Sputum eosinophilia is a determinant of FEV1 decline in occupational asthma: results of an observational study

<table>
<thead>
<tr>
<th>Journal:</th>
<th>BMJ Open</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID:</td>
<td>bmjopen-2014-005748</td>
</tr>
<tr>
<td>Article Type:</td>
<td>Research</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>21-May-2014</td>
</tr>
<tr>
<td>Complete List of Authors:</td>
<td>Talini, Donatella; CeRIMP, Regione Toscana</td>
</tr>
<tr>
<td></td>
<td>Novelli, Federica; Department of Surgery, Medicine, Molecular Biology and Critical Care, University of Pisa</td>
</tr>
<tr>
<td></td>
<td>Bacci, Elena; Department of Surgery, Medicine, Molecular Biology and Critical Care, University of Pisa</td>
</tr>
<tr>
<td></td>
<td>Bartoli, Marialaura; Department of Surgery, Medicine, Molecular Biology and Critical Care, University of Pisa</td>
</tr>
<tr>
<td></td>
<td>Silvana, Cianchetti; Department of Surgery, Medicine, Molecular Biology and Critical Care, University of Pisa</td>
</tr>
<tr>
<td></td>
<td>Costa, Francesca; Department of Surgery, Medicine, Molecular Biology and Critical Care, University of Pisa</td>
</tr>
<tr>
<td></td>
<td>Dente, Federico; Department of Surgery, Medicine, Molecular Biology and Critical Care, University of Pisa</td>
</tr>
<tr>
<td></td>
<td>Di Franco, Antonella; Department of Surgery, Medicine, Molecular Biology and Critical Care, University of Pisa</td>
</tr>
<tr>
<td></td>
<td>Latorre, Manuela; Department of Surgery, Medicine, Molecular Biology and Critical Care, University of Pisa</td>
</tr>
<tr>
<td></td>
<td>Malagrinò, Laura; Department of Surgery, Medicine, Molecular Biology and Critical Care, University of Pisa</td>
</tr>
<tr>
<td></td>
<td>Vagaggini, Barbara; Department of Surgery, Medicine, Molecular Biology and Critical Care, University of Pisa</td>
</tr>
<tr>
<td></td>
<td>Celi, Alessandro; Department of Surgery, Medicine, Molecular Biology and Critical Care, University of Pisa</td>
</tr>
<tr>
<td></td>
<td>Paggiaro, PierLUIGI; Department of Surgery, Medicine, Molecular Biology and Critical Care, University of Pisa</td>
</tr>
<tr>
<td>Primary Subject Heading:</td>
<td>Respiratory medicine</td>
</tr>
<tr>
<td>Secondary Subject Heading:</td>
<td>Occupational and environmental medicine, Epidemiology</td>
</tr>
<tr>
<td>Keywords:</td>
<td>Asthma &lt; THORACIC MEDICINE, OCCUPATIONAL &amp; INDUSTRIAL MEDICINE, Epidemiology &lt; THORACIC MEDICINE, Cell biology &lt; BASIC SCIENCES</td>
</tr>
</tbody>
</table>
Sputum eosinophilia is a determinant of FEV1 decline in occupational asthma: results of an observational study

Donatella Talini, MD *, Federica Novelli, MD, Elena Bacci, MD, Marialaura Bartoli, PhD, Silvana Cianchetti, PhD, Francesco Costa, MD, Federico L Dente, MD, Antonella Di Franco, MD, Manuela Latorre, MD, Laura Malagrinò, MD, Barbara Vagaggini, MD, Alessandro Celi, MD, and Pierluigi Paggiaro, Prof

Department of Surgery, Medicine, Molecular Biology and Critical Care; University of Pisa; *
CERIMP, Regione Toscana; Pisa, Italy

Correspondence:
Donatella Talini
CERIMP, Regione Toscana, Dipartimento della Prevenzione ASL 5 di Pisa
Galleria Gerace 14, 56124, Pisa (Italy)
Tel. +39050954436, Fax +39050954454, E-Mail: d.talini@usl5.toscana.it

Key words: occupational – asthma, eosinophils, sputum, FEV1 decline

Word count: 2815
ABSTRACT

Background: The outcome of occupational asthma (OA) after diagnosis is often poor, with large heterogeneity according to clinical or inflammatory characteristics measured at the diagnosis.

Objective: To evaluate the potential determinants of FEV1 decline in workers with OA still exposed to the causative agent, routinely followed up at a clinical laboratory of the University Hospital of Pisa between 1990 and 2009.

Methods: Estimates of the decline of Forced Expiratory Volume in 1 second (FEV1) were obtained by means of simple regression analysis in 39 subjects (28 males and 11 females) with OA during the period of occupational exposure after diagnosis. Logistic regression was used to analyse the effects of factors (baseline FEV1, sputum inflammatory cells, duration and type of exposure) that may potentially influence FEV1 decline.

Results: At follow-up (5.7±3.7 yrs), most subjects were symptomatic, had high sputum eosinophil levels and low FEV1 values. Subjects with higher sputum eosinophils (>3%) had significantly greater decline of FEV1 (–52.5 ml/yr vs -18.6 ml/yr, p=0.012). Logistic regression showed that persistent exposure and sputum eosinophilia were significantly associated with greater decline of FEV1.

Conclusions: Sputum eosinophilia at diagnosis may contribute to greater decline in FEV1 in patients with occupational asthma still at work.
ARTICLE SUMMARY

Strengths and limitations of this study

- In our study we found a significant relationship between baseline sputum eosinophil levels and FEV1 decline, suggesting that higher levels of inflammation at baseline may cause accelerated decline in FEV1.

- No previous paper have considered this biomarker as possible determinants of the decline in FEV1 in patients with occupational asthma who continued to work.

- The number of patients examined is relatively small. However, apart few studies enrolling large number of patients, several other published studies have included similar number of patients.

- The type of exposure (LMWC vs HMWC) was heterogeneous, but again this is frequently reported in many previous studies.

- The distinction between persistence and reduction of exposure is not based on specific environmental measurements, but the majority of previous studies used the same rough distinction we did between patients who continued and patients who reduced occupational exposure to the specific sensitizer.

STATEMENTS

- Not additional data available

- This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors

- All authors don't have any competing interest
INTRODUCTION

Subjects with occupational asthma often experience permanent sequelae after removal from exposure, and the outcome of occupational asthma after diagnosis is often poor. A substantial body of data indicates that lower lung volumes, greater nonspecific bronchial hyperresponsiveness (NSBHR) and stronger asthmatic response to specific inhalation challenge (SIC) at diagnosis are risk factors for the poor outcome of occupational asthma.[1] Ideally, the worker should be removed from the work environment causing asthma, in order to prevent the deterioration of respiratory condition. However, complete cessation of exposure has high socio-economic consequences and is thus rarely feasible.[2] Recent systematic literature research suggests that longer symptomatic exposure is associated with worse occupational asthma outcome, in terms of persistence of symptoms and NSBHR and greater decline in FEV1.[3-4] Some studies have evaluated the outcome of functional parameters after diagnosis.[5] Two retrospective cohort studies documented accelerated FEV1 decline in patients with occupational asthma before removal from the causal agents;[5-6] in one of these studies,[6] FEV1 continued to decline after removal from exposure, but at slower rate, similar to the rate of decline observed in healthy adults. Data about the effect of anti-asthma medication on symptoms and lung function in patients with persistent exposure are contradictory.[6-7]

However, occupational asthma may have different outcomes, and it is unknown whether these differences depend upon the underlying inflammatory process and structural changes in the airways. Different phenotypic categories have been reported according to clinical or inflammatory characteristics, or to the triggers inducing or aggravating asthma.[8] Eosinophilic and noneosinophilic phenotypes have been described.[9] A recent study reported rapid decrease in eosinophilic inflammation after removal from exposure, but subjects with noneosinophilic asthmatic reaction during SIC seemed to have poorer prognosis than subjects with SIC-induced eosinophilic airway inflammation at diagnosis.[10]

The aim of this study was to evaluate the possible determinants of FEV1 decline in workers with occupational asthma still exposed to the causative agent. In particular, we hypothesized that sputum eosinophilia might be a predictor of poor asthma outcome after diagnosis.
PATIENTS AND METHODS

We studied 39 subjects previously diagnosed as having occupational asthma (OA), routinely followed up at the Cardio-Thoracic and Vascular Department of the University Hospital of Pisa between 1990 and 2009. The diagnosis of OA was made at the first evaluation, according to the positive response to specific inhalation challenge (SIC) test: twenty-three subjects were sensitized to low molecular weight compounds (LMWC: isocyanates and persulfate salts) and 16 subjects to high molecular weight compounds (HMWC: flour dusts, wood dusts, latex and tobacco dusts).

All subjects were routinely evaluated every 6 months after diagnosis, while still exposed to the specific sensitizer at work. The duration of follow-up was 5.7±3.7 yrs.

At the first evaluation, all patients were interviewed about asthmatic symptoms and occupational exposure, and underwent spirometry and methacholine challenge test, prick test to common airborne allergens, collection of sputum for inflammatory cells count, and SIC test with the specific occupational agent.

At each follow-up examination, asthmatic symptoms, pharmacologic therapy, type of occupational exposure (persistent vs reduced exposure) and spirometric data were collected. Work exposure was considered as persistent when the patient continued working with the same job title and in the same environment, whereas it was considered as reduced when the patient had been relocated in another area of the same factory where the specific sensitizer was not used, with occasional short-term direct exposure to the specific sensitizer.[11]

Antiasthma treatment was withdrawn 48 hrs before spirometry.

Pulmonary function tests, atopy and symptoms evaluation.

FEV1 and FVC were measured by a computerized water-sealed spirometer (Biomedin, Padova, Italy) using predicted values approved by the European Respiratory Society. The details of methacholine challenge test have been reported previously.[12] The cumulative dose of methacholine producing a 20% fall in FEV1 (PD20FEV1) was computed; a PD20FEV1 value <1000 µg of methacholine was considered as positive for NSBH.

SIC was performed using two different methods: a) for diisocyanates, subjects were exposed to vapours generated by blowing air on the surface of a small amount of freshly prepared
toluenediisocyanate (TDI) or methylendiisocyanate (MDI) in a challenge chamber, and monitoring isocyanate concentrations with a specific TDI/MDI detector (MDA model 7005 isocyanate detection equipment, MDA Scientific Inc., Glenview, IL); the duration of the exposure was the same (30 min) for all subjects. FEV1 was measured before and immediately after exposure, then hourly for 8 hours;[12] b) for dusts, subjects were asked to breath through a mouthpiece connected to a small box where a suspension of the dust was obtained by blowing compressed air on a small amount of fine dust of the compound (flour, wood, persulfate or latex), and the concentration of dust in the box was measured by sucking the air of the box through a 0.8 µm cellulose nitrate filter by means of a vacuum pump.[13] A positive response was defined as a decrease in FEV1 greater than 15% from baseline within the first hour (immediate response) or between the second and the 8th hour (late response), and in absence of a greater than 10% decrease in FEV1 during a control test performed with diluent (for diisocyanates) or with lactose dust (for other sensitizers).

Current asthma symptoms (more than 2 times/week) and antiasthma therapy (defined as regular use of inhaled corticosteroids) were recorded as qualitative parameters (yes / no) at each visit.

