RESPIRATORY MEDICINE | RESEARCH ARTICLE

Quantitative assessment of specific serum IgGs may verify source of environmental exposure in extrinsic allergic alveolitis (EAA)

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Abstract: Assessment of humoral response to inhalation antigens is currently the most frequently used method to confirm exposure. It may be useful in extrinsic allergic alveolitis (EAA) patients, especially in cases that are unaware of the source of exposure. However, commercially available test may not include relevant antigens, which may lead to false negativity of the test. We proposed that testing patient serological responses to antigens from respectively environments might be useful in showing the relevance of these exposures. Ten patients diagnosed with EAA were included in the case-control study. Samples from potentially harmful environments were collected, and antigenic extracts were prepared and used for enzyme-linked immunosorbent assays (ELISA) to investigate serological responses to suspected antigens. Plasma samples of unexposed volunteers were used as controls. The results were interpreted in the context of other clinical findings when known (e.g., radiologic patterns, bronchoalveolar lavage fluid findings, histology results) and patient history. We suggest that environmental sampling may provide more information than previous history assessment and commercially available specific IgGs tests and helps to either reveal hidden exposures or find relevant exposure in cases with multiple potential sources. The results of these tests must be interpreted carefully in the context of other clinical data.

ABOUT THE AUTHOR
Our group is focused on interstitial lung diseases, namely extrinsic allergic alveolitis and idiopathic pulmonary fibrosis. The presented project concerns a very important part of our research devoted to environmental exposure and its effect on immune response and disease course modulation. Closely related are projects involving genetic predisposition to abnormal reaction of immune cells and respiratory system to inhalation antigens. A better understanding of factors predisposing to interstitial lung diseases, what drives the immune response and which factor predicts patients outcomes, would help us to choose a proper therapeutic approach.

PUBLIC INTEREST STATEMENT
Interstitial lung diseases including extrinsic allergic alveolitis are thought to share both genetic predisposition and environmental challenge. In patients with mainly inflammatory presentation of extrinsic allergic alveolitis, exposure might be the driver of the disease. While source of exposure is obvious in most of our patients, there is still no negligible group where we are not able to detect causative inhalation antigen. Taken into account that avoiding exposure remains the first and most important take-home message a patient should take away from a medical appointment, new methods how to find the source of exposure in these “hidden” cases must be sought. Presented study shows a possible way, how to approach to antigen detection in patients with unknown source of exposure.
**Subjects:** Interstitial lung diseases; environmental exposure; occupational medicine; antigens; antigen detection methods

**Keywords:** extrinsic allergic alveolitis; exposure; antigen; immunoglobulin G; humoral response

### 1. Introduction

Currently, there are no internationally accepted guidelines for the diagnosis of extrinsic allergic alveolitis (EAA). However, most authors define EAA as interstitial lung involvement evolving in susceptible individuals after repeated exposure to mostly organic inhalation antigens. Despite this definition, the source of an inhalation antigen cannot be found in 20% of EAA patients (Hanak et al., 2007). There are several ways to test whether the patient was exposed to environmental challenge. Based on the proposed pathogenetic events leading to EAA development, one of the most convenient and safe ways to determine environmental challenge is to test the humoral response using serum-specific immunoglobulin G (IgG) concentrations (Sterclova et al., 2011).

It is crucial to remember that testing specific serum IgGs has a high burden of nonspecific results. There are no physiological concentrations that can be used, and obtaining a low concentration in individual patients might be because we do not test the response to the proper antigen.

Patients with more potentially relevant sources of exposure may also present difficulties even though according to our knowledge, there are no large clinical studies aimed at the history of possible sources of exposure in consecutive EAA patients. Our experience shows that some patients claim more than one exposure. It was found that at least some orthologous immunogenic proteins of inhalation antigens possess common epitopes (Rouzet et al., 2017); however, these epitopes have been described only in several antigens thus far. Currently, if EAA patients claim more potential sources of exposure, we are not sure whether all of them are clinically relevant and therefore should be avoided.

These challenges of identifying possible sources of exposure in EAA patients led us to hypothesize that the assessment of patients’ serological responses to antigens from different environments with either unknown or multiple potential sources of exposure might be useful for showing if all of them are clinically relevant.

