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METHODS

Cohort Recruitment

This retrospective cross-sectional study was conducted in the context of the multicentre cohort of the European network for the PHenotyping of OCcupational Asthma (E-PHOCAS) [1-5]. This cohort aimed at recruiting all patients with occupational asthma (OA) due to various occupational agents documented by a specific inhalation challenge (SIC) completed in 20 European centers between 2006 and 2018. Overall, these centers reported 1,518 subjects with OA due to various occupational agents. From these initially reported subjects who fulfilled the criteria for a positive SIC (see below), 221 subjects were excluded because of incomplete information pertaining to key asthma outcomes (i.e., detailed asthma medications while exposed at work and at the time of the SIC and number of severe asthma exacerbations over the last 12 months at work). Eligible subjects for this analysis were those with available induced sputum samples collected both before and 24 hours after the SIC procedure.

Eight of the 20 participating centers used the induced sputum technique for periods ranging from one to 13 years (median: 6 years) during the 2006-2018 study period. Over the years during which induced sputum was performed, a total of 651 subjects underwent an SIC procedure in these eight centers. Of these, 361 (55.4%) subjects completed at least one sputum induction while 290 subjects had no sputum data. Two hundred ninety-six subjects had complete data on eosinophil and neutrophil percentages from both the pre- and post-challenge sputum samples with a median of 18 (range: 1-142) subjects per center.

Data Collection

Anonymized information on demographic, clinical, occupational, and physiological characteristics of the subjects collected at the time of the diagnostic evaluation was entered in a standardized spreadsheet in each participating center. These local databases were then checked for inconsistencies and missing data by three investigators (CR, NM, and OV), pooled together and centralized at the Strasbourg University.

The database gathered information on the following items: 1) causal agent and job; 2) demographic and clinical characteristics; 3) timing of work-related respiratory symptoms in relation to occupational exposure; 4) co-existing conditions (i.e. work-related rhinitis, dysphonia, contact urticaria and/or dermatitis, and chronic rhinosinusitis); 5) asthma medication, including frequency of short-acting β_2-agonist use, while exposed at work and at the time of the SIC procedure; and 6) severe asthma exacerbations during the last 12 months at work.

Since most of the participating centers failed to use validated instruments for the assessment of asthma control throughout the study period, "poor symptom control" was defined by the need for an inhaled short-acting β_2-agonist (SABA) once or more a day as proposed in the recommendations of the American Thoracic Society (ATS) issued in 2000 [6]. Severe asthma exacerbations were defined as those requiring oral corticosteroids for at least three consecutive days or an emergency room visit or a hospitalization [7].

The definition of severe asthma was adapted from the European Respiratory Society/American Thoracic Society criteria [7] and required a high-level treatment according to the Global Initiative for Asthma (GINA) [8] (i.e., treatment step 4-5 including a high dose of inhaled corticosteroid [ICS] and a second controller or systemic corticosteroid use >50% of the previous year) together with any one of the following criteria indicating uncontrolled asthma: 1) "poor symptom control"; 2) two or more severe exacerbations in the previous year; or 3) airflow obstruction defined by a forced expiratory volume in 1 sec (FEV_1) <80% predicted value together with a FEV_1/forced vital capacity (FVC) ratio <0.70 [2].

Assessment of Nonspecific Bronchial Hyperresponsiveness

The level of nonspecific bronchial hyperresponsiveness (NSBH) was expressed as the concentration or dose of the pharmacological agent inducing a 15% or 20 % fall in FEV_1 ($PC/PD_{15\text{-}20\%}$) according to the bronchoprovocation method used in each center. Since participating centers used different methods, the level of NSBH was only categorized as "absent", "mild", or "moderate-to-severe" based on the available recommendations [9-11] or a consensus Delphi approach among investigators [1].

The bronchoprovocation methods and threshold values used for defining the level of NSBH in the 296 subjects included in this analysis are detailed in Table E1.