*Sputum induction and processing*

Sputum was induced according to European Respiratory Society Task Force recommendations.[14] Hypertonic saline solution (NaCl 4.5%) was nebulized by means of an ultrasonic nebulizer (Ultraneb 2000, DeVilbiss, Somerset, Pa, USA) with 2.8 mL·min−1 output, and were inhaled for three 5-min periods for up to 15 min. Every 5 min, after the start of nebulization, subjects were asked to rinse their mouth and throat carefully, to discard saliva, and to try to cough sputum into a container; FEV1 was then measured. Nebulization was stopped after 15 min or when FEV1 fell by ≥20% from baseline value. Saline-induced bronchoconstriction was promptly relieved by short-acting β2-agonist inhalation. Sputum samples were diluted with an equal volume of 0.1% dithiotreithol (Sputasol, Unipath; Basingstoke, UK). Samples were treated as previously reported.[15] Macrophages, lymphocytes, neutrophils and eosinophils were expressed as percentage of total inflammatory cells, excluding squamous cells. The upper limit of normal range for sputum eosinophils was set at 3% as derived from a group of normal subjects, whereas high sputum neutrophils were defined when sputum neutrophil percentage was > 63%. [16]
Statistical analysis

FEV1 is expressed as mean ± SD. PD20FEV1 is expressed as geometric mean and is log-transformed for comparisons. Sputum cell count are expressed as median (range). Simple regression analysis was used to provide estimates of the decline of FEV1 (expressed as annual average change in FEV1) during the period of occupational exposure. Categorical data were compared by using Pearson’s $\chi^2$ statistics. Continuous data were compared using unpaired Student's t-test or Mann-Whitney test.

Logistic regression was used to analyse the effects of potential factors measured at baseline (independent variables: baseline FEV1, PD20FEV1 methacholine, sputum eosinophils and neutrophils and other general or clinical characteristics such as atopy, smoking habit and duration of exposure) on the dependent variables (decline > -57.5 ml/yr, the lowest tertile of the distribution of single FEV1 declines). In the analysis we also included two variables that may potentially modify FEV1 decline during follow-up: a) persistence or reduction of occupational exposure to sensitizer (we considered as reduced the exposure to sensitizer for less than 100% but more than 50% of the follow-up period); b) ICS therapy (we considered as ICS-treated patients those who used ICS for more than 50% of the follow-up period including the last visit).

The results are given in terms of odds ratios (ORs) with 95% confidence intervals (95% CI). Dependent and independent categorical variables were binary (high vs low sputum eosinophils, high vs low sputum neutrophils, hyperreactivity vs no hyperreactivity, persistence vs reduction of exposure, smoker and ex-smoker vs non-smoker, LMWC vs HMWC, ICS therapy vs no therapy), whereas continuous variables (age, baseline FEV1, duration of exposure) were transformed into categorical variables with the tertile distribution obtained for all patients; in particular, the highest tertile (for age, duration of exposure and time to removal) or the lowest tertile (for FEV1) were associated with the presence of the condition.

RESULTS

Table 1 shows the general characteristics of patients at the time of diagnosis. Low molecular weight compounds were the main agents causing OA, with isocyanates causing asthma in 19 patients (48%).
Table 1. General characteristics of patients at the time of diagnosis

<table>
<thead>
<tr>
<th>Number</th>
<th>39</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yrs</td>
<td>43.5±11.8</td>
</tr>
<tr>
<td>Sex, M/F</td>
<td>28/11</td>
</tr>
<tr>
<td>Smoke, Y/Ex/No</td>
<td>3/20/16</td>
</tr>
<tr>
<td>Atopy, n (%)</td>
<td>13 (33.3)</td>
</tr>
<tr>
<td>Duration of exposure, yrs</td>
<td>18.6±11.1</td>
</tr>
<tr>
<td>Latency, yrs</td>
<td>12.8±10.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Agents</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>LMWC</td>
<td>23</td>
</tr>
<tr>
<td>Isocyanates</td>
<td>19</td>
</tr>
<tr>
<td>Persulfate salts</td>
<td>4</td>
</tr>
<tr>
<td>HMWC</td>
<td>16</td>
</tr>
<tr>
<td>Flour dusts</td>
<td>11</td>
</tr>
<tr>
<td>Wood dusts</td>
<td>2</td>
</tr>
<tr>
<td>Latex</td>
<td>2</td>
</tr>
<tr>
<td>Tobacco dusts</td>
<td>1</td>
</tr>
</tbody>
</table>

Clinical and functional findings of patients at the time of diagnosis and at the last visit of follow-up are reported in Table 2. At that time, all but two patients (who had already reduced their occupational exposure to the specific sensitizer) had current asthma symptoms and NSBH, whereas mean FEV1 was normal and sputum eosinophilia was observed in less than 50% of patients.

Table 2. Clinical and functional findings at baseline and at the last visit of follow-up (* p<0.001)

<table>
<thead>
<tr>
<th></th>
<th>Diagnosis</th>
<th>Follow-up (5.7±3.7 yrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduction of exposure</td>
<td>6 (15.4)</td>
<td>26 (66.7) *</td>
</tr>
<tr>
<td>FEV1, mL</td>
<td>3.12±0.57</td>
<td>2.92±0.55 *</td>
</tr>
<tr>
<td>FEV1, % of predicted</td>
<td>94.1±16.1</td>
<td>92.2±14.8</td>
</tr>
<tr>
<td>PD20FEV1, mcg</td>
<td>227 (1437)</td>
<td>228 (1757)</td>
</tr>
<tr>
<td>NSBH, n (%)</td>
<td>33 (84.6)</td>
<td>28 (71.8)</td>
</tr>
<tr>
<td>ICS therapy, n (%)</td>
<td>14 (35.9)</td>
<td>36 (92.3) *</td>
</tr>
<tr>
<td>Patients with current symptoms, n (%)</td>
<td>32 (82.1)</td>
<td>17 (43.6) *</td>
</tr>
<tr>
<td>Sputum eosinophils (median, range)</td>
<td>2.9 (0-43.1)</td>
<td>n.a.</td>
</tr>
<tr>
<td>Sputum eosinophils &gt;3%, n (%)</td>
<td>19 (48.7)</td>
<td>n.a.</td>
</tr>
</tbody>
</table>
At follow-up, FEV1 (in L) was lower than that measured at diagnosis (p<0.001), but FEV1 expressed as % of predicted and PD20FEV1 methacholine were no different from baseline; also, the number of patients with NSBH was not significantly different between diagnosis and follow-up. At the time of diagnosis, 6 patients had already reduced their occupational exposure to the specific sensitizer, because they had been relocated to another job in the same factory with occasional, indirect exposure to the sensitizer. At follow-up, a further 20 patients had reduced their exposure to sensitizing agent. More patients were treated with inhaled corticosteroids (ICS) at the last visit of follow-up in comparison with baseline (p<0.001), but 43.6% still reported asthma symptoms, although generally of mild severity, and in lower percentage than at diagnosis (p<0.001).

Using simple regression analysis we provided estimates of the decline of FEV1 (expressed as annual average change in FEV1) during the period of occupational exposure in all subjects. The median annual decline in FEV1 in all subjects was -30.9 ml/yr (range: -188.3 ml/yr to +57.9 ml/yr). When we compared patients with different decline in FEV1 (according to the tertile distribution, from patients with lowest decline, < -16.9 ml/yr, to patients with highest decline, > -57.5ml/yr) as regards baseline characteristics, we did not observe any significant difference among different decliners except for baseline FEV1, which was significantly higher in patients with the highest decline (Table 3). The group with the lowest FEV1 decline included only patients with persistent exposure. Atopic patients were more represented in the group with the highest decline, although the difference was not significant.

Table 3. Characteristics of patients at baseline, grouped by FEV1 decline during the follow-up (*p<0.05)

<table>
<thead>
<tr>
<th></th>
<th>&lt; -16.9 ml/yr</th>
<th>-16.9 to -57.5 ml/yr</th>
<th>&gt; -57.5 ml/yr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>13</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Age, yrs</td>
<td>43.8+12.7</td>
<td>44.2+11.9</td>
<td>42.4+11.6</td>
</tr>
<tr>
<td>Gender, M/F</td>
<td>10/3</td>
<td>8/5</td>
<td>10/3</td>
</tr>
<tr>
<td>Smoke, No/Ex/Yes</td>
<td>4/7/2</td>
<td>7/5/1</td>
<td>5/8/0</td>
</tr>
<tr>
<td>Atopy, n (%)</td>
<td>2 (15.4)</td>
<td>4 (30.8)</td>
<td>7 (53.8)</td>
</tr>
<tr>
<td>LMWC/HMWC</td>
<td>8/5</td>
<td>7/6</td>
<td>8/5</td>
</tr>
<tr>
<td>FEV1, % predicted</td>
<td>84.8+15.2</td>
<td>97.4+14.3</td>
<td>100.9+14.6   *</td>
</tr>
</tbody>
</table>
Subjects with higher sputum eosinophils (>3%) had significantly greater decline in FEV1 (Figure 1) (p=0.012), as well as subjects with persistent exposure when compared to those who had reduced their exposure to the occupational sensitizer (Figure 2) (p= 0.03).

Using a multivariate analysis, we estimated the decline in FEV1 (using as independent variable the highest tertile of FEV1 decline: -57.5 ml/yr) in relationship to baseline FEV1, baseline eosinophil (≥3% vs <3%) and neutrophil (≥ 63% vs <63%) levels, level of exposure at follow-up (persistence vs reduction of exposure), smoking habit (ex or current smokers vs non smokers), molecular weight of the sensitizer (LMWC vs HMWC), duration of exposure and use of inhaled corticosteroids during the follow-up. Persistent exposure and higher baseline sputum eosinophil levels significantly correlated with greater decline in FEV1 (Table 4). The analysis also showed a trend for smoking habit (p=0.08) and duration of exposure (p=0.06), which however did not enter in the relationship. Adding bronchial hyperreactivity as an independent variable did not change the results, except for the inclusion of baseline FEV1 (data not shown).

Table 4. Independent variables significantly related to FEV1 decline in a multivariate analysis

<table>
<thead>
<tr>
<th>Variables</th>
<th>Odd ratios</th>
<th>95% C.I.</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exposure, persistence vs reduction</td>
<td>11.5</td>
<td>1.8 - 71.4</td>
<td>0.009</td>
</tr>
<tr>
<td>Sputum eosinophils ≥ 3%</td>
<td>6.7</td>
<td>1.1 - 41.7</td>
<td>0.042</td>
</tr>
</tbody>
</table>

**DISCUSSION**
The present study confirms that the prognosis of occupational asthma is poor. This is shown by the persistence of symptoms and airway hyperresponsiveness in many patients, as found in most follow-up studies. However, little information is available on how rapidly lung function declines in those who continue to be exposed and how, on the other hand, this decline is affected by removal from exposure and by the presence of airway inflammation, as shown by high levels of eosinophils or neutrophils in induced sputum.

In 280 patients with red cedar asthma with persistent exposure, a greater rate of decline in FEV1 was observed in comparison with asymptomatic sawmill workers.[5] Piriila et al.[17] reported a mean rate of decline of 40 mL/year in 91 selected subjects with isocyanate-induced occupational asthma, although only 12 of these continued to be exposed to the causative agent in the workplace during the period of follow-up. Anees studied 90 subjects undergoing FEV1 measurements at least once yearly before removal from exposure.[6] In this study, FEV1 rapidly declined in exposed workers with occupational asthma; after removal from exposure, FEV1 continued to decline but at a slower rate, similar to the rate of decline observed in healthy adults. The nature of causative agent, current smoking, or treatment with inhaled corticosteroids did not seem to affect the rate of decline in FEV1. However, the authors could not estimate the decline in FEV1 any further because follow-up assessments after removal from exposure were less close and the model of linear decline following the step-up period might have masked intra-individual variation in the pattern of recovery.

In our study we investigated the determinants of FEV1 decline in subjects with occupational asthma, after diagnosis and during the follow-up period, when they were either fully or partially exposed to the specific sensitizer. We observed that baseline FEV1 was inversely related with its own decline, in agreement with what had already been published in asthmatic patients and in the general population.[5,18] On the contrary, we found no significant relationship between bronchial hyperresponsiveness and FEV1 decline, in agreement with the observation that there was no difference between PD20FEV1 at diagnosis and at follow-up, nor did we confirm what observed in a previous paper [19] regarding the relationship between smoking habit or atopy and FEV1 decline.