Innovative potential of the presented method consists in more appropriate and thus more accurate use of antigens used for assessment of humoral response. Even though more arduous compared to commercially available tests, the presented method may give relevant results dealing with patient-specific environment and antigen mixtures in it. We recommend the use of the presented approach in patients with nonfibrotic EAA and multiple possible sources of inhalation antigens triggering their disease, as elimination of further exposure has the highest clinical significance in this group.

### 2. Materials and methods

#### 2.1. Patients

Ten patients diagnosed with EAA in the past with unrevealed or multiple sources of exposure to organic inhalation antigens were enrolled in the case-control pilot study after they signed an informed consent form. The study was approved by the ethical committee of Thomayer Hospital and Institute for Clinical and Experimental Medicine in 2016. The study of flowchart is summarized in Figure 1. All patients underwent a history assessment, physical examination, blood tests including screening for autoimmune diseases, lung function tests, high-resolution computed tomography (HRCT) of the chest, bronchoscopy with bronchoalveolar lavage (BAL) and transbronchial biopsy. Cases were discussed at a multidisciplinary team meeting, and the patients for
whom a diagnosis could not be established with known data underwent either a surgical lung biopsy or cryobiopsy. These cases were discussed at another multidisciplinary meeting to confirm the final diagnosis. When needed, the diagnosis of EAA was based on a history of exposure to organic inhalation antigens or laboratory proof of exposure (e.g., serum-specific immunoglobulin G), BAL fluid (BALF) lymphocytosis, and HRCT and histology findings compatible with EAA (Vasakova et al., 2017). Plasma samples from healthy volunteers (V1 = 39 years old, female, nonsmoker; V2 = 30 years old, male, nonsmoker) with no history of significant environmental exposures were used as controls.

2.2. Suspected source material collection
A manual for suspected material collection was created. Suspected source material was collected by the patient's spouses, as patients were instructed not to collect the material themselves because it may cause deterioration of their disease. Sterile containers were provided for each sample. Patients with an unknown source of exposure were asked to collect samples from these locations: flower beds,
evaporator, refrigerator, bedding, carpets, and upholstered furniture. If there was any other suspected or possible source of exposure or multiple sources of possible exposure, especially involving molds, bird feathers/droppings, bacteria, or a farming environment, patients were asked to collect these suspected samples as well and bring them to our department the next day.

2.3. Preparation of antigenic extracts and measurement of protein content

Samples were frozen at −80°C for storage. Antigenic extracts were prepared according to previously published studies (Ojanguren et al., 2015) and lyophilized (Telstar Cryodos 80, Telstar, Spain). Five milligrams of lyophilized extract was dissolved in 100 µl of deionized water. A BCA protein Assay Kit (Pierce, Rockford, IL) was used to assess protein concentration in lyophilized samples according to the manufacturer’s instructions.

2.4. Custom ELISA of antigenic extracts and patient plasma samples

According to the protein concentrations assessed in the previous step, amine-binding buffer was mixed with the antigenic extract solution prepared for previous analysis; the final concentration of protein in the binding buffer (Na2CO3/NaHCO3 0.2 M; pH 9.6) was 12.5 µg/ml. Two hundred microliters of binding buffer and antigen extract solution were needed for one microplate well. For each antigenic extract, 4 wells were precoated (2 wells to test patient serum reactivity and 2 for control serum). Precoated plates were incubated at +4°C for 16 hours. After three washes with wash buffer (phosphate-buffered saline (PBS)/Tween-20), plates were incubated for 1 hour at 37°C with blocking buffer (PBS/1% bovine serum albumin (BSA); 300 µl/well), washed three times and incubated for 1 hour at 37°C with diluted sera of patients and controls (serum dilution 1:1000 in PBS/0.1% gelatin/0.02% Tween-20; 200 µl/well). Following another washing step (3 times), 200 µl of anti-human IgG horseradish peroxidase (HRP) (Clone JDC-10:1:1000) was added, and plates were incubated for 1 hour at 37°C and washed again 3 times. Two hundred microliters of tetramethylbenzidine (TMB) solution (acetate buffer pH 5.5/H2O2/TMB) was added to each well. The reaction was terminated after 20 minutes of incubation in darkness at 25°C by adding 60 µl of stop solution (sulfuric acid). Absorbance was measured at 450 nm using a plate reader.

