.0)	6 (33.3)	14 (60.9)	6 (11.1)	
Mixed granulocytic		10 (5.0)	4 (22.2)	3 (13.0)	1 (1.9)	
Paucigranulocytic		78 (38.8)	8 (44.4)	5 (21.7)	40 (74.1)	

Legend: *FeNO*, fractional exhaled nitric oxide; *FEV_1*, forced expiratory volume in one-second; *FVC*, forced vital capacity; *ND*, not done; *NSBH*, nonspecific bronchial hyperresponsiveness; *SIC,* specific inhalation challenge. The sputum inflammatory patterns were characterized as "eosinophilic" (i.e. ≥3% eosinophils and <76% neutrophils); "neutrophilic" (i.e. ≥76% and <3% eosinophils); "paucigranulocytic" (i.e. <3% eosinophils and <76% neutrophils); and "mixed granulocytic" (i.e. ≥76% neutrophils and ≥3% eosinophils. Data are presented as n (% of available data) unless otherwise specified. Bold indicates statistical significance (*P*<0.05).

* Median value with interquartile range (IQR) within parentheses.

† See Table S1 for the grading of nonspecific bronchial hyperresponsiveness.

‡ Late asthmatic reactions including isolated late and dual reactions.

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§ Difference from baseline value

Table 3. Univariate associations with post-challenge sputum eosinophilia

Characteristics	Mising values	Sputum eosinophils ≥3% (n=219)	Sputum eosinophils <3% (n=77)	Univariate analysis	
				OR (95% CI)	*P* value
Age, yr [a]	0/0	43 (34-51)	45 (35-51)	1.00 (0.97-1.02)	0.891
Sex, male	0/0	147 (67)	47 (61)	1.30 (0.76-2.22)	0.335
Smoking habit	0/0				
Never smoker		110 (50)	31 (40)	-	
Ex-smoker		24 (31)	60 (27)	0.70 (0.38-1.32)	0.267
Current smoker		22 (29)	49 (22)	0.63 (0.33-1.20)	0.155
Body mass index, ≥30 kg/m^2 [a]		63 (29)	25 (32)	0.84 (0.48-1.28)	0.541
Atopy [b]	1/1	106 (49)	38 (50)	0.95 (0.56-1.60)	0.836
Chronic rhinosinusitis	1/1	16 (7)	10 (13)	0.52 (0.23-1.25)	0.129
Childhood asthma	0/0	15 (7)	10 (13)	0.49 (0.21-1.18)	0.101
Exposure before symptom onset, mo[a]	1/1	120 (58-216)	108 (36-212)	1.00 (1.00-1.00)	0.814
Duration of asthma symptoms at work, mo[a]	1/1	36 (16-84)	36 (15-58)	1.00 (1.00-1.01)	0.163
Interval since last work exposure, mo[a]	0/1	3 .0 (0.1-12.0)	3.0 (0.03-12.0)	0.90 (0.72-1.12)	0.344
HMW causal agent (vs. LMW agent)	0/0	147 (67)	41 (53)	1.79 (1.05-3.05)	**0.031**
Associated work-related rhinitis	0/0	178 (81)	51 (66)	2.21 (1.23-3.95)	**0.007**
Asthma treatment at work:					
ICS use	0/0	170 (78)	47 (61)	2.21 (1.26-3.86)	**0.005**
Daily dose of ICS, µg[a, c]	0/0	500 (250-1000)	500 (0-800)	1.20 (1.03-1.40)	**0.021**
SABA ≥ 1/day at work	0/0	50 (23)	29 (38)	0.49 (0.28-0.86)	**0.012**
≥2 severe exacerbations last 12 mo at work	0/0	20 (9)	3 (4)	2.48 (0.82-10.74)	0.152
Baseline spirometry:	0/0				
FVC, % pred[a]		102 (91-110)	102 (91-109)	1.01 (0.99-1.03)	0.259
FEV_1, % pred[a]		89 (77-97)	92 (80-101)	1.26 (0.71-2.33)	0.445
FEV_1/FVC <0.70		85 (39)	20 (26)	0.96 (0.93-0.99)	**0.021**
Baseline level of NSBH[d]	4/16				
Absent		32 (16)	22 (30)	-	
Mild		109 (54)	35 (48)	2.14 (1.10-4.16)	**0.024**
Moderate-to-severe		62 (31)	16 (22)	2.66 (1.24-5.85)	**0.013**
Pre/post-SIC NSBH ratio >2 [a]	13/77	94 (66)	35 (55)	1.62 (0.89-2.97)	0.115
Maximum fall in FEV_1, % baseline [a]	0/0	24 (19-33)	22 (19-27)	1.27 (0.99-1.65)	0.067
Isolated immediate reaction during the SIC[e]	8/16	76 (37)	30 (43)	0.78 (0.45-1.36)	0.375
Baseline blood eosinophil count:	18/36				
cells/µl [a]		300 (200-410)	200 (100-296)	1.00 (1.00-1.01)	**<0.001**
>300/µl		98 (54)	15 (25)	3.38 (1.79-6.68)	**<0.001**
Baseline FeNO, ppb [a]	30/142	25 (15-40)	17 (8-28)	1.02 (1.00-1.04)	**0.042**
Post-SIC change in FeNO, ppb [a]	36/145	18 (5-46)	2 (0-13)	1.03 (1.01-1.05)	**0.002**