Previous studies [20-21] have shown an association between high eosinophil levels and accelerated FEV1 decline in adult asthma patients, thereby postulating a role for eosinophils in the progression of the disease. On the other hand, Lemièr et al [10] studied a sample of 24 subjects with occupational asthma removed from exposure after diagnosis, and identified a
noneosinophilic phenotype that showed significant FEV1 decline, along with a lack of improvement in airway responsiveness during the four-year follow-up period after cessation of exposure. By contrast, the eosinophilic phenotype showed less marked FEV1 decline, significant decrease in ICS use over time and a trend toward improvement in airway responsiveness. Based on these findings, it is tempting to speculate that accelerated FEV1 decline in asthma may result from progressive airway remodeling process in which neutrophilic inflammation is also likely to play an important role.

In our study we found a significant relationship between baseline sputum eosinophil levels and FEV1 decline, suggesting that higher levels of inflammation at baseline may cause accelerated decline in FEV1. At our knowledge, no previous paper have considered this biomarker as possible determinants of the decline in FEV1 in patients with occupational asthma who continued to work. Broekema et al.[20] showed that asthmatic patients with accelerated FEV1 decline had high sputum eosinophil levels, but sputum was analyzed at the end of follow-up, and we therefore cannot exclude that sputum eosinophilic inflammation is not the cause but rather the consequence of accelerated FEV1 decline in this study. Unfortunately, in our experience, sputum analysis at the end of occupational exposure was available in only eight subjects.

As in the study by Lemière et al.,[10] we found no significant relationship between ICS therapy and FEV1 decline. Noneosinophilic asthma may be less responsive to ICS treatment than eosinophilic asthma: although Dijkstra et al. found that oral or inhaled corticosteroids reduced FEV1 decline in asthma,[22] some authors have suggested that the lack of eosinophilic inflammation may be a characteristic of refractory asthma.[23] This fact may suggest the need for specific adjustment of asthma treatment according to the characteristics of airway inflammation.

Our study has some limitations. Firstly, the number of patients examined is relatively small. In effect several patients with a diagnosis of occupational asthma ceased work in the six months after diagnosis, or were lost at the first follow-up visit. However, apart few studies enrolling large number of patients, several other published studies have included similar number of patients.[10] Secondly, the type of exposure (LMWC vs HMWC) was heterogeneous, but again this is frequently reported in many previous studies. Finally, the distinction between persistence and reduction of exposure is not based on specific environmental measurements, but the majority of previous studies used the same rough distinction we did between patients who continued and patients who reduced occupational exposure to the specific sensitizer.[24]
In conclusion, we demonstrated that in a well characterized group of patients with occupational asthma, sputum eosinophilia at diagnosis is one determinant of the accelerated decline in FEV1 when patients are still at work. Further long-term studies are required as to whether intensive ICS treatment may be beneficial for patients with occupational asthma and increased eosinophilic inflammation.

**Contributorship Statement**

Contributors: DT, FN, conceived and designed the original paper. All authors were involved in amending protocol. DT coordinated the study throughout. Data entry was carried out by DT and FN. MB and SC have analyzed the biological determinants. EB, FC, FLD, AF, ML, LM, BV, AC dealt with the clinical assessment on patients. PP did the work supervision and paper review.

**Data sharing**

No additional data available.

**Competing Interests**

None.
REFERENCES


FEV1 decline according to baseline sputum eosinophilia

254x190mm (96 x 96 DPI)
Figure 2

FEV1 decline according to level of exposure at follow-up
254x190mm (96 x 96 DPI)
LEGENDS OF THE FIGURES

Figure 1. FEV1 decline according to baseline sputum eosinophilia

Figure 2. FEV1 decline according to level of exposure at follow-up
STROBE Statement—checklist of items that should be included in reports of observational studies

<table>
<thead>
<tr>
<th>Item No</th>
<th>Recommendation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Title and abstract</strong> (V)</td>
<td>1</td>
</tr>
<tr>
<td>(a) pg. 1</td>
<td>(a) Indicate the study’s design with a commonly used term in the title or the abstract</td>
</tr>
<tr>
<td>(b) pg. 3</td>
<td>(b) Provide in the abstract an informative and balanced summary of what was done and what was found</td>
</tr>
<tr>
<td><strong>Introduction</strong></td>
<td></td>
</tr>
<tr>
<td>Background/rationale (V) pg. 4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Explain the scientific background and rationale for the investigation being reported</td>
</tr>
<tr>
<td><strong>Objectives (V) pg. 4</strong></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>State specific objectives, including any prespecified hypotheses</td>
</tr>
<tr>
<td><strong>Methods</strong></td>
<td></td>
</tr>
<tr>
<td>Study design (V) pg. 5</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Present key elements of study design early in the paper</td>
</tr>
<tr>
<td>Setting (V) pg. 5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection</td>
</tr>
<tr>
<td>Participants (V) pg. 5-6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>(a) <em>Cohort study</em>—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up</td>
</tr>
<tr>
<td></td>
<td><em>Case-control study</em>—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls</td>
</tr>
<tr>
<td></td>
<td><em>Cross-sectional study</em>—Give the eligibility criteria, and the sources and methods of selection of participants</td>
</tr>
<tr>
<td></td>
<td>(b) <em>Cohort study</em>—For matched studies, give matching criteria and number of exposed and unexposed</td>
</tr>
<tr>
<td></td>
<td><em>Case-control study</em>—For matched studies, give matching criteria and the number of controls per case</td>
</tr>
<tr>
<td>Variables (V) pg. 5-6</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable</td>
</tr>
<tr>
<td>Data sources/ measurement (V) pg. 5-6</td>
<td>8*</td>
</tr>
<tr>
<td></td>
<td>For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group</td>
</tr>
<tr>
<td>Bias (V) pg. 5-6-7</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Describe any efforts to address potential sources of bias</td>
</tr>
<tr>
<td>Study size (V) pg. 5</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Explain how the study size was arrived at</td>
</tr>
<tr>
<td>Quantitative variables (V) pg. 5-6</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why</td>
</tr>
<tr>
<td>Statistical methods (V) pg. 7</td>
<td>12</td>
</tr>
<tr>
<td>(V)</td>
<td>(a) Describe all statistical methods, including those used to control for confounding</td>
</tr>
<tr>
<td></td>
<td>(b) Describe any methods used to examine subgroups and interactions</td>
</tr>
<tr>
<td></td>
<td>(c) Explain how missing data were addressed</td>
</tr>
</tbody>
</table>
(NA)

(V) pg. 7
Continued on next page

(d) Cohort study—If applicable, explain how loss to follow-up was addressed

Case-control study—If applicable, explain how matching of cases and controls was addressed

Cross-sectional study—If applicable, describe analytical methods taking account of sampling strategy

(e) Describe any sensitivity analyses
Results

Participants (V)  Pg. 7, 8, 9, 10  13*  (a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed
(b) Give reasons for non-participation at each stage
(c) Consider use of a flow diagram

Descriptive (V) data  pg. 8  14*  (a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
(b) Indicate number of participants with missing data for each variable of interest
(c) Consider use of a flow diagram

Outcome data (V)  pg. 8  15*  Cohort study—Report numbers of outcome events or summary measures over time
Case-control study—Report numbers in each exposure category, or summary measures of exposure
Cross-sectional study—Report numbers of outcome events or summary measures

Main results (V)  pg. 9-10, Fig. 1-2  16  (a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included
(b) Report category boundaries when continuous variables were categorized
(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period

Other analyses (V)  pg. 10  17  Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses

Discussion

Key results (V)  pg. 10, 11, 12  18  Summarise key results with reference to study objectives

Limitations (V)  pg. 12  19  Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias

Interpretation (V)  pg. 11-12  20  Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence

Generalisability (V)  pg. 12-13  21  Discuss the generalisability (external validity) of the study results

Other information

Funding (NA)  22  Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

**Sputum eosinophilia is a determinant of FEV1 decline in occupational asthma: results of an observational study**

<table>
<thead>
<tr>
<th>Journal:</th>
<th><em>BMJ Open</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID:</td>
<td>bmjopen-2014-005748.R1</td>
</tr>
<tr>
<td>Article Type:</td>
<td>Research</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>15-Jul-2014</td>
</tr>
<tr>
<td>Complete List of Authors:</td>
<td>Talini, Donatella; CeRIMP, Regione Toscana; Novelli, Federica; Department of Surgery, Medicine, Molecular Biology and Critical Care, University of Pisa; Bacci, Elena; Department of Surgery, Medicine, Molecular Biology and Critical Care, University of Pisa; Bortoli, Marialaura; Department of Surgery, Medicine, Molecular Biology and Critical Care, University of Pisa; Silvani, Cianchetti; Department of Surgery, Medicine, Molecular Biology and Critical Care, University of Pisa; Costa, Francesca; Department of Surgery, Medicine, Molecular Biology and Critical Care, University of Pisa; Dente, Federico; Department of Surgery, Medicine, Molecular Biology and Critical Care, University of Pisa; Di Franco, Antonella; Department of Surgery, Medicine, Molecular Biology and Critical Care, University of Pisa; Latorre, Manuela; Department of Surgery, Medicine, Molecular Biology and Critical Care, University of Pisa; Malagrinò, Laura; Department of Surgery, Medicine, Molecular Biology and Critical Care, University of Pisa; Vagaggini, Barbara; Department of Surgery, Medicine, Molecular Biology and Critical Care, University of Pisa; Celi, Alessandro; Department of Surgery, Medicine, Molecular Biology and Critical Care, University of Pisa; Paggiaro, Pierluigi; Department of Surgery, Medicine, Molecular Biology and Critical Care, University of Pisa</td>
</tr>
<tr>
<td>Primary Subject Heading:</td>
<td>Respiratory medicine</td>
</tr>
<tr>
<td>Secondary Subject Heading:</td>
<td>Occupational and environmental medicine, Epidemiology</td>
</tr>
<tr>
<td>Keywords:</td>
<td>Asthma &lt; THORACIC MEDICINE, OCCUPATIONAL &amp; INDUSTRIAL MEDICINE, Epidemiology &lt; THORACIC MEDICINE, Cell biology &lt; BASIC SCIENCES</td>
</tr>
</tbody>
</table>
Sputum eosinophilia is a determinant of FEV1 decline in occupational asthma: results of an observational study

Donatella Talini, MD *, Federica Novelli, MD, Elena Bacci, MD, Marialaura Bartoli, PhD, Silvana Cianchetti, PhD, Francesco Costa, MD, Federico L Dente, MD, Antonella Di Franco, MD, Manuela Latorre, MD, Laura Malagrinò, MD, Barbara Vagaggini, MD, Alessandro Celi, MD, and Pierluigi Paggiaro, Prof

Department of Surgery, Medicine, Molecular Biology and Critical Care; University of Pisa; * CERIMP, Regione Toscana; Pisa, Italy

Correspondence: Donatella Talini
CERIMP, Regione Toscana, Dipartimento della Prevenzione ASL 5 di Pisa
Galleria Gerace 14, 56124, Pisa (Italy)
Tel. +39050954436, Fax +39050954454, E-Mail: d.talini@usl5.toscana.it

Key words: occupational – asthma, eosinophils, sputum, FEV1 decline

Word count: 2893
ABSTRACT

Objective: To evaluate the potential determinants of Forced Expiratory Volume in 1 second (FEV1) decline in workers with OA still exposed to the causative agent. We hypothesized that sputum eosinophilia might be a predictor of poor asthma outcome after diagnosis.

Setting, Design and Participants: In a specialist clinical center of the University Hospital of Pisa we studied 39 subjects (28 M, 11 F) diagnosed as having OA, routinely followed up between 1990 and 2009. They were a subgroup of 94 subjects diagnosed as affected by OA in that period: 9 had been removed from work at the diagnosis, 21 were excluded for having ceased occupational exposure after few months from diagnosis, and 25 were lost at the follow-up or had no acceptable sputum measurements at the diagnosis.