2.5. Evaluation of results

The absorbance of the control samples was regarded as 100%, and the percentage increase/decrease in the absorbance of the patient samples was calculated. The results of individual patient serum reactivity were evaluated in the context of other clinical and laboratory data (e.g., BAL lymphocyte percentages, HRCT pattern, repeated history of possible source of exposure, total serum IgG concentrations and serum concentrations of specific IgGs). Each case was evaluated on an individual basis. Concentrations of specific serum IgGs were evaluated as previously published (Sterclova et al., 2011).

3. Results

Source of samples from the patients’ environments, the protein concentration in each sample, ELISA results (optical density of antigenic extract vs. patient plasma (OD sample) and optical density of antigenic extract vs. control plasma (OD controls)) are summarized in Table 1.

3.1. Case reports

Demographic data, BALF cell profiles, HRCT patterns, histology patterns, both total serum IgG and specific IgG concentrations and individual histories of exposure to all possible and unsure sources are listed in Table 2. Maximal serological reactivities to tested environmental sources are listed in the table as well.

4. Discussion

The present study focuses on EAA patients with either an unknown source or multiple sources of inhalation antigens exposure. It shows that assessing the serological response to mixture of antigens from the expected source may be more useful than assessing concentrations of specific
| Sample | Protein concentration (µg/ml) | Mean OD Sample | Mean OD Control | OD sample/ control *100 (%) | Antigen source |
|--------|-------------------------------|----------------|----------------|-----------------------------|----------------|
| 1      | 2000                          | 2.6            | 2.8265         | 92                          | Dust, carpet, flat |
| 2      | 2000                          | 2.4575         | 2.265          | 108                         | Dust, upholstered furniture, cottage |
| 3      | 1000                          | 3.1015         | 2.7415         | 113                         | Soil, flower pots, flat |
| 4      | 800                           | 1.1125         | 2.779          | 40                          | Swimming pool, cottage |
| 5      | 360                           | 2.224          | 2.779          | 80                          | Evaporator, flat |
| 6      | 40                            | 1.403          | 1.4395         | 97                          | Refrigerator, flat |
| 7      | 93                            | 1.659          | 1.663          | 100                         | Refrigerator, cottage |
| 8      | 1000                          | 2.729          | 3.0725         | 89                          | Flower bed, cottage |
| 9      | 450                           | 1.395          | 0.5235         | 266                         | Garage |
| 10     | 2000                          | 2.8875         | 3.1215         | 93                          | Workshop |
| 11     | 810                           | 1.2195         | 0.7685         | 159                         | Shower |
| 12     | 80                            | 1.0035         | 0.7            | 143                         | Bathroom |
| 13     | 40                            | 0.2675         | 0.1975         | 135                         | Bedroom |
| 14     | 2000                          | 1.998          | 0.8205         | 244                         | Poultry house |
| 15     | 2000                          | 2.7555         | 2.675          | 103                         | Stable |
| 16     | 2000                          | 2.641          | 0.6175         | 428                         | Dust, office, workshop |
| 17     | 900                           | 2.459          | 2.246          | 109                         | Bedding, home |
| 18     | 2000                          | 2.111          | 1.6595         | 127                         | Dust around laptop—home |
| 19     | 950                           | 2.47           | 2.433          | 102                         | Refrigerator—home |
| 20     | 870                           | 2.2675         | 1.2275         | 185                         | Workshop—frant |
| 21     | 1000                          | 2.3555         | 0.7495         | 314                         | Workshop—door in the middle |