The database collected information on the methacholine/histamine $PC/PD_{15-20\%}$ value measured at baseline and 24 hours post-challenge. Among the subjects included in this analysis, baseline NSBH was not assessed in 20 subjects; the diagnosis of asthma was documented by reversible airflow obstruction on spirometry (n=14) or daily variations in peak expiratory flow (n=2), and was not formally documented in four subjects.

A significant increase in post-challenge NSBH was defined by a ≥2-fold decrease in the level of NSBH measured 24 hours after the challenge exposure as compared to the baseline value [12].

Methodology of Specific Inhalation Challenges

In order to evaluate the compliance with international recommendations on SIC with occupational agents [12], the investigators completed a questionnaire on the following items prior to participating in the E-PHOCAS cohort: 1) absence of respiratory tract infection or asthma exacerbation within the previous 4 weeks; 2) duration of ICS withdrawal before the SIC procedure; 3) performance of a control (placebo) test on a separate day before challenging the subjects with occupational agents; 4) lower limit of FEV_1 value considered a contra-indication for performing a SIC procedure 5) method used for delivering challenge exposures with workplace agents (i.e., “realistic” challenge or inhalation of an “allergen extract”; and 6) functional monitoring of at least 6 hours after the end of challenge exposure.

All participating centers conformed with safety and reliability requirements. The lower limit of FEV_1 was 70% of the predicted value in three centers, 65% in one center; 60% in four centers. In all centers, ICS were withheld 2 or 3 days before the SIC procedure. Challenge exposure to the workplace agent was carried out using a “realistic” method aimed at reproducing the condition of exposure at the workplace in 282 subjects and by nebulizing “allergen extracts” in 14 subjects included in this analysis [13].

The database collected information on the maximum fall in FEV_1 expressed as percent from baseline value that was recorded during: 1) the period between the end of the challenge exposure and the 60th minute post-exposure (i.e., the "early component" of the bronchial response) and 2) the period between the 60th minute post-challenge and the end of the post-SIC follow-up (i.e., the "late component" of the bronchial response). The results of the SICs were interpreted *a posteriori* according to standardized criteria. A positive SIC result was defined by either a ≥15% fall in FEV_1 at any time during the post-challenge monitoring or a twofold or greater increase in the post-challenge level of NSBH (i.e., a pre/post $PC/PD_{15-20\%}$ ratio ≥2) in the absence of a ≥15% fall in FEV_1 [12]. Among the 296 subjects included in this analysis on induced sputum data, the diagnosis of OA was ascertained by a ≥15% decline in FEV_1 during SIC in 272 subjects or a significant increase in the post-challenge level of NSBH with <15% fall in FEV_1 in 24 subjects.

Based on the presence of an immediate and/or a late asthmatic component, the pattern of the FEV_1 bronchial response was categorized as an "isolated immediate", "isolated late", or "dual" reaction. In this analysis, we compared isolated immediate reactions with late-component reactions, including isolated late and dual responses.

Sputum Induction and Processing

The eight participating centers completed a detailed questionnaire pertaining to the method used for the induction and analysis of sputum samples. Sputum was induced through different methods, including the inhalation of nebulized isotonic saline (n=1), a single concentration of hypertonic solutions (i.e., 3%; n=1) or increasing concentrations of hypertonic solutions ranging (i.e., 3%, 4%, and 5%; n=7) for a maximum cumulative duration of 15 to 40 minutes [14]. The processing of sputum samples was carried out either by selecting viscid portions from the expectorate (3 centers) [15] or using the whole expectorate (5 centers) [16]. Homogenization of the sample was achieved by adding dithiothreitol (0.1%). All centers applied quality criteria based on the cell viability (i.e. at least 40%) and the level of contamination by squamous cells [14]. The accepted squamous cell contamination was <20% in five centers, <30% in one center, and <50% in two centers. The differential cell count

was determined by counting a minimum of 400 nonsquamous cells. Sputum eosinophil and neutrophil counts collected at baseline and 24 hours after the challenge exposure were expressed as a percentage of nonsquamous cells. Available information indicates that using viscid portions from the expectorate or the whole expectorate as well as different nebulizers and saline concentrations does not significantly affect differential sputum cell counts [14, 17].