Estimates of the decline of FEV1 were obtained by means of simple regression analysis during the period of occupational exposure after diagnosis. Logistic regression was used to analyse the effects of factors (baseline FEV1 and sputum inflammatory cells, duration and type of exposure) that may potentially influence FEV1 decline.

Results: At follow-up (5.7±3.7 yrs), most subjects were still symptomatic despite ICS treatment and had their occupational exposure reduced. Subjects with higher sputum eosinophils (>3%) at baseline had significantly greater decline of FEV1 (−52.5 ml/yr vs -18.6 ml/yr, p=0.012). Logistic regression showed that persistent exposure and sputum eosinophilia were significantly associated with greater decline of FEV1 (OR 11.5, 95% CI 1.8-71.4, p=0.009 and OR 6.7, 95% CI 1.1-41.7, p=0.042 respectively).

Conclusions: Sputum eosinophilia at diagnosis, together with the persistence of occupational exposure during follow-up, may contribute to greater decline in FEV1 in patients with OA still at work. Further long-term studies are required as to whether intensive ICS treatment may be beneficial for patients with occupational asthma and increased eosinophilic inflammation.
ARTICLE SUMMARY

Strengths and limitations of this study

- In our study we found a significant relationship between baseline sputum eosinophil levels and FEV1 decline, suggesting that higher levels of inflammation at baseline may cause accelerated decline in FEV1

- No previous paper have considered this biomarker as possible determinants of the decline in FEV1 in patients with occupational asthma who continued to work

- The number of patients examined is relatively small. However, apart few studies enrolling large number of patients, several other published studies have included similar number of patients

- The type of exposure (LMWC vs HMWC) was heterogeneous, but again this is frequently reported in many previous studies

- The distinction between persistence and reduction of exposure is not based on specific environmental measurements, but the majority of previous studies used the same rough distinction we did between patients who continued and patients who reduced occupational exposure to specific sensitizer

Statements

- Not additional data available

- This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors

- All authors don’t have any competing interest
INTRODUCTION

Subjects with occupational asthma often experience permanent sequelae after removal from exposure, and the outcome of occupational asthma after diagnosis is often poor. A substantial body of data indicates that lower lung volumes, greater nonspecific bronchial hyperresponsiveness (NSBH) and stronger asthmatic response to specific inhalation challenge (SIC) at diagnosis are risk factors for the poor outcome of occupational asthma (1). Ideally, the worker should be removed from the work environment causing asthma, in order to prevent the deterioration of respiratory condition. However, complete cessation of exposure has high socioeconomic consequences and is thus rarely feasible (2). Recent systematic literature research suggests that longer symptomatic exposure is associated with worse occupational asthma outcome, in terms of persistence of symptoms and NSBHR and greater decline in FEV1 (3,4). Some studies have evaluated the outcome of functional parameters after diagnosis (revised in 5). Two retrospective cohort studies documented accelerated FEV1 decline in patients with occupational asthma before removal from the causal agents (5, 6); in one of these studies (6), FEV1 continued to decline after removal from exposure, but at slower rate, similar to the rate of decline observed in healthy adults. Data about the effect of anti-asthma medication on symptoms and lung function in patients with persistent exposure are contradictory (6,7).

However, occupational asthma may have different outcomes, and it is unknown whether these differences depend upon the underlying inflammatory process and structural changes in the airways. Different phenotypic categories have been reported according to clinical or inflammatory characteristics, or to the triggers inducing or aggravating asthma (8). Eosinophilic and noneosinophilic phenotypes have been described (9). A recent study reported rapid decrease in eosinophilic inflammation after removal from exposure, but subjects with noneosinophilic asthmatic reaction during SIC seemed to have poorer prognosis than subjects with SIC-induced eosinophilic airway inflammation at diagnosis (10).

The aim of this study was to evaluate the possible determinants of FEV1 decline in workers with occupational asthma still exposed to the causative agent.
PATIENTS AND METHODS

We studied 39 subjects previously diagnosed as having occupational asthma (OA), routinely followed up at the Cardio-Thoracic and Vascular Department of the University Hospital of Pisa between 1990 and 2009. They were only a subgroup of the 94 subjects diagnosed as affected by OA in that period. Of the whole group, 9 subjects had been completely removed from work at the time of diagnosis, while 21 subjects were excluded for having ceased occupational exposure after few months from diagnosis, and 25 subjects were lost at the follow-up or had no acceptable sputum measurements at the diagnosis. There was no difference in the main clinical and functional data between 39 subjects included in this study and the other 46 subjects still at work at the time of the diagnosis and not included in this study (see Table 1 in the Appendix).

The diagnosis of OA was made at the first evaluation, according to the positive response to specific inhalation challenge (SIC) test: twenty-three subjects were sensitized to low molecular weight compounds (LMWC: isocyanates and persulfate salts) and 16 subjects to high molecular weight compounds (HMWC: flour dusts, wood dusts, latex and tobacco dusts).

All subjects were routinely evaluated every 6 months after diagnosis, while still exposed to the specific sensitizer at work. The duration of follow-up was 5.7±3.7 yrs.

At the first evaluation, all patients were interviewed about asthmatic symptoms and occupational exposure, and underwent spirometry and methacholine challenge test, prick test to common airborne allergens, collection of sputum for inflammatory cells count, and SIC test with the specific occupational agent.

At each follow-up examination, asthmatic symptoms, pharmacologic therapy, type of occupational exposure (persistent vs reduced exposure) and spirometric data were collected. Work exposure was considered as persistent when the patient continued working with the same job title and in the same environment, whereas it was considered as reduced when the patient had been relocated in another area of the same factory where the specific sensitizer was not used, with occasional short-term direct exposure to the specific sensitizer (11).

Antiasthma treatment was withdrawn 48 hrs before spirometry.

*Pulmonary function tests, atopy and symptoms evaluation.*

FEV1 and FVC were measured by a computerized water-sealed spirometer (Biomedin, Padova, Italy) using predicted values approved by the European Respiratory Society. The details of
methacholine challenge test have been reported previously (12). The cumulative dose of methacholine producing a 20% fall in FEV1 (PD20FEV1) was computed; a PD20FEV1 value <1000 µg of methacholine was considered as positive for NSBH.

SIC was performed using two different methods: a) for diisocyanates, subjects were exposed to vapours of toluenediisocyanate (TDI) or methylendiisocyanate (MDI), and the duration of the exposure was the same (30 min) for all subjects. FEV1 was measured before and immediately after exposure, then hourly for 8 hours (12); b) for dusts, subjects were asked to breath through a mouthpiece connected to a small box where a measured suspension of the dust was obtained (13). A positive response was defined as a decrease in FEV1 greater than 15% from baseline within the first hour (immediate response) or between the second and the 8th hour (late response), and in absence of a greater than 10% decrease in FEV1 during a control test performed with diluent (for diisocyanates) or with lactose dust (for other sensitizers).

Current asthma symptoms (more than 2 times/week) and antiasthma therapy (defined as regular use of inhaled corticosteroids) were recorded as qualitative parameters (yes / no) at each visit.

**Sputum induction and processing**

Sputum was induced according to European Respiratory Society Task Force recommendations (14). Hypertonic saline solution (NaCl 4.5%) was nebulized by means of an ultrasonic nebulizer (Ultraneb 2000, DeVilbiss, Somerset, Pa, USA) with 2.8 mL·min⁻¹ output, and was inhaled for three 5-min periods for up to 15 min. Every 5 min, after the start of nebulization, subjects were asked to rinse their mouth and throat carefully, to discard saliva, and to try to cough sputum into a container; FEV1 was then measured. Nebulization was stopped after 15 min or when FEV1 fell by ≥20% from baseline value. Saline-induced bronchoconstriction was promptly relieved by short-acting β2-agonist inhalation. Sputum samples were diluted with an equal volume of 0.1% dithiotreithol (Sputasol, Unipath; Basingstoke, UK). Samples were treated as previously reported (15). Macrophages, lymphocytes, neutrophils and eosinophils were expressed as percentage of total inflammatory cells, excluding squamous cells. The upper limit of normal range for sputum eosinophils was set at 3% as derived from a group of normal subjects, whereas high sputum neutrophils were defined when sputum neutrophil percentage was > 63% (16).
**Statistical analysis**

FEV1 is expressed as mean ± SD. PD20FEV1 is expressed as geometric mean and is log-transformed for comparisons. Sputum cell count are expressed as median (range). Simple regression analysis was used to provide estimates of the decline of FEV1 (expressed as annual average change in FEV1) during the period of occupational exposure. Categorical data were compared by using Pearson’s $\chi^2$ statistics. Continuous data were compared using unpaired Student’s t-test or Mann-Whitney test.

Logistic regression was used to analyse the effects of potential factors measured at baseline (independent variables: baseline FEV1, PD20FEV1 methacholine, sputum eosinophils and neutrophils and other general or clinical characteristics such as atopy, smoking habit and duration of exposure) on the dependent variables (decline > -57.5 ml/yr, the lowest tertile of the distribution of single FEV1 declines). In the analysis we also included two variables that may potentially modify FEV1 decline during follow-up: a) persistence or reduction of occupational exposure to sensitizer (we considered as reduced the exposure to sensitizer for less than 100% but more than 50% of the follow-up period); b) ICS therapy (we considered as ICS-treated patients those who used ICS for more than 50% of the follow-up period including the last visit).

The results are given in terms of odds ratios (ORs) with 95% confidence intervals (95% CI).

Dependent and independent categorical variables were binary (high vs low sputum eosinophils, high vs low sputum neutrophils, hyperreactivity vs no hyperreactivity, persistence vs reduction of exposure, smoker and ex-smoker vs non-smoker, LMWC vs HMWC, ICS therapy vs no therapy), whereas continuous variables (age, baseline FEV1, duration of exposure) were transformed into categorical variables with the tertile distribution obtained for all patients; in particular, the highest tertile (for age, duration of exposure and time to removal) or the lowest tertile (for FEV1) were associated with the presence of the condition.

**RESULTS**

Table 1 shows the general characteristics of patients at the time of diagnosis. Low molecular weight compounds were the main agents causing OA, with isocyanates causing asthma in 19 patients (48%).
Table 1. General characteristics of the patients at the time of diagnosis

<table>
<thead>
<tr>
<th>Number</th>
<th>39</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yrs</td>
<td>43.5±11.8</td>
</tr>
<tr>
<td>Sex, M/F</td>
<td>28/11</td>
</tr>
<tr>
<td>Smoke, Y/Ex/No</td>
<td>3/20/16</td>
</tr>
<tr>
<td>Atopy, n (%)</td>
<td>13 (33.3)</td>
</tr>
<tr>
<td>Duration of exposure, yrs</td>
<td>18.6±11.1</td>
</tr>
<tr>
<td>Latency, yrs</td>
<td>12.8±10.9</td>
</tr>
</tbody>
</table>

Agents

LMWC | 23 |
Isocyanates | 19 |
Persulfate salts | 4 |
HMWC | 16 |
Flour dusts | 11 |
Wood dusts | 2 |
Latex | 2 |
Tobacco dusts | 1 |

LMWC= low molecular weight compounds; HMWC=high molecular weight compounds

Clinical and functional findings of patients at the time of diagnosis and at the last visit of follow-up are reported in Table 2. At the time of diagnosis, all but two out of the 6 patients who had already reduced their occupational exposure to the specific sensitizer, had current asthma symptoms and NSBH, whereas mean FEV1 was normal and sputum eosinophilia was observed in less than 50% of patients.
Table 2. Clinical and functional findings at baseline and at the last visit of follow-up

(* p<0.001 )