Abbreviations: *FeNO*, fractional exhaled nitric oxide; *FEV_1*, forced expiratory volume in one-second; *FVC*, forced vital capacity; *HMW,* high-molecular-weight; *ICS,* inhaled corticosteroid; *LMW*, low-molecular-weight; *NSBH*, nonspecific bronchial hyperresponsiveness; *OR,* odds ratio; *SABA,* short-acting β_2-agonist*; SIC,* specific inhalation challenge. Data are presented as n (% of available data) unless otherwise specified. Bold indicates statistical significance (P<0.05).

[a] Median value with interquartile range within parentheses.

[b] Atopy defined by the presence of at least one positive skin prick test result to common allergens.

[c] Daily dose of inhaled corticosteroid expressed as beclomethasone dipropionate equivalent.

[d] See Table S1 for the grading of nonspecific bronchial hyperresponsiveness.

[e] The SIC was considered positive based on a significant increase in the post-challenge level of NSBH (i.e., pre/post ratio >2) while the changes in FEV_1 remained <15% in 24 subjects.

[f]

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Table 4. Multivariate models for sputum eosinophilia

Independent variables	Post-challenge sputum eosinophils ≥3% (n=219/296)			Pre-challenge sputum eosinophils ≥3% [a] (n=60/123)		
	OR	**(95% CI)**	***P* value**	**OR**	**(95% CI)**	***P* value**
Final model 1:						
Causal agent, HMW vs. LMW	1.68	(0.87-3.24)	0.119	2.43	(0.86-7.47)	0.104
Work-related rhinitis	1.69	(0.83-3.41)	0.146	0.35	(0.10-1.15)	0.095
ICS daily dose, per 250 µg	1.31	(1.11-1.55)	**0.002**	1.36	(1.09-1.76)	**0.010**
SABA ≥1/day	0.28	(0.14-0.55)	**<0.001**	0.28	(0.09-0.84)	**0.028**
Level of baseline NSBH, vs. no NSBH[b]						
Mild	2.48	(1.21-5.08)	**0.012**	3.64	(0.97-18.01)	0.075
Moderate/severe	3.40	(1.44-8.29)	**0.006**	7.74	(1.85-41.72)	**0.008**
Final model 2:						
HMW causal agent *plus* rhinitis at work	2.78	(1.55-5.08)	**0.001**	1.07	(0.45-2.53)	0.881
ICS daily dose, per 250 µg	1.32	(1.12-1.57)	**0.001**	1.30	(1.05-1.67)	**0.024**
SABA ≥1/day	0.28	(0.14-0.54)	**<0.001**	0.32	(0.10-0.93)	**0.042**
Level of baseline NSBH, vs. no NSBH[b]						
Mild	2.57	(1.25-5.29)	**0.010**	3.54	(0.97-17.10)	0.075
Moderate/severe	3.54	(1.48-8.73)	**0.005**	8.83	(2.18-46.42)	**0.004**