In this study, we used the sputum cell counts obtained 24 hours after the SIC as the primary outcomes since Lemière et al. [18] found that sputum eosinophil counts were already decreased to normal values in more than half of the subjects within two weeks after removal from exposure. One hundred seventy-three of the 296 (58.4%) subjects included in this analysis were already removed from the causal exposure for more than one week at the time of the SIC procedure.

Fractional Exhaled Nitric Oxide

The FeNO level was measured at baseline and 24 hours post-SIC at a flow rate of 50 ml/s using different devices in compliance with the recommendations of the European Respiratory Society and the American Thoracic Society [19].

RESULTS

Clinical Characteristics Associated with Sputum Inflammatory Patterns

Paucigranulocytic pattern. The paucigranulocytic pattern showed the highest median FEV_1/FVC ratio, but the difference was significant only when compared with the eosinophilic (*P*=0.036) patterns. Subjects with a paucigranulocytic pattern showed less often significant NSBH (66.7% vs. 85.1% vs. 88.9% vs. 82.6% for paucigranulocytic, eosinophilic, mixed and neutrophilic patterns, respectively; across-group *P*=0.020).

Neutrophilic pattern. The neutrophilic pattern was characterized by the highest rate (69.6%) of poor asthma control compared with the eosinophilic (22.4%; *P*=0.016), mixed granulocytic (27.8%; *P*=0.059), and paucigranulocytic (24.1%; *P*=0.016) patterns. Subjects with a neutrophilic pattern had the lowest rate of treatment with ICS (47.8%), but the difference reached statistical significance only when compared with the eosinophilic pattern (79.6%; *P*=0.016). They showed a higher rate (68.4%) of isolated immediate reactions after challenge exposure to the causal agent compared with the eosinophilic (35.4%; *P*=0.036) and paucigranulocytic patterns (34.0%; *P*=0.061), but this rate was similar to that recorded in the mixed granulocytic pattern (64.3%).

Mixed granulocytic pattern. The mixed granulocytic pattern exhibited the highest proportion (22.2%) of subjects who experienced two or more severe exacerbations during the last 12 months at work, but this proportion did not differ significantly from the paucigranulocytic (1.7%; *P*=0.059), eosinophilic (8.0%; *P*=0.178), and neutrophilic (8.7%; *P*=0.609) patterns.

Eosinophilic pattern. Subjects with a post-challenge eosinophilic pattern showed a trend toward the highest use of ICS while exposed at work. These subjects were treated with an ICS significantly more frequently (79.6%) than those with a neutrophilic (47.8%; *P*=0.016), but their ICS use did not differ from the subjects with a mixed granulocytic (55.6%, *P*=0.119) or a paucigranulocytic pattern (66.7%; *P*=0.178). The eosinophilic pattern was associated with the highest baseline blood eosinophil count and the greatest post-challenge increase in FeNO compared to the neutrophilic

(*P*=0.088 and *P*=0.016, respectively) and paucigranulocytic patterns (*P*=0.016 and *P*=0.016, respectively), but these indices were not significantly different from the mixed granulocytic pattern.

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Table S1. Methods used for measuring and grading the level of nonspecific bronchial hyperresponsiveness

Method (pharmacological agent)	No. of centers (subjects)*	Threshold values for nonspecific bronchial hyperresponsiveness		
		Moderate-to-severe	Mild	Absent
Tidal breath method (histamine/methacholine) [9, 10]	2 (212)	PC_{20} <1 mg/ml	PC_{20} :1-16 mg/ml	PC_{20} >16 mg/ml
Five-breath dosimeter method (methacholine) [9, 10]	5 (43)	PD_{20} <0.1 mg PC_{20} <1 mg/ml	PD_{20}: 0.1-1.5 mg PC_{20}:1-16 mg/ml	PD_{20} >1.5 mg PC_{20} >16 mg/ml
Rapid dosimeter method (histamine) [11]	1 (17)	PD_{15} <0.4 mg	PD_{15} : 0.4-1.6 mg	PD_{15} >1.6 mg
Reservoir bag dosimeter method (methacholine) [21]	1 (4)	PD_{20} or PD_{100} sRt <0.1 mg	PD_{20} or PD_{100} sRt: 0.1-0.3 mg	PD_{20} or PD_{100} sRt >0.3 mg