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Follow-up (5.7±3.7 yrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduction of exposure, n (%)</td>
<td>6 (15.4)</td>
</tr>
<tr>
<td>FEV1, mL, mean ± SD</td>
<td>3.12±0.57</td>
</tr>
<tr>
<td>FEV1, % of predicted, mean ± SD</td>
<td>94.1±16.1</td>
</tr>
<tr>
<td>PD20FEV1, mcg, GM (SD)</td>
<td>227 (1437)</td>
</tr>
<tr>
<td>NSBH, n (%)</td>
<td>33 (84.6)</td>
</tr>
<tr>
<td>ICS therapy, n (%)</td>
<td>14 (35.9)</td>
</tr>
<tr>
<td>Patients with current symptoms, n (%)</td>
<td>32 (82.1)</td>
</tr>
<tr>
<td>Sputum eosinophils %, median (range)</td>
<td>2.9 (0-43.1)</td>
</tr>
<tr>
<td>Sputum eosinophils &gt;3%, n (%)</td>
<td>19 (48.7)</td>
</tr>
</tbody>
</table>

SD= standard deviation; FEV1= forced expiratory volume in the first second; GM= geometric mean; PD20FEV1= provocative dose of methacholine causing a 20% reduction of FEV1; NSBH= nonspecific bronchial hyperresponsiveness; ICS= inhaled corticosteroids

* p<0.05 between diagnosis and follow-up

At follow-up, FEV1 (in L) was lower than that measured at diagnosis (p<0.001), but FEV1 expressed as % of predicted and PD20FEV1 methacholine were no different from baseline; also, the number of patients with NSBH was not significantly different between diagnosis and follow-up. At the time of diagnosis, 6 patients had already reduced their occupational exposure to the specific sensitizer, because they had been relocated to another job in the same factory with occasional, indirect exposure to the sensitizer. At follow-up, a further 20 patients had reduced their exposure to sensitizing agent. More patients were treated with inhaled corticosteroids (ICS) at the last visit of follow-up in comparison with baseline (p<0.001), but 43.6% still reported asthma symptoms, although generally of mild severity, and in lower percentage than at diagnosis (p<0.001). All these changes were similar between patients who had persistent or reduced exposure to the occupational sensitizer, except for PD20FEV1 increased in subjects who had reduced exposure.

Using simple regression analysis we provided estimates of the decline of FEV1 (expressed as annual average change in FEV1) during the period of occupational exposure in all subjects. The median annual decline in FEV1 in all subjects was -30.9 ml/yr (range: -188.3 ml/yr to +57.9 ml/yr). Using separate regression lines to separate periods of reduced exposure from periods of original
exposure (available data only in 9 patients), the median estimate FEV1 slope during the period of persistent exposure was much higher than in the period of reduced exposure to the causative agent (-166.4 [-354.9, -57.7] vs -7.9 [-129.3, 55.8] mL/yr, p= 0.001). When we compared patients with different decline in FEV1 (according to the tertile distribution, from patients with lowest decline, < -16.9 ml/yr, to patients with highest decline, > -57.5ml/yr) as regards baseline characteristics, we did not observe any significant difference among different decliners except for baseline FEV1, which was significantly higher in patients with the highest decline (Table 3). The group with the lowest FEV1 decline included only patients with persistent exposure. Atopic patients were more represented in the group with the highest decline, although the difference was not significant.

Table 3. Characteristics of patients at baseline, grouped by FEV1 decline during the follow-up (*p<0.05)

<table>
<thead>
<tr>
<th></th>
<th>&lt;-16.9 ml/yr</th>
<th>-16.9 to -57.5 ml/yr</th>
<th>&gt;-57.5 ml/yr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>13</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Age, yrs, mean + SD</td>
<td>43.8+12.7</td>
<td>44.2+11.9</td>
<td>42.4+11.6</td>
</tr>
<tr>
<td>Gender, M/F</td>
<td>10/3</td>
<td>8/5</td>
<td>10/3</td>
</tr>
<tr>
<td>Smoke, No/Ex/Yes</td>
<td>4/7/2</td>
<td>7/5/1</td>
<td>5/8/0</td>
</tr>
<tr>
<td>Atopy, n (%)</td>
<td>2 (15.4)</td>
<td>4 (30.8)</td>
<td>7 (53.8)</td>
</tr>
<tr>
<td>LMWC/HMWC</td>
<td>8/5</td>
<td>7/6</td>
<td>8/5</td>
</tr>
<tr>
<td>FEV1, % predicted, mean + SD</td>
<td>84.8+15.2</td>
<td>97.4+14.3</td>
<td>100.9+14.6 *</td>
</tr>
<tr>
<td>PD20FEV1, mcg, GM (SD)</td>
<td>196</td>
<td>149</td>
<td>399</td>
</tr>
<tr>
<td>Bronchial hyperreactivity, n (%)</td>
<td>12 (92.3)</td>
<td>11 (84.6)</td>
<td>10 (76.9)</td>
</tr>
<tr>
<td>Reduction of exposure, n (%)</td>
<td>0</td>
<td>5 (38.5)</td>
<td>1 (7.7)</td>
</tr>
<tr>
<td>Sputum eosinophil %, median (range)</td>
<td>2.3 (0-15.5)</td>
<td>2.0 (0.43.1)</td>
<td>4.9 (0-24.4)</td>
</tr>
<tr>
<td>Sputum neutrophil %, median (range)</td>
<td>59.5 (30.8-86.7)</td>
<td>37.0 (11.1-85.0)</td>
<td>47.2 (19.4-96.0)</td>
</tr>
</tbody>
</table>

SD= standard deviation; LOWC=low molecular weight compounds; HMWC= high molecular weight compounds; FEV1= forced expiratory volume in the first second; GM= geometric mean; PD20FEV1= provocative dose of methacholine causing a 20% reduction of FEV1; ICS= inhaled corticosteroids

*p<0.05 among the three groups
Subjects with higher sputum eosinophils (>3%) had significantly greater decline in FEV1 (Figure 1) \( (p=0.012) \), as well as subjects with persistent exposure when compared to those who had reduced their exposure to the occupational sensitizer (Figure 2) \( (p=0.03) \).

Using an univariate analysis, we estimated the decline in FEV1 (using as independent variable the highest tertile of FEV1 decline: -57.5 ml/yr) in relationship to baseline FEV1, baseline eosinophil \( (\geq 3\% \text{ vs } <3\%) \) and neutrophil \( (\geq 63\% \text{ vs } <63\%) \) levels, level of exposure at follow-up \( (\text{persistence vs reduction of exposure}) \), smoking habit \( (\text{ex or current smokers vs non smokers}) \), molecular weight of the sensitizer \( (\text{LMWC vs HMWC}) \), duration of exposure and use of inhaled corticosteroids during the follow-up. Persistent exposure \( (\text{OR: 6.7 (1.5-29.6), } p=0.012) \), higher baseline sputum eosinophil levels \( (\text{OR: 3.6 (0.8-14.8), } p=0.07) \) and baseline FEV1 \( (\% \text{ of predicted}) \) \( (\text{OR: 1.04 (0.99-1.1), } p=0.07) \) significantly correlated with greater decline in FEV1. Table 4 shows results of a multivariate analysis when persistent exposure, higher baseline sputum eosinophil levels and baseline FEV1 \( (\% \text{ of predicted}) \) were used as independent variables. Persistent exposure and higher baseline sputum eosinophil levels significantly correlated with greater decline in FEV1.

Table 4. Results of the multivariate analysis on the main determinants of the FEV1 decline, including as independent variables only those variables significantly related to the FEV1 decline in the univariate analysis.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Odd ratios</th>
<th>95% C.I.</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Persistent exposure vs reduction</td>
<td>12.7</td>
<td>1.8</td>
<td>90.8</td>
</tr>
<tr>
<td>Sputum eosinophilia &gt;= 3%</td>
<td>7.6</td>
<td>1.1</td>
<td>52.9</td>
</tr>
<tr>
<td>Baseline FEV1 (% of predicted)</td>
<td>1.06</td>
<td>0.99</td>
<td>1.13</td>
</tr>
</tbody>
</table>
DISCUSSION

The present study confirms that the prognosis of occupational asthma is poor in subjects who remain exposed to the sensitizing agent, as shown by the persistence of symptoms and airway hyperresponsiveness in many patients. Furthermore, it demonstrates for the first time that the highest rate of decline in FEV1 during the follow-up was associated with a high (>3%) sputum eosinophil count at the initial evaluation and with the persistence of exposure to the causal agent, suggesting that baseline eosinophilic airway inflammation may contribute, together with the persistence of occupational exposure, to a poor asthma outcome.

In 280 patients with red cedar asthma with persistent exposure, a greater rate of decline in FEV1 was observed in comparison with asymptomatic sawmill workers (5). Piriila et al. (17) reported a mean rate of decline of 40 mL/year in 91 selected subjects with isocyanate-induced occupational asthma, although only 12 of these continued to be exposed to the causative agent in the workplace during the period of follow-up. Anees studied 90 subjects undergoing FEV1 measurements at least once yearly before removal from exposure (6). In this study, FEV1 rapidly declined in exposed workers with occupational asthma; after removal from exposure, FEV1 continued to decline but at a slower rate, similar to the rate of decline observed in healthy adults. The nature of causative agent, current smoking, or treatment with inhaled corticosteroids did not seem to affect the rate of decline in FEV1. However, the authors could not estimate the decline in FEV1 any further because follow-up assessments after removal from exposure were less close and the model of linear decline following the step-up period might have masked intra-individual variation in the pattern of recovery.

In our study we investigated the determinants of FEV1 decline in subjects with occupational asthma, after diagnosis and during the follow-up period, when they were either fully or partially exposed to the specific sensitizer. We observed that baseline FEV1 was inversely related with its own decline, in agreement with what had already been published in asthmatic patients and in the general population (5,18). On the contrary, we found no significant relationship between bronchial hyperresponsiveness and FEV1 decline, in agreement with the observation that there was no difference between PD20FEV1 at diagnosis and at follow-up, nor did we confirm what observed in a previous paper (19) regarding the relationship between smoking habit or atopy and FEV1 decline.
Previous studies (20,21) have shown an association between high eosinophil levels and accelerated FEV1 decline in adult asthma patients, thereby postulating a role for eosinophils in the progression of the disease. On the other hand, Lemière et al (10) studied a sample of 24 subjects with occupational asthma removed from exposure after diagnosis, and identified a noneosinophilic phenotype that showed significant FEV1 decline, along with a lack of improvement in airway responsiveness during the four-year follow-up period after cessation of exposure. By contrast, the eosinophilic phenotype showed less marked FEV1 decline, significant decrease in ICS use over time and a trend toward improvement in airway responsiveness. Based on these findings, it is tempting to speculate that accelerated FEV1 decline in asthma may result from progressive airway remodeling process in which neutrophilic inflammation is also likely to play an important role.

In our study we found a significant relationship between baseline sputum eosinophil levels and FEV1 decline, suggesting that higher levels of inflammation at baseline may cause accelerated decline in FEV1. At our knowledge, no previous paper have considered this biomarker as possible determinants of the decline in FEV1 in patients with occupational asthma who continued to work. Broekema et al. (20) showed that asthmatic patients with accelerated FEV1 decline had high sputum eosinophil levels, but sputum was analyzed at the end of follow-up, and we therefore cannot exclude that sputum eosinophilic inflammation is not the cause but rather the consequence of accelerated FEV1 decline in this study. Unfortunately, in our experience, sputum analysis at the end of occupational exposure was available in only eight subjects.

As in the study by Lemière et al. (10), we found no significant relationship between ICS therapy and FEV1 decline. Noneosinophilic asthma may be less responsive to ICS treatment than eosinophilic asthma: although Dijkstra et al. found that oral or inhaled corticosteroids reduced FEV1 decline in asthma (22), some authors have suggested that the lack of eosinophilic inflammation may be a characteristic of refractory asthma (23). This fact may suggest the need for specific adjustment of asthma treatment according to the characteristics of airway inflammation.