(Continued)
| Sample | Protein concentration (µg/ml) | Mean OD Sample | Mean OD Control | OD sample/control *100 (%) | Antigen source |
|--------|-----------------------------|----------------|----------------|--------------------------|----------------|
| 22     | 1800                        | 2.659          | 0.5245         | 507                      | Skin sebum—sphynx cat |
| 23     | 550                         | 2.362          | 1.432          | 165                      | Gap, bathroom—home |
| 24     | 2000                        | 2.3685         | 0.7735         | 306                      | Workshop floor |
| 25     | 500                         | 3              | 1.0425         | 288                      | Workshop toilets |
| 26     | 2000                        | 2.263          | 1.1145         | 203                      | Bedroom—bedding |
| 27     | 1100                        | 2.1455         | 1.525          | 141                      | Flowers in room |
| 28     | 850                         | 2.998          | 1.3545         | 221                      | Mold above windows |
| 29     | 2000                        | 2.365          | 1.6215         | 146                      | Upholstered furniture—chair, couch |
| 30     | 1700                        | 2.981          | 0.9075         | 328                      | Washed inhalator |
| 31     | 600                         | 3              | 2.9965         | 100                      | Workshop—production of wooden door frames and door covers |
| 32     | 310                         | 3              | 1.744          | 172                      | Office—upholstering |
| 33     | 310                         | 2.2865         | 0.945          | 242                      | Air cleaner filter—home (ionic care) |
| 34     | 680                         | 2.712          | 2.5535         | 106                      | Carpet—under the table, living room |
| 35     | 95                          | 2.378          | 2.5555         | 93                       | Upholstered living room |
| 38     | 1600                        | 2.503          | 1.3885         | 180                      | Finished products—warehouse |
| 39     | 2000                        | 2.789          | 2.2515         | 124                      | Soil from flower pots—home |
| 40     | 50                          | 1.8155         | 1.053          | 172                      | Surface of furniture—office |
| 41     | 1300                        | 2.3815         | 1.8405         | 129                      | Bathroom + shower |
| 42     | 2000                        | 2.7045         | 2.7935         | 97                       | Duvet + blanket |

(Continued)
| Sample | Protein concentration (µg/ml) | Mean OD Sample | Mean OD Control | OD sample/control *100 (%) | Antigen source |
|--------|-----------------------------|----------------|----------------|---------------------------|----------------|
| 43     | 2000                        | 1.995          | 2.839          | 70                        | Garden + house exterior |
| 44     | 2000                        | 1.708          | 2.49           | 69                        | Flower pots—indoor |
| 45     | 2000                        | 0.6625         | 1.9115         | 35                        | Mattress (under the bed) |
| 46     | 1100                        | 2.0975         | 1.2975         | 162                       | Job—Inside, outside |
| 48     | 450                         | 2.856          | 2.9755         | 96                        | Dust from fan coil filter—bedroom |
| 49     | 2000                        | 3              | 3              | 100                       | Dust—bedroom floor |
| 50     | 1500                        | 2.8325         | 2.5405         | 111                       | CPAP machine |
| 51     | 2000                        | 2.8125         | 2.089          | 135                       | Room flowers |
| 52     | 1300                        | 3              | 2.976          | 101                       | Indoor stainless steel swimming pool |
| 53     | 610                         | 2.942          | 2.539          | 116                       | Outdoor whirlpool |
| 55     | 2000                        | 2.462          | 1.533          | 161                       | Job—Dust from the fan coil air filter |
| 56     | 2000                        | 2.762          | 1.888          | 146                       | Bedroom—plaster |
| 57     | 2000                        | 2.552          | 0.6265         | 407                       | Garage—plaster |
| 58     | 2000                        | 2.5235         | 2.39           | 106                       | Bedroom mattress |
| 59     | 80                          | 2.34           | 0.118          | 1983                      | Cellar—plaster |
| 60     | 1060                        | 2.5235         | 1.5775         | 160                       | Soil from flower pots |
| 61     | 580                         | 2.5115         | 1.0125         | 248                       | Parrot |
| 62     | 550                         | 2.5245         | 1.976          | 128                       | Bathroom—washbasin faucet |
| 63     | 720                         | 1.249          | 0.6735         | 185                       | Dust from personal computer |
| 64     | 650                         | 2.5785         | 0.9275         | 278                       | Working table |
| Sample | Protein concentration (µg/ml) | Mean OD Sample | Mean OD Control | OD sample/control *100 (%) | Antigen source |
|--------|-------------------------------|----------------|----------------|-----------------------------|----------------|
| 65     | 180                           | 2.5095         | 0.9465         | 265                         | Upper couch cloth |
| 66     | 140                           | 2.3895         | 1.9365         | 123                         | Bed—duvet, blanket, sheet |
| 67     | 180                           | 2.15           | 1.207          | 178                         | Refrigerator |
| 68     | 250                           | 2.661          | 1.2755         | 209                         | Cage with ferrets |
| 69     | 1000                          | 2.1785         | 0.4025         | 541                         | Carpet |
| 70     | 120                           | 1.411          | 1.344          | 105                         | Refrigerator |
| 72     | 2000                          | 0.9195         | 0.9665         | 95                          | Car—cabin air filter |
| 73     | 55                            | 0.0905         | 0.08           | 113                         | Bedroom—plaster |
| 74     | 1000                          | 2.312          | 2.451          | 94                          | Living room couch |
| 75     | 2000                          | 1.0925         | 1.22           | 90                          | Dust—bedroom |
| 76     | 2000                          | 1.012          | 1.1045         | 92                          | Bedroom |
| 77     | 250                           | 0.281          | 0.2895         | 97                          | Office—plaster |
### Table 2. Patient data (sex, age, smoking history), BAL findings (cell count + CD4/CD8), total serum IgG and specific IgG concentrations, histology results, HRCT patterns, and exposure evaluations