Legend: *$PC_/PD_{15-20}$*, provocative concentration/dose of pharmacological agent inducing a 15 or 20% fall in FEV_1; *PD_{100} sRt*: provocative concentration of pharmacological agent inducing a doubling of specific airway resistance (sRt).

Table S2. Workplace agents causing occupational asthma

High-molecular-weight agents	n (%)*	Low-molecular-weight agents	n (%)*
Flour/grains	148 (50.2)	Isocyanates	28 (9.5)
Latex	11 (3.7)	Various cleaning products/disinfectants†	17 (5.8)
Enzymes	5 (1.7)	Metals	11 (3.7)
Storage mites	4 (1.4)	Wood dusts	10 (3.4)
Fish/seafood	4 (1.4)	Persulfate salts	10 (3.4)
Cow dander	2 (0.7)	Quaternary ammonium compounds†	7 (2.4)
Rodents	2 (0.7)	Acrylate compounds	6 (2.0)
Molds	2 (0.7)	Welding fumes	5 (1.7)
Insects (parasitoid wasps)	1 (0.3)	Metal working fluids	3 (1.1)
Various plant-derived products	6 (2.0)	Amines	3 (1.1)
Various animals and derived products	3 (1.1)	Colophony	2 (0.7)
		Resins/glues/paints (NOS)	2 (0.7)
		Various low-molecular-weight agents	4 (1.4)
Total:	188 (63.5)	Total:	108 (36.5)

Legend:

* Expressed as % of total identified agents (n=296).
† Cleaning products contained mixtures of various chemicals; seven subjects were challenged only with quaternary ammonium compounds {Migueres, 2021 #9778}.

Table S3. Univariate associations with pre-challenge sputum eosinophilia and neutrophilia among subjects still exposed at work