Our study has some limitations. Firstly, the number of patients examined is relatively small. In effect several patients with a diagnosis of occupational asthma ceased work in the six months after diagnosis, or were lost at the first follow-up visit. However, apart few studies enrolling large number of patients, several other published studies have included similar number of patients (10). Secondly, the type of exposure (LMWC vs HMWC) was heterogeneous, but again this is frequently reported in many previous studies. Finally, the distinction between persistence and reduction of
exposure is not based on specific environmental measurements, but the majority of previous studies used the same rough distinction we did between patients who continued and patients who reduced occupational exposure to the specific sensitizer (reviewed in 24).

In conclusion, we demonstrated that in a well characterized group of patients with occupational asthma, sputum eosinophilia at diagnosis is one determinant of the accelerated decline in FEV1 when patients are still at work. Further long-term studies are required as to whether intensive ICS treatment may be beneficial for patients with occupational asthma and increased eosinophilic inflammation.
**FOOTNOTES**

**Contributorship Statement**

Contributors: DT, FN, conceived and designed the original paper. All authors were involved in amending protocol. DT coordinated the study throughout. Data entry was carried out by DT and FN. MB ans SC have analysed the biological determinants. EB, FC, FLD, AF, ML, LM, BV, AC dealt with the clinical assessment on patients. PP did the work supervision and the paper review.

**Data sharing:** No additional data available

**Competing Interests:** None

**Funding:** None


LEGENDS OF THE FIGURES

Figure 1. FEV1 decline according to baseline sputum eosinophilia

Figure 2. FEV1 decline according to level of exposure at follow-up
Sputum eosinophilia is a determinant of FEV1 decline in occupational asthma: results of an observational study

Donatella Talini, MD *, Federica Novelli, MD, Elena Bacci, MD, Marialaura Bartoli, PhD, Silvana Cianchetti, PhD, Francesco Costa, MD, Federico L Dente, MD, Antonella Di Franco, MD, Manuela Latorre, MD, Laura Malagrinò, MD, Barbara Vagaggini, MD, Alessandro Celi, MD, and Pierluigi Paggiaro, Prof

Department of Surgery, Medicine, Molecular Biology and Critical Care; University of Pisa; * CERIMP, Regione Toscana; Pisa, Italy

Correspondence: Donatella Talini
CERIMP, Regione Toscana, Dipartimento della Prevenzione ASL 5 di Pisa
Galleria Gerace 14, 56124, Pisa (Italy)
Tel. +39050954436, Fax +39050954454, E-Mail: d.talini@usl5.toscana.it

Key words: occupational – asthma, eosinophils, sputum, FEV1 decline

Word count: 2893
ABSTRACT

Objective: To evaluate the potential determinants of Forced Expiratory Volume in 1 second (FEV1) decline in workers with OA still exposed to the causative agent. We hypothesized that sputum eosinophilia might be a predictor of poor asthma outcome after diagnosis.

Setting, Design and Participants: In a specialist clinical center of the University Hospital of Pisa we studied 39 subjects (28 M, 11 F) diagnosed as having OA, routinely followed up between 1990 and 2009. They were a subgroup of 94 subjects diagnosed as affected by OA in that period: 9 had been removed from work at the diagnosis, 21 were excluded for having ceased occupational exposure after few months from diagnosis, and 25 were lost at the follow-up or had no acceptable sputum measurements at the diagnosis.

Estimates of the decline of FEV1 were obtained by means of simple regression analysis during the period of occupational exposure after diagnosis. Logistic regression was used to analyse the effects of factors (baseline FEV1 and sputum inflammatory cells, duration and type of exposure) that may potentially influence FEV1 decline.

Results: At follow-up (5.7+3.7 yrs), most subjects were still symptomatic despite ICS treatment and had their occupational exposure reduced. Subjects with higher sputum eosinophils (>3%) at baseline had significantly greater decline of FEV1 (−52.5 ml/yr vs -18.6 ml/yr, p=0.012). Logistic regression showed that persistent exposure and sputum eosinophilia were significantly associated with greater decline of FEV1 (OR 11.5, 95% CI 1.8-71.4, p=0.009 and OR 6.7, 95% CI 1.1-41.7, p= 0.042 respectively).

Conclusions: Sputum eosinophilia at diagnosis, together with the persistence of occupational exposure during follow-up, may contribute to greater decline in FEV1 in patients with OA still at work. Further long-term studies are required as to whether intensive ICS treatment may be beneficial for patients with occupational asthma and increased eosinophilic inflammation.

ARTICLE SUMMARY
Strengths and limitations of this study

- In our study we found a significant relationship between baseline sputum eosinophil levels and FEV1 decline, suggesting that higher levels of inflammation at baseline may cause accelerated decline in FEV1.

- No previous paper have considered this biomarker as possible determinants of the decline in FEV1 in patients with occupational asthma who continued to work.

- The number of patients examined is relatively small. However, apart few studies enrolling large number of patients, several other published studies have included similar number of patients.

- The type of exposure (LMWC vs HMWC) was heterogeneous, but again this is frequently reported in many previous studies.

- The distinction between persistence and reduction of exposure is not based on specific environmental measurements, but the majority of previous studies used the same rough distinction we did between patients who continued and patients who reduced occupational exposure to specific sensitizer.

Statements

- Not additional data available.

- This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

- All authors don't have any competing interest.
INTRODUCTION

Subjects with occupational asthma often experience permanent sequelae after removal from exposure, and the outcome of occupational asthma after diagnosis is often poor. A substantial body of data indicates that lower lung volumes, greater nonspecific bronchial hyperresponsiveness (NSBH) and stronger asthmatic response to specific inhalation challenge (SIC) at diagnosis are risk factors for the poor outcome of occupational asthma (1). Ideally, the worker should be removed from the work environment causing asthma, in order to prevent the deterioration of respiratory condition. However, complete cessation of exposure has high socio-economic consequences and is thus rarely feasible (2). Recent systematic literature research suggests that longer symptomatic exposure is associated with worse occupational asthma outcome, in terms of persistence of symptoms and NSBHR and greater decline in FEV1 (3,4). Some studies have evaluated the outcome of functional parameters after diagnosis (revised in 5). Two retrospective cohort studies documented accelerated FEV1 decline in patients with occupational asthma before removal from the causal agents (5, 6); in one of these studies (6), FEV1 continued to decline after removal from exposure, but at slower rate, similar to the rate of decline observed in healthy adults. Data about the effect of anti-asthma medication on symptoms and lung function in patients with persistent exposure are contradictory (6,7).

However, occupational asthma may have different outcomes, and it is unknown whether these differences depend upon the underlying inflammatory process and structural changes in the airways. Different phenotypic categories have been reported according to clinical or inflammatory characteristics, or to the triggers inducing or aggravating asthma (8). Eosinophilic and noneosinophilic phenotypes have been described (9). A recent study reported rapid decrease in eosinophilic inflammation after removal from exposure, but subjects with noneosinophilic asthmatic reaction during SIC seemed to have poorer prognosis than subjects with SIC-induced eosinophilic airway inflammation at diagnosis (10).

The aim of this study was to evaluate the possible determinants of FEV1 decline in workers with occupational asthma still exposed to the causative agent.
PATIENTS AND METHODS

We studied 39 subjects previously diagnosed as having occupational asthma (OA), routinely followed up at the Cardio-Thoracic and Vascular Department of the University Hospital of Pisa between 1990 and 2009. They were only a subgroup of the 94 subjects diagnosed as affected by OA in that period. Of the whole group, 9 subjects had been completely removed from work at the time of diagnosis, while 21 subjects were excluded for having ceased occupational exposure after few months from diagnosis, and 25 subjects were lost at the follow-up or had no acceptable sputum measurements at the diagnosis. There was no difference in the main clinical and functional data between 39 subjects included in this study and the other 46 subjects still at work at the time of the diagnosis and not included in this study (see Table 1 in the Appendix).

The diagnosis of OA was made at the first evaluation, according to the positive response to specific inhalation challenge (SIC) test: twenty-three subjects were sensitized to low molecular weight compounds (LMWC: isocyanates and persulfate salts) and 16 subjects to high molecular weight compounds (HMWC: flour dusts, wood dusts, latex and tobacco dusts).

All subjects were routinely evaluated every 6 months after diagnosis, while still exposed to the specific sensitizer at work. The duration of follow-up was 5.7±3.7 yrs.

At the first evaluation, all patients were interviewed about asthmatic symptoms and occupational exposure, and underwent spirometry and methacholine challenge test, prick test to common airborne allergens, collection of sputum for inflammatory cells count, and SIC test with the specific occupational agent.

At each follow-up examination, asthmatic symptoms, pharmacologic therapy, type of occupational exposure (persistent vs reduced exposure) and spirometric data were collected. Work exposure was considered as persistent when the patient continued working with the same job title and in the same environment, whereas it was considered as reduced when the patient had been relocated in another area of the same factory where the specific sensitizer was not used, with occasional short-term direct exposure to the specific sensitizer (11).

Antiasthma treatment was withdrawn 48 hrs before spirometry.

Pulmonary function tests, atopy and symptoms evaluation.

FEV1 and FVC were measured by a computerized water-sealed spirometer (Biomedin, Padova, Italy) using predicted values approved by the European Respiratory Society. The details of
methacholine challenge test have been reported previously (12). The cumulative dose of methacholine producing a 20% fall in FEV1 (PD20FEV1) was computed; a PD20FEV1 value <1000 µg of methacholine was considered as positive for NSBH.

**SIC was performed using two different methods:** a) for diisocyanates, subjects were exposed to vapours of toluenediisocyanate (TDI) or methylendiisocyanate (MDI), and the duration of the exposure was the same (30 min) for all subjects. FEV1 was measured before and immediately after exposure, then hourly for 8 hours (12); b) for dusts, subjects were asked to breath through a mouthpiece connected to a small box where a measured suspension of the dust was obtained (13). A positive response was defined as a decrease in FEV1 greater than 15% from baseline within the first hour (immediate response) or between the second and the 8th hour (late response), and in absence of a greater than 10% decrease in FEV1 during a control test performed with diluent (for diisocyanates) or with lactose dust (for other sensitizers).

Current asthma symptoms (more than 2 times/week) and antiasthma therapy (defined as regular use of inhaled corticosteroids) were recorded as qualitative parameters (yes / no) at each visit.

**Sputum induction and processing**

Sputum was induced according to European Respiratory Society Task Force recommendations (14). Hypertonic saline solution (NaCl 4.5%) was nebulized by means of an ultrasonic nebulizer (Ultraneb 2000, DeVilbiss, Somerset, Pa, USA) with 2.8 mL·min⁻¹ output, and was inhaled for three 5-min periods for up to 15 min. Every 5 min, after the start of nebulization, subjects were asked to rinse their mouth and throat carefully, to discard saliva, and to try to cough sputum into a container; FEV1 was then measured. Nebulization was stopped after 15 min or when FEV1 fell by ≥20% from baseline value. Saline-induced bronchoconstriction was promptly relieved by short-acting β2-agonist inhalation. Sputum samples were diluted with an equal volume of 0.1% dithiotreithol (Sputasol, Unipath; Basingstoke, UK). Samples were treated as previously reported (15). Macrophages, lymphocytes, neutrophils and eosinophils were expressed as percentage of total inflammatory cells, excluding squamous cells. The upper limit of normal range for sputum eosinophils was set at 3% as derived from a group of normal subjects, whereas high sputum neutrophils were defined when sputum neutrophil percentage was > 63% (16).
Statistical analysis

FEV1 is expressed as mean ± SD. PD20FEV1 is expressed as geometric mean and is log-transformed for comparisons. Sputum cell count are expressed as median (range). Simple regression analysis was used to provide estimates of the decline of FEV1 (expressed as annual average change in FEV1) during the period of occupational exposure. Categorical data were compared by using Pearson’s χ² statistics. Continuous data were compared using unpaired Student’s t-test or Mann-Whitney test.