Legend: *HMW,* high-molecular-weight; *ICS*, inhaled corticosteroid; *LMW*, low-molecular-weight; *NSBH*, nonspecific bronchial hyperresponsiveness; *OR*, odds ratio; *SABA,* inhaled short-acting β_2-agonist.

The multivariate model 1 incorporated HMW causal agents vs. LMW agents and work-related rhinitis (yes/no) as independent variables, whereas model 2 included only the association between a HMW agent and work-related rhinitis. The multivariate models included 275 subjects for the analysis of post-challenge sputum eosinophilia and 111 subjects for pre-challenge sputum eosinophilia. Bold indicates statistical significance (P<0.05).

[a] Multivariate analysis conducted among 123 subjects still at work at the time of assessment (see Table S3 in the supplementary materials for univariate analyses of pre-challenge eosinophilia).

[b] SeeTable S1 for the grading of nonspecific bronchial hyperresponsiveness.

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Table 5. Univariate associations with post-challenge sputum neutrophilia

Characteristics	Missing values	Sputum neutrophils ≥76% (n=41)	Sputum neutrophils <76% (n=255)	Univariate analysis	
				OR (95% CI)	*P* value
Age, yr [a]	0/0	46 (37-55)	43 (34, 51)	1.02 (0.99-1.06)	0.154
Sex, male	0/0	31 (76)	163 (64)	1.75 (0.85-3.91)	0.148
Smoking habit	0/0				
Never smoker		17 (41)	124 (49)	-	
Ex-smoker		13 (32)	71 (28)	1.34 (0.60-2.90)	0.467
Current smoker		11 (27)	60 (24)	1.34 (0.58-3.00)	0.487
Body mass index, ≥30 kg/m^2 [a]	0/0	12 (29)	76 (30)	0.97 (0.46-1.97)	0.944
Atopy [b]	0/2	24 (59)	120 (47)	1.56 (0.81-3.10)	0.189
Chronic rhinosinusitis		6 (15)	20 (8)	2.00 (0.69-5.06)	0.166
Childhood asthma	0/0	7 (17)	18 (7)	2.71 (0.99-6.74)	**0.038**
Exposure before symptom onset, mo [a]	0/2	120 (48-240)	120 (48, 204)	1.00 (1.00-1.00)	0.560
Duration of asthma symptoms at work, mo [a]	1/2	36 (12-52)	36 (16, 84)	1.00 (0.99-1.00)	0.738
Interval since last work exposure, mo [a]	0/1	5 (0-15)	3 (0, 11)219	1.19 (0.90-1.59)	0.225
HMW causal agent (vs. LMW agent)	0/0	24 (59)	164 (64)	0.78 (0.40-1.55)	0.476
Associated work-related rhinitis	0/0	28 (68)	201 (79)	0.58 (0.29-1.22)	0.138
Asthma treatment at work:	0/0				
ICS use		21 (51)	196 (77)	0.32 (0.16-0.62)	**0.001**
Daily dose of ICS, µg [a,c]		250 (0-1000)	500 (250, 1000)	0.90 (0.74-1.07)	0.249
SABA ≥ 1/day at work		21 (51)	58 (23)	3.57 (1.81-7.08)	**<0.001**
≥2 severe exacerbations last 12 mo at work	0/0	6 (15)	17 (7)	2.40 (0.82-6.22)	0.085
Baseline spirometry:	0/0				
FVC, % pred [a]		103 (91-110)	102 (91, 110)	1.00 (0.98-1.02)	0.906
FEV_1, % pred [a]		88 (73-96)	90 (79, 98)	1.59 (0.78-3.14)	0.192
FEV_1/FVC <0.70		16 (39)	89 (35)	0.97 (0.94-1.0)	0.163
Baseline level of NSBH [d]	2/18				
Absent		6 (15)	48 (20)	-	
Mild		22 (56)	122 (51)	1.44 (0.58-4.11)	0.456
Moderate-to-severe				1.31 (0.47-4.04)	0.615
Pre/post-SIC NSBH ratio >2	8/82	15 (45)	114 (66)	0.43 (0.20-0.92)	**0.029**
Maximum fall in FEV_1, % baseline [a]				0.94 (0.68-1.26)	0.693
Isolated immediate reaction during the SIC [e]	8/16	22 (67)	84 (35)	3.69 (1.74-8.25)	**0.001**
Baseline blood eosinophil count:	7/47				
cells/µl [a]		207 (145-300)	292 (200, 400)	1.00 (1.00-1.00)	0.065
>300/µl		11 (32)	102 (49)	0.50 (0.22-1.05)	0.074
Baseline FeNO, ppb [a]	11/161	16 (9-38)	23 (10, 37)	1.00 (0.98-1.01)	0.717
Post-SIC change in FeNO, ppb [a]	11/170	4 (1-15)	13 (2, 38)	0.99 (0.97-1.00)	0.154