| Patient (Number, gender, age (years), smoking history) | BALT (AM, PMN, Eos, Ly (%) | IgG (g/l) (total; specific) | Histology | HRCT | Exposures | Evaluation of serological response |
|-------------------------------------------------------|-----------------------------|-----------------------------|-----------|------|-----------|-----------------------------------|
| 1, F, 65, nonsmoker                                    | 67; 5; 17; 11; 1.85         | 9.55; nonsignificant        | NSIP, no granulomas | NSIP | Air conditioner, swimming-pool    | No convincing serological response to tested environmental sources found |
| 2, M, 66, ex-smoker                                   | 50; 30; 2; 18; 0.55         | 12.5; high concentration mites, molds, bird feathers | Fibrosis, emphysema, no granulomas | CPFE | Electrainingal—construction work (exposure to dust due to masonry) | Maximal serological response—poultry house, garage |
| 3, M, 62, nonsmoker                                   | 60; 5; 0; 35; 3.05          | 12.0; NA                    | NA        | NSIP | Metal production—steel, stainless steel, welding, brewery | Maximal serological response—workshop floor, workshop office, cat skin sebum |
| 4, F, 66, nonsmoker                                   | 40; 0; 0; 60; 0.18          | NA; mites, molds, bird feathers | NA       | EAA  | Molds in office, geese in neighborhood | Maximal serum reactivity—mold above windows, inhalator |
| 5, F, 43, nonsmoker                                   | 98; 2; 0; 0; 1.78           | 11.4; mites, molds          | NSIP + vaguely formed epithelioid granulomas | NSIP | Feather duvets, hamster, chipboard dust | Maximal serological response—air filter at home, storage of finished products at work |
| 6, M, 40, nonsmoker                                   | 34; 10; 3; 53; 2.5          | 7.95; mites, feathers       | Vaguely formed epithelioid granulomas | EAA  | Bird feathers, agriculture machines | Maximal serological reactivity—work inside, outside |
| 7, M, 56, nonsmoker                                   | 42; 7; 3; 48; 0.54          | NA, molds                   | Vaguely formed epithelioid granulomas | EAA  | Air conditioner, scuba, whirlpool, swimming pool, parrot | Maximal serological response—air cleaner fan coil filter at work |
| 8, F, 49, nonsmoker                                   | 40; 22; 0; 38; 1.5          | 22.5; bird feathers         | NA        | EAA  | Molds, parrot, air conditioner    | Maximal serological reactivity—parrot, garage plaster |

(Continued)
| Patient (Number, gender, age (years), smoking history) | BALT (AM, PMN, Eos, Ly (%), CD4/CD8) | IgG (g/l) (total; specific) | Histology | HRCT | Exposures | Evaluation of serological response |
|------------------------------------------------------|-------------------------------------|----------------------------|-----------|------|-----------|----------------------------------|
| 9, M, 69, ex-smoker                                  | 59; 12; 8; 2; 21; 1.0               | NA; nonsignificant          | NA        | NSIP | Chinchillas, ferrets, molds       | Maximal serological reactivity—working table, carpet |
| 10, F, 43, nonsmoker                                 | 80; 8; 2; 10; 1.6                   | NA; NA                     | NA        | NSIP | Shop assistant—tea, nuts, herbs  | No convincing serological response |

F—female, M—male, AM alveolar macrophages, PMN polymorphonuclear cells; Eos—eosinophils, Ly—lymphocytes, IgG—immunoglobulin G, NA—not assessed, NSIP—nonspecific interstitial pneumonitis, CPFE—combined pulmonary fibrosis and emphysema, EAA—extrinsic allergic alveolitis.
IgGs with commercially available kits. Although this approach might be helpful in some cases, there are still many points for discussion.