Logistic regression was used to analyse the effects of potential factors measured at baseline (independent variables: baseline FEV1, PD20FEV1 methacholine, sputum eosinophils and neutrophils and other general or clinical characteristics such as atopy, smoking habit and duration of exposure) on the dependent variables (decline > -57.5 ml/yr, the lowest tertile of the distribution of single FEV1 declines). In the analysis we also included two variables that may potentially modify FEV1 decline during follow-up: a) persistence or reduction of occupational exposure to sensitizer (we considered as reduced the exposure to sensitizer for less than 100% but more than 50% of the follow-up period); b) ICS therapy (we considered as ICS-treated patients those who used ICS for more than 50% of the follow-up period including the last visit).

The results are given in terms of odds ratios (ORs) with 95% confidence intervals (95% CI).

Dependent and independent categorical variables were binary (high vs low sputum eosinophils, high vs low sputum neutrophils, hyperreactivity vs no hyperreactivity, persistence vs reduction of exposure, smoker and ex-smoker vs non-smoker, LMWC vs HMWC, ICS therapy vs no therapy), whereas continuous variables (age, baseline FEV1, duration of exposure) were transformed into categorical variables with the tertile distribution obtained for all patients; in particular, the highest tertile (for age, duration of exposure and time to removal) or the lowest tertile (for FEV1) were associated with the presence of the condition.

RESULTS

Table 1 shows the general characteristics of patients at the time of diagnosis. Low molecular weight compounds were the main agents causing OA, with isocyanates causing asthma in 19 patients (48%).
Table 1. General characteristics of the patients at the time of diagnosis

<table>
<thead>
<tr>
<th>Number</th>
<th>39</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yrs</td>
<td>43.5±11.8</td>
</tr>
<tr>
<td>Sex, M/F</td>
<td>28/11</td>
</tr>
<tr>
<td>Smoke, Y/Ex/No</td>
<td>3/20/16</td>
</tr>
<tr>
<td>Atopy, n (%)</td>
<td>13 (33.3)</td>
</tr>
<tr>
<td>Duration of exposure, yrs</td>
<td>18.6±11.1</td>
</tr>
<tr>
<td>Latency, yrs</td>
<td>12.8±10.9</td>
</tr>
</tbody>
</table>

Agents

<table>
<thead>
<tr>
<th>LMWC</th>
<th>23</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isocyanates</td>
<td>19</td>
</tr>
<tr>
<td>Persulfate salts</td>
<td>4</td>
</tr>
<tr>
<td>HMWC</td>
<td>16</td>
</tr>
<tr>
<td>Flour dusts</td>
<td>11</td>
</tr>
<tr>
<td>Wood dusts</td>
<td>2</td>
</tr>
<tr>
<td>Latex</td>
<td>2</td>
</tr>
<tr>
<td>Tobacco dusts</td>
<td>1</td>
</tr>
</tbody>
</table>

LMWC= low molecular weight compounds; HMWC=high molecular weight compounds

Clinical and functional findings of patients at the time of diagnosis and at the last visit of follow-up are reported in Table 2. At the time of diagnosis, all but two out of the 6 patients who had already reduced their occupational exposure to the specific sensitizer, had current asthma symptoms and NSBH, whereas mean FEV1 was normal and sputum eosinophilia was observed in less than 50% of patients.
Table 2. Clinical and functional findings at baseline and at the last visit of follow-up
(* p<0.001 )

<table>
<thead>
<tr>
<th></th>
<th>Diagnosis</th>
<th>Follow-up (5.7±3.7 yrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduction of exposure, n (%)</td>
<td>6 (15.4)</td>
<td>26 (66.7) *</td>
</tr>
<tr>
<td>FEV1, mL, mean ± SD</td>
<td>3.12±0.57</td>
<td>2.92±0.55 *</td>
</tr>
<tr>
<td>FEV1, % of predicted, mean ± SD</td>
<td>94.1±16.1</td>
<td>92.2±14.8</td>
</tr>
<tr>
<td>PD20FEV1, mcg, GM (SD)</td>
<td>227 (1437)</td>
<td>228 (1757)</td>
</tr>
<tr>
<td>NSBH, n (%)</td>
<td>33 (84.6)</td>
<td>28 (71.8)</td>
</tr>
<tr>
<td>ICS therapy, n (%)</td>
<td>14 (35.9)</td>
<td>36 (92.3) *</td>
</tr>
<tr>
<td>Patients with current symptoms, n (%)</td>
<td>32 (82.1)</td>
<td>17 (43.6) *</td>
</tr>
<tr>
<td>Sputum eosinophils %, median (range)</td>
<td>2.9 (0-43.1)</td>
<td>n.a.</td>
</tr>
<tr>
<td>Sputum eosinophils &gt;3%, n (%)</td>
<td>19 (48.7)</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

SD= standard deviation; FEV1= forced expiratory volume in the first second; GM= geometric mean; PD20FEV1= provocative dose of methacholine causing a 20% reduction of FEV1; NSBH= nonspecific bronchial hyperresponsiveness; ICS= inhaled corticosteroids

At follow-up, FEV1 (in L) was lower than that measured at diagnosis (p<0.001), but FEV1 expressed as % of predicted and PD20FEV1 methacholine were no different from baseline; also, the number of patients with NSBH was not significantly different between diagnosis and follow-up. At the time of diagnosis, 6 patients had already reduced their occupational exposure to the specific sensitizer, because they had been relocated to another job in the same factory with occasional, indirect exposure to the sensitizer. At follow-up, a further 20 patients had reduced their exposure to sensitizing agent. More patients were treated with inhaled corticosteroids (ICS) at the last visit of follow-up in comparison with baseline (p<0.001), but 43.6% still reported asthma symptoms, although generally of mild severity, and in lower percentage than at diagnosis (p<0.001). All these changes were similar between patients who had persistent or reduced exposure to the occupational sensitizer, except for PD20FEV1 increased in subjects who had reduced exposure.

Using simple regression analysis we provided estimates of the decline of FEV1 (expressed as annual average change in FEV1) during the period of occupational exposure in all subjects. The median annual decline in FEV1 in all subjects was -30.9 ml/yr (range: -188.3 ml/yr to +57.9 ml/yr). Using separate regression lines to separate periods of reduced exposure from periods of original...
exposure (available data only in 9 patients), the median estimate FEV1 slope during the period of persistent exposure was much higher than in the period of reduced exposure to the causative agent (-166.4 [-354.9, -57.7] vs -7.9 [-129.3, 55.8] mL/yr, p= 0.001). When we compared patients with different decline in FEV1 (according to the tertile distribution, from patients with lowest decline, < -16.9 ml/yr, to patients with highest decline, > -57.5ml/yr) as regards baseline characteristics, we did not observe any significant difference among different decliners except for baseline FEV1, which was significantly higher in patients with the highest decline (Table 3). The group with the lowest FEV1 decline included only patients with persistent exposure. Atopic patients were more represented in the group with the highest decline, although the difference was not significant.

Table 3. Characteristics of patients at baseline, grouped by FEV1 decline during the follow-up (*p<0.05)

<table>
<thead>
<tr>
<th></th>
<th>&lt;-16.9 ml/yr</th>
<th>-16.9 to -57.5 ml/yr</th>
<th>&gt;-57.5 ml/yr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>13</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Age, yrs, mean ± SD</td>
<td>43.8±12.7</td>
<td>44.2±11.9</td>
<td>42.4±11.6</td>
</tr>
<tr>
<td>Gender, M/F</td>
<td>10/3</td>
<td>8/5</td>
<td>10/3</td>
</tr>
<tr>
<td>Smoke, No/Ex/Yes</td>
<td>4/7/2</td>
<td>7/5/1</td>
<td>5/8/0</td>
</tr>
<tr>
<td>Atopy, n (%)</td>
<td>2 (15.4)</td>
<td>4 (30.8)</td>
<td>7 (53.8)</td>
</tr>
<tr>
<td>LMWC/HMWC</td>
<td>8/5</td>
<td>7/6</td>
<td>8/5</td>
</tr>
<tr>
<td>FEV1, % predicted, mean ± SD</td>
<td>84.8±15.2</td>
<td>97.4±14.3</td>
<td>100.9±14.6 *</td>
</tr>
<tr>
<td>PD20FEV1, mcg, GM (SD)</td>
<td>196</td>
<td>149</td>
<td>399</td>
</tr>
<tr>
<td>Bronchial hyperreactivity, n (%)</td>
<td>12 (92.3)</td>
<td>11 (84.6)</td>
<td>10 (76.9)</td>
</tr>
<tr>
<td>Reduction of exposure, n (%)</td>
<td>0</td>
<td>5 (38.5)</td>
<td>1 (7.7)</td>
</tr>
<tr>
<td>Sputum eosinophil %, median (range)</td>
<td>2.3 (0-15.5)</td>
<td>2.0 (0.43.1)</td>
<td>4.9 (0-24.4)</td>
</tr>
<tr>
<td>Sputum neutrophil %, median (range)</td>
<td>59.5 (30.8-86.7)</td>
<td>37.0 (11.1-85.0)</td>
<td>47.2 (19.4-96.0)</td>
</tr>
</tbody>
</table>

SD= standard deviation; LOWC=low molecular weight compounds; HMWC= high molecular weight compounds; FEV1= forced expiratory volume in the first second; GM= geometric mean; PD20FEV1= provocative dose of methacholine causing a 20% reduction of FEV1; ICS= inhaled corticosteroids

*p<0.05 among the three groups
Subjects with higher sputum eosinophils (>3%) had significantly greater decline in FEV1 (Figure 1) (p=0.012), as well as subjects with persistent exposure when compared to those who had reduced their exposure to the occupational sensitizer (Figure 2) (p= 0.03).

Using an univariate analysis, we estimated the decline in FEV1 (using as independent variable the highest tertile of FEV1 decline: -57.5 ml/yr) in relationship to baseline FEV1, baseline eosinophil (> 3% vs <3%) and neutrophil (> 63% vs < 63%) levels, level of exposure at follow-up (persistence vs reduction of exposure), smoking habit (ex or current smokers vs non smokers), molecular weight of the sensitizer (LMWC vs HMWC), duration of exposure and use of inhaled corticosteroids during the follow-up. Persistent exposure (OR: 6.7 (1.5-29.6), p=0.012), higher baseline sputum eosinophil levels (OR: 3.6 (0.8-14.8), p=0.07) and baseline FEV1 (% of predicted) (OR: 1.04 (0.99-1.1), p=0.07) significantly correlated with greater decline in FEV1. Table 4 shows results of a multivariate analysis when persistent exposure, higher baseline sputum eosinophil levels and baseline FEV1 (% of predicted) were used as independent variables. Persistent exposure and higher baseline sputum eosinophil levels significantly correlated with greater decline in FEV1.

Table 4. Results of the multivariate analysis on the main determinants of the FEV1 decline, including as independent variables only those variables significantly related to the FEV1 decline in the univariate analysis.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Odd ratios</th>
<th>95% C.I.</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lower</td>
<td>Upper</td>
</tr>
<tr>
<td>Persistent exposure vs reduction</td>
<td>12.7</td>
<td>1.8</td>
<td>90.8</td>
</tr>
<tr>
<td>Sputum eosinophilia &gt;= 3%</td>
<td>7.6</td>
<td>1.1</td>
<td>52.9</td>
</tr>
<tr>
<td>Baseline FEV1 (% of predicted)</td>
<td>1.06</td>
<td>0.99</td>
<td>1.13</td>
</tr>
</tbody>
</table>
DISCUSSION

The present study confirms that the prognosis of occupational asthma is poor in subjects who remain exposed to the sensitizing agent, as shown by the persistence of symptoms and airway hyperresponsiveness in many patients. Furthermore, it demonstrates for the first time that the highest rate of decline in FEV1 during the follow-up was associated with a high (>3%) sputum eosinophil count at the initial evaluation and with the persistence of exposure to the causal agent, suggesting that baseline eosinophilic airway inflammation may contribute, together with the persistence of occupational exposure, to a poor asthma outcome.