Legend: *FeNO*, fractional exhaled nitric oxide; *FEV_1*, forced expiratory volume in one-second; *FVC*, forced vital capacity; *HMW,* high-molecular-weight; *ICS,* inhaled corticosteroid; *LMW*, low-molecular-weight; *NSBH*, nonspecific bronchial hyperresponsiveness; *OR,* odds ratio; *SABA,* short-acting β_2-agonist*; SIC,* specific inhalation challenge. Data are presented as n (% of available data) unless otherwise specified. Bold indicates statistical significance (P<0.05).

[a] Median value with interquartile range within parentheses.

[b] Atopy defined by the presence of at least one positive skin prick test result to common allergens.

[c] Daily dose of inhaled corticosteroid expressed as beclomethasone dipropionate equivalent.

[d] See Table S1 for the grading of nonspecific bronchial hyperresponsiveness.

[e] The SIC was considered positive based on a significant increase in the post-challenge level of NSBH (i.e., pre/post ratio >2) while the changes

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Table 6. Multivariate models for sputum neutrophilia

Independent variables	Post-challenge sputum neutrophils ≥76% (n=41/296)			Pre-challenge sputum neutrophils ≥76%[a] (n=30/123)		
	OR	**(95% CI)**	***P* value**	**OR**	**(95% CI)**	***P* value**
Final model:						
Age, yr	1.06	(1.01-1.11)	**0.014**	1.05	(1.00-1.11)	0.073
Gender, male	3.34	(1.29-9.99)	**0.019**	2.75	(0.92-9.77)	0.089
Body mass index ≥30 kg/m^2	-			0.32	(0.08-1.00)	0.064
No ICS use	5.47	2.09-15.16	**<0.001**	5.09	(1.82-15.22)	**0.002**
SABA ≥1/day	4.09	(1.71-10.01)	**0.024**	-		
Severe exacerbation, ≥2 last 12 mo at work	4.22	(1.14-14.99)	**0.025**	-		
Isolated early reaction vs. late reaction	4.45	(1.85-11.59)	**0.001**	-		

Legend: *ICS*, inhaled corticosteroid; *OR*, odds ratio; *SABA*, short-acting β_2-agonist.

The multivariate models included 271 subjects for the analysis of post-challenge sputum neutrophilia and 123 subjects for pre-challenge sputum neutrophilia among subjects still exposed at work. Bold indicates statistical significance (*P*<0.05).

[a] Multivariate analysis conducted among subjects still at work at the time of assessment (see Table S3 in the online supplementary materials for univariate analyses of pre-challenge neutrophilia).

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