It is widely admitted that EAA may radiologically retain the phenotype of usual interstitial pneumonia (UIP) or nonspecific interstitial pneumonia (NSIP) patterns without typical centrilobular granulomas (Bhattacharyya et al., 2018). In these patients, namely, those with uncertain antigenic exposure, we may diagnostically hesitate between EAA and idiopathic interstitial pneumonias. This situation is documented in cases No. 1 and 10, in which there may have been an exposure in the patient’s environment, but the BALF results were not suggestive of EAA (low lymphocyte counts), and no granulomas were found in the histology specimen or with HRCT. Samples from suspected environments were collected, and negative serological responses, together with the abovementioned areas of uncertainty, led us to designate both cases as non-EAA. Recent studies prioritize surgical lung biopsy to cryobiopsy (preferred at our site) in inconclusive cases (Bondue et al., 2020), which may help to refine diagnosis.

In contrast, in most patient environments, more possible sources of antigens were detected (e.g., Patient No. 9: exposure to ferrets, chinchillas and possibly molds). Specific IgGs were low, and serological responses to material obtained from environmental sources indicated the suspected antigens (i.e., Patient No. 9: mold samples to which serologic reactivity was high were taken from a building wall that was very wet after flooding and had visible mold but then underwent reconstruction and currently shows no visible mold. The patient’s working table was constantly near the wall before and after the reconstruction). To our knowledge, excluding studies from the farming environment (May et al., 2012), there have been no studies of patients with more possible environmental exposure sources. However, it has been accepted that serological response may not be limited to one antigen in patients with household EAA (Millerick-May et al., 2016). Currently, the percentages of EAA patients having more than one source of possible exposure to organic inhalation antigen are not known.

In another case (No. 2), the patient claimed no significant exposure and then brought samples from a horse stall and poultry house. High concentrations of specific IgGs to molds, mites and bird feathers were detected in his serum, and only the environmental samples illustrated his exposure history. Detecting the source of exposure in EAA patients may be critical because avoiding exposure improves the outcomes of patients with this disease (Fernández-Pérez et al., 2013).

In other patients, tests confirmed the suspected hypothesis: in Patient No. 3, the antigen likely came from his work environment. For Patient No. 4, mold exposure was confirmed, and the patient was instructed to sterilize her nebulizer. Patient No. 5 was advised to remove a feather duvet (as it may be a source of bird feather, mold, and mite antigens) and change the work environment as well. In Patient No. 6, we detected high concentrations of IgGs specific against molds and bird feathers, and the work environment was confirmed as a possible source of these antigens. Molds originating from the air conditioning system at the workplace of Patient No. 7 were suggested to be offending agents, and serological reactivities to both parrots and molds (garage wall plaster) were confirmed in Patient No. 8.

All patients included in the study were instructed to avoid further exposure to environmental sources to which high serological reactivity was detected. This should be useful in patients with “active” or “acute” disease with high inflammatory activity according to BALF (high percentages of lymphocytes), HRCT (ground glass opacities (GGO), centrilobular nodules) and biopsy results (vaguely formed epithelioid granulomas, lymphoid infiltrates) as well as patients with “chronic” or “fibrotic” disease [9]. It is not known whether continuous exposure to inhalation antigens would lead to the development of a fibrotic response in all exposed patients; however, the prognosis of patients for whom avoidance could be achieved is better compared to patients with further exposure (Ojanguren et al., 2019).
Serum-specific immunoglobulin measurement is proposed as a diagnostic “rule-in” confirmatory test for EAA, pooled sensitivity for identifying patients with probable HP was reported 83% and sensitivity 68% (Johannsson et al., 2020). Selection of commercially available IgG antigens is limited especially for moulds and bacteria, leading to false negativity of the performed test (Szturmowicz et al., 2019). Published studies suggested microbial analysis of samples from suspected environment, followed by antigen preparation (Belanger et al., 2019). This approach involves time-consuming cultivation of samples with the use of multiple specific media followed by biochemical identification of individual IgG-binding proteins. Antigenic extracts based on workplace samples were used to test serologic response also in EAA triggered water-based metalworking fluid exposure (Kespolh et al., 2020). Metal working fluid-specific bacteria were until now unavailable for routine tests and the presented method similiary to our approach is suggested to use in cases with new or rare antigen sources. This approach was used also in cases of farmer’s lung (Tjalvin et al., 2020). To our knowledge, except for sporadic use of antigenic extract to test humoral response in occupational EAA this method was not used in other EAA cases.