In 280 patients with red cedar asthma with persistent exposure, a greater rate of decline in FEV1 was observed in comparison with asymptomatic sawmill workers (5). Piriila et al. (17) reported a mean rate of decline of 40 mL/year in 91 selected subjects with isocyanate-induced occupational asthma, although only 12 of these continued to be exposed to the causative agent in the workplace during the period of follow-up. Anees studied 90 subjects undergoing FEV1 measurements at least once yearly before removal from exposure (6). In this study, FEV1 rapidly declined in exposed workers with occupational asthma; after removal from exposure, FEV1 continued to decline but at a slower rate, similar to the rate of decline observed in healthy adults. The nature of causative agent, current smoking, or treatment with inhaled corticosteroids did not seem to affect the rate of decline in FEV1. However, the authors could not estimate the decline in FEV1 any further because follow-up assessments after removal from exposure were less close and the model of linear decline following the step-up period might have masked intra-individual variation in the pattern of recovery.

In our study we investigated the determinants of FEV1 decline in subjects with occupational asthma, after diagnosis and during the follow-up period, when they were either fully or partially exposed to the specific sensitizer. We observed that baseline FEV1 was inversely related with its own decline, in agreement with what had already been published in asthmatic patients and in the general population (5,18). On the contrary, we found no significant relationship between bronchial hyperresponsiveness and FEV1 decline, in agreement with the observation that there was no difference between PD20FEV1 at diagnosis and at follow-up, nor did we confirm what observed in a previous paper (19) regarding the relationship between smoking habit or atopy and FEV1 decline.
Previous studies (20,21) have shown an association between high eosinophil levels and accelerated FEV1 decline in adult asthma patients, thereby postulating a role for eosinophils in the progression of the disease. On the other hand, Lemière et al (10) studied a sample of 24 subjects with occupational asthma removed from exposure after diagnosis, and identified a noneosinophilic phenotype that showed significant FEV1 decline, along with a lack of improvement in airway responsiveness during the four-year follow-up period after cessation of exposure. By contrast, the eosinophilic phenotype showed less marked FEV1 decline, significant decrease in ICS use over time and a trend toward improvement in airway responsiveness. Based on these findings, it is tempting to speculate that accelerated FEV1 decline in asthma may result from progressive airway remodeling process in which neutrophilic inflammation is also likely to play an important role.

In our study we found a significant relationship between baseline sputum eosinophil levels and FEV1 decline, suggesting that higher levels of inflammation at baseline may cause accelerated decline in FEV1. At our knowledge, no previous paper have considered this biomarker as possible determinants of the decline in FEV1 in patients with occupational asthma who continued to work. Broekema et al. (20) showed that asthmatic patients with accelerated FEV1 decline had high sputum eosinophil levels, but sputum was analyzed at the end of follow-up, and we therefore cannot exclude that sputum eosinophilic inflammation is not the cause but rather the consequence of accelerated FEV1 decline in this study. Unfortunately, in our experience, sputum analysis at the end of occupational exposure was available in only eight subjects.

As in the study by Lemière et al. (10), we found no significant relationship between ICS therapy and FEV1 decline. Noneosinophilic asthma may be less responsive to ICS treatment than eosinophilic asthma: although Dijkstra et al. found that oral or inhaled corticosteroids reduced FEV1 decline in asthma (22), some authors have suggested that the lack of eosinophilic inflammation may be a characteristic of refractory asthma (23). This fact may suggest the need for specific adjustment of asthma treatment according to the characteristics of airway inflammation.

Our study has some limitations. Firstly, the number of patients examined is relatively small. In effect several patients with a diagnosis of occupational asthma ceased work in the six months after diagnosis, or were lost at the first follow-up visit. However, apart few studies enrolling large number of patients, several other published studies have included similar number of patients (10). Secondly, the type of exposure (LMWC vs HMWC) was heterogeneous, but again this is frequently reported in many previous studies. Finally, the distinction between persistence and reduction of
exposure is not based on specific environmental measurements, but the majority of previous
studies used the same rough distinction we did between patients who continued and patients who
reduced occupational exposure to the specific sensitizer (reviewed in 24).

In conclusion, we demonstrated that in a well characterized group of patients with
occupational asthma, sputum eosinophilia at diagnosis is one determinant of the accelerated
decline in FEV1 when patients are still at work. Further long-term studies are required as to
whether intensive ICS treatment may be beneficial for patients with occupational asthma and
increased eosinophilic inflammation.
REFERENCES


FEV1 decline according to baseline sputum eosinophilia

210x297mm (300 x 300 DPI)
FEV1 decline according to level of exposure at follow-up

210x297mm (300 x 300 DPI)
## Appendix

Table 1A: Main characteristics of the subjects included in the present study in comparison with the remaining 46 subjects still at work at diagnosis but not included in the study for several reasons (reported in the text).

<table>
<thead>
<tr>
<th></th>
<th>Subjects included (N=39)</th>
<th>Subjects not included (N=46)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yrs, mean ±SD</td>
<td>43.5±11.8</td>
<td>40.2±12.2</td>
</tr>
<tr>
<td>Gender, M/F</td>
<td>28/11</td>
<td>35/11</td>
</tr>
<tr>
<td>High molecular weight compound, n (%)</td>
<td>14 (35.9)</td>
<td>19 (41.3)</td>
</tr>
<tr>
<td>Duration of exposure, yrs, mean ± SD</td>
<td>18.6±11.1</td>
<td>16.8±12.9</td>
</tr>
<tr>
<td>Reduction of exposure during f.u., yrs, n (%)</td>
<td>6 (15.4)</td>
<td>13 (28.3)</td>
</tr>
<tr>
<td>FEV1, lt, mean ±SD</td>
<td>3.12±0.57</td>
<td>3.27±0.82</td>
</tr>
<tr>
<td>FEV1, % of pred, mean±SD</td>
<td>94.1±16.1</td>
<td>92.0±15.9</td>
</tr>
<tr>
<td>PD20FEV1, mcg, GM (SD)</td>
<td>227 (1437)</td>
<td>195 (1270)</td>
</tr>
<tr>
<td>ICS therapy, n (%)</td>
<td>14 (39.5)</td>
<td>25 (54.3)</td>
</tr>
<tr>
<td>Sputum eosinophil %, median (range)</td>
<td>2.9 (0-43.1)</td>
<td>1.6 (0-26.5)</td>
</tr>
</tbody>
</table>

SD= standard deviation; f.u.= follow-up; FEV1= forced expiratory volume in the first second; GM= geometric mean; PD20FEV1= provocative dose of methacholine causing a 20% reduction of FEV1; ICS= inhaled corticosteroids
### STROBE Statement—checklist of items that should be included in reports of observational studies

<table>
<thead>
<tr>
<th>Item No</th>
<th>Recommendation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Title and abstract</strong></td>
<td></td>
</tr>
<tr>
<td>1 (V)</td>
<td>(a) Indicate the study’s design with a commonly used term in the title or the abstract</td>
</tr>
<tr>
<td>(a) pg. 1</td>
<td>(b) Provide in the abstract an informative and balanced summary of what was done and what was found</td>
</tr>
<tr>
<td>2 (V)</td>
<td>Explain the scientific background and rationale for the investigation being reported</td>
</tr>
<tr>
<td><strong>Introduction</strong></td>
<td></td>
</tr>
<tr>
<td>3 (V)</td>
<td>State specific objectives, including any prespecified hypotheses</td>
</tr>
<tr>
<td><strong>Methods</strong></td>
<td></td>
</tr>
<tr>
<td>4 (V)</td>
<td>Present key elements of study design early in the paper</td>
</tr>
<tr>
<td>5 (V)</td>
<td>Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection</td>
</tr>
<tr>
<td>6 (V)</td>
<td>(a) Cohort study—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up</td>
</tr>
<tr>
<td>(a) pg. 5-6</td>
<td>Case-control study—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls</td>
</tr>
<tr>
<td>(a) Cohort study—For matched studies, give matching criteria and number of exposed and unexposed</td>
<td></td>
</tr>
<tr>
<td>(b) Case-control study—For matched studies, give matching criteria and the number of controls per case</td>
<td></td>
</tr>
<tr>
<td><strong>Participants (V)</strong></td>
<td></td>
</tr>
<tr>
<td>7 (V)</td>
<td>Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable</td>
</tr>
<tr>
<td><strong>Variables (V)</strong></td>
<td></td>
</tr>
<tr>
<td>8 (V)</td>
<td>For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group</td>
</tr>
<tr>
<td>9 (V)</td>
<td>Describe any efforts to address potential sources of bias</td>
</tr>
<tr>
<td><strong>Data sources/measurement (V)</strong></td>
<td></td>
</tr>
<tr>
<td>10 (V)</td>
<td>Explain how the study size was arrived at</td>
</tr>
<tr>
<td><strong>Quantitative variables (V)</strong></td>
<td></td>
</tr>
<tr>
<td>11 (V)</td>
<td>Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why</td>
</tr>
<tr>
<td><strong>Statistical methods (V)</strong></td>
<td></td>
</tr>
<tr>
<td>12 (V)</td>
<td>(a) Describe all statistical methods, including those used to control for confounding</td>
</tr>
<tr>
<td>pg. 7</td>
<td>(b) Describe any methods used to examine subgroups and interactions</td>
</tr>
<tr>
<td>13 (V)</td>
<td>(c) Explain how missing data were addressed</td>
</tr>
</tbody>
</table>
(d) Cohort study—If applicable, explain how loss to follow-up was addressed
Case-control study—If applicable, explain how matching of cases and controls was addressed
Cross-sectional study—If applicable, describe analytical methods taking account of sampling strategy

(e) Describe any sensitivity analyses
## Results

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Participants (V)</td>
<td>(NA)</td>
</tr>
<tr>
<td>Pg. 7, 8, 9, 10</td>
<td>(NA)</td>
</tr>
<tr>
<td></td>
<td>(NA)</td>
</tr>
<tr>
<td></td>
<td>(NA)</td>
</tr>
<tr>
<td>Descriptive (V) data pg. 8</td>
<td>(NA)</td>
</tr>
<tr>
<td></td>
<td>(NA)</td>
</tr>
<tr>
<td>Outcome data (V) pg. 8</td>
<td>(NA)</td>
</tr>
<tr>
<td></td>
<td>(NA)</td>
</tr>
<tr>
<td>Main results (V) pg. 9-10, Fig. 1-2</td>
<td>(NA)</td>
</tr>
<tr>
<td></td>
<td>(NA)</td>
</tr>
<tr>
<td>Other analyses (V) pg. 10</td>
<td>(NA)</td>
</tr>
<tr>
<td></td>
<td>(NA)</td>
</tr>
</tbody>
</table>

### 13* (a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed

### 13* (b) Give reasons for non-participation at each stage

### 13* (c) Consider use of a flow diagram

### 14* (a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders

### 14* (b) Indicate number of participants with missing data for each variable of interest

### 14* (c) Consider use of a flow diagram

### 15* (a) Cohort study—Report numbers of outcome events or summary measures over time

### 15* (b) Case-control study—Report numbers in each exposure category, or summary measures of exposure

### 15* (c) Cross-sectional study—Report numbers of outcome events or summary measures

### 16* (a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included

### 16* (b) Report category boundaries when continuous variables were categorized

### 16* (c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period

### 17 Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses

### Discussion

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Key results (V) pg. 10, 11, 12</td>
<td>(NA)</td>
</tr>
<tr>
<td>Limitations (V) pg. 12</td>
<td>(NA)</td>
</tr>
<tr>
<td>Interpretation (V) pg. 11-12</td>
<td>(NA)</td>
</tr>
<tr>
<td>Generalisability (V) pg. 12-13</td>
<td>(NA)</td>
</tr>
</tbody>
</table>

### 18 Summarise key results with reference to study objectives

### 19 Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias

### 20 Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence

### 21 Discuss the generalisability (external validity) of the study results

### Other information

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Funding (NA) 22</td>
<td>(NA)</td>
</tr>
</tbody>
</table>

### 22 Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based

---

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.