Characteristics	Pre-SIC sputum eosinophilia ≥3%* (n=60)		Pre-SIC sputum neutrophils ≥76% [a] (n=30)	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Age, yr [a]	0.99 (0.95-1.03)	0.675	1.01 (0.97-1.06)	0.588
Sex, male	0.86 (0.40-1.85)	0.705	1.72 (0.69-4.73)	0.260
Smoking habit				
Never smoker	-		-	
Ex-smoker	0.64 (0.27-1.46)	0.290	1.06 (0.40-2.73)	0.903
Current smoker	0.68 (0.27-1.68)	0.404	0.88 (0.28-2.49)	0.810
Body mass index, ≥30 kg/m^2 [a]	1.07 (0.47-2.44)	0.878	0.40 (0.11-1.14)	0.114
Atopy [b]	0.94 (0.46-1.92)	0.866	1.09 (0.48-2.52)	0.831
Chronic rhinosinusitis	0.87 (0.27-2.79)	0.817	0.91 (0.19-3.24)	0.893
Childhood asthma	0.68 (0.17-2.50)	0.564	2.23 (0.54-8.42)	0.240
Exposure before symptom onset, mo [a]	1.00 (1.00-1.00)	0.631	1.00 (1.00-1.01)	0.373
Duration of asthma symptoms at work, mo	1.00 (0.99-1.01)	0.830	1.00 (0.99-1.01)	0.784
HMW causal agent (vs. LMW agent)	1.73 (0.80-3.81)	0.169	1.64 (0.66-4.52)	0.306
Associated work-related rhinitis	0.62 (0.25-1.52)	0.299	2.62 (0.82-11.74)	0.142
Asthma treatment at work:				
ICS use	2.07 (0.91-4.94)	0.090	0.31 (0.13-0.77)	**0.010**
Daily dose of ICS, µg [a, c]	1.27 (1.05-1.57)	**0.019**	0.92 (0.73-1.13)	0.479
SABA ≥ 1/day at work	0.97 (0.42-2.25)	0.950	1.56 (0.60-3.89)	0.343
≥2 severe exacerbations last 12 mo at work	3.39 (0.75-23.80)	0.145	0.42 (0.02-2.52)	0.431
Severe asthma at work[d]	2.00 (0.74-5.75)	0.178	1.54 (0.50-4.36)	0.430
Baseline spirometry:				
FVC, % pred [a]	1.00 (0.98-1.02)	0.967	1.01 (0.99-1.04)	0.301
FEV_1, % pred [a]	2.03 (0.93-4.56)	0.081	0.84 (0.32-2.07)	0.719
FEV_1/FVC, %	0.94 (0.91-0.98)	**0.004**	1.01 (0.97-1.05	0.664
Baseline level of NSBH [e]				
Absent	-		-	
Mild	3.61 (1.03-16.97)	0.063	0.31 (0.09-1.11)	0.067
Moderate-to-severe	8.33 (2.26-40.87)	**0.003**	0.92 (0.28-3.17	0.887
Pre/post-SIC NSBH ratio >2 [a]	0.60 (0.24-1.50)	0.274	0.65 (0.24-1.83)	0.407
Maximum fall in FEV_1 during the SIC, % baseline [a]	1.28 (0.96-1.76)	0.105	1.30 (0.94-1.80)	0.107
Isolated immediate vs late reaction [f]	0.73 (0.34-1.53)	0.403	1.79 (0.71-4.66)	0.220
Baseline blood eosinophil count,				
cells/µl [a]	1.00 (1.00-1.01)	**0.030**	1.00 (1.00-1.00)	0.329
>300/µl	1.64 (0.76-3.57)	0.212	0.64 (0.25-1.57	0.335
Baseline FeNO, ppb [a]	1.00 (0.99-1.02)	0.616	1.00 (0.97-1.02)	0.754
Post-SIC change in FeNO, ppb [a]	1.00 (0.98-1.03)	0.664	1.00 (0.98-1.02)	0.929

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. Univariate analyses of prechallenge sputum cells were performed among 123 subjects who were still exposed at work at the time of the evaluation (within two weeks). Data are presented as n (% of available data) unless otherwise specified. Bold indicates statistical significance (P<0.05). Bold indicates statistical significance (*P*<0.05)

[a] Median value with interquartile range (IQR) 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] Multidimensional definition of severe asthma adapted from the European Respiratory Society/American Thoracic Society guidelines [29].

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

[f] 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.

Table S4. Workplace agents involved in post-challenge sputum neutrophilia

Causal agents	**Post-challenge neutrophilic pattern** (N=23)			**Post-challenge mixed granulocytic pattern** (N=18)		
	N	**Positive SPT**	**Positive sIgE**	**N**	**Positive SPT**	**Positive sIgE**
High-molecular-weight agents:						
Flour/grains	14	11/14	11/14	5	5/5	5/5
Latex	1	1/1	0/1	1	1/1	1/1
Chamomile flowers	1	1/1	1/1	-	-	-
Tomato flowers and leaves	-	-	-	1	1/1	1/1
Animal fur (fox, mink)	-	-	-	1	ND	ND
Low-molecular-weight agnents:						
Isocyanates	2	0/1	0/1	3	ND	1/2
Cleaning products[a]	2	ND	ND	4	ND	ND
Metal dust containing cobalt	1	0/1	ND	-	-	-
Metal working fluids	1	ND	ND	1	ND	ND
Persulfates salts	1	ND	ND	1	ND	ND
Drug (erythromycine)	-	-	-	1	0/1	ND

Legend: *ND*, not done; *sIgE*, specific IgE antibody assessment; *SPT*, skin-prick test.

[a] Cleaning agents contained various chemical compounds, including an amine surfactant and peracetic acid in the two subjects with a neutrophilic pattern, peracetic acid in one subject with a mixed granulocytic pattern, and multiple potential sensitizers in the other 3 subjects with a mixed granulocytic pattern.

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