The present study has several limitations. The serological response is the hallmark of exposure, yet it does not prove causality. Inhalation exposure tests designed to prove causality between a suspected antigen and disease in EAA patients have been described (Muñoz et al., 2014). We also plan to perform inhalation challenges in enrolled patients with preserved lung functions, as it might not be the procedure of choice in patients with severe lung impairment.

The control group might be another issue because strict avoidance of any exposure to an inhalation antigen is impossible, and there might be some hidden inhalation exposure in control patients. However, it is accepted that inhalation exposure is not enough to develop EAA. There are data confirming a genetic predisposition to this disease that involve differences in antigen presentation, the cytokine milieu and exhausted self-repair processes (Riario Sforza & Marinou, 2017). If we assess the humoral response via specific IgGs, the gained information would also be of limited value because of a lack of information concerning physiologic concentrations in healthy patients. Evaluation of the serological response helps us find possible sources of exposure but must be interpreted carefully in the context of other clinical and history data.

A genetic response may affect not only susceptibility to EAA but also the overall response to different stimuli, which is also observed in our patient group (Spagnolo et al., 2015). While Patient No. 8 presented with high concentrations of serum IgGs and both inflammatory and fibrotic changes detected by HRCT, the serum IgG concentrations of Patient No. 6 were in the lower limit of the normal range, active involvement was represented by epithelioid granulomas, and HRCT patterns of centrilobular nodules were found. Some of the patients involved in the present study had a history of smoking, which may further affect the immune response; aging may also affect the response. Smoking has been suggested to promote antigen-specific immune responses, which are profoundly influenced by host genetic factors (Nemery et al., 2001). Further studies with more patients are needed to reveal associations between genetic background and immune response and thus help us understand and interpret the results of serological tests in EAA patients.

It might be objected that immunological results (Table 1) are not expressed in contexts with “normal” range—we have to bear in mind, that currently there is no widely accepted “normal” range for the humoral response (specific IgGs) to inhalation antigens in EAA patients. To set “normal” range general screening for the most common antigens in the respective population is recommended (Soumagne & Dalphin, 2018). This type of screening includes sampling from suspected environmental sources, cultivation of achieved samples in case of suspected bacterial or mold antigen and setting physiologic values for specific IgGs in nonexposed population (Bellanger et al., 2019). However, achieved values can not be used in any different population, because different environments may lead to different microbiome structures. The above mentioned approach does not cover other sources of antigen, including bird feathers or droppings.
We decided to test subject’s serological response to each potential harmful environment separately and compare it to serological response of a control, who had no contact with the tested environment. Achieved result can not be considered as a proof of causality, strong serologic response should be interpreted as a proof of previous repeated contact with tested environment, not as reliable detection of cause. Despite that in these cases prevention of further exposure to potentially harmful environment should be recommended to patients with a clear diagnosis of EAA, as immunologic response plus clinical criteria of EAA fulfillment were suggested helpful (Johansson et al., 2020).

The strength of the suggested method applies before all in cases with multiple potential sources of exposure. As effect of antigen avoidance on lung functions may be expected in subjects with mostly inflammatory response, it should be preferentially aimed at patients with clinically evident nonfibrotic EAA, who report more than one potential source of offending antigens.

However, even in patients with fibrotic lung involvement, persisting exuberant humoral immune response to inhalation antigens may lead clinicians to different therapeutic approaches. Because management of EAA patients from both diagnostical and therapeutic point of view are currently widely discussed, we believe that proposed method might be useful in some cases, even if definitely more data are needed to validate it for more common use.

5. Conclusions
The presented method is useful in the identification of potentially harmful environments for EAA patients. Based on test results, patients may be advised to avoid specific exposures, which would substantially improve their prognoses, especially in those with nonfibrotic EAA. In some patients, environmental sampling may provide more information than previous history assessment and thus reveal hidden exposures. In cases with multiple sources of potential inhalation agents, the presented method may give more accurate results compared to commercially available specific IgG tests. We suggest further validation of our results in subjects with nonfibrotic EAA a multiple potential sources of exposure to inhalation antigens. Compared to published studies, the presented method is more simple and thus more feasible for wider use and shows a novel approach to EAA management.

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Competing interest
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