Determination of ragweed allergen Amb a 1 distribution in aerosols using ELISA and immunogold scanning electron microscopy

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Background: Ragweed as an invasive species in Europe has become more important for allergy sufferers in the last decade. Because pollen fractions can be found in the respirable fraction of aerosols, they can generate severe disease progressions.
Objective: To obtain information about the concentration and distribution of 1 of the main ragweed allergens *Ambrosia artemisiifolia* 1 in the air of Vienna, PM_{10} and $PM_{2.5}$ fine dust filters were analyzed.
Methods: Standard fine dust filters used for air quality monitoring were analyzed via ELISA and immunogold scanning electron microscopy.
Results: Via ELISA it was possible to show that already at pollen season start in August a recognizably high *A artemisiifolia* 1 concentration can be found. In addition, the allergen concentration in the air stays comparatively high after the peak season has ended even when the pollen concentration drops to a moderate level. The immunogold electron microscopy investigation directly applied on filters shows that the allergen can be found on organic as well as on mixtures of organic and inorganic particles. A first semistatistical analysis of the labeled particle sizes indicates that a large number of the allergen carriers can be found within the smallest particle size range. Nevertheless, further investigations are needed to obtain enough particle counts for a significant statistical analysis.
Conclusions: It was possible to show that reliable results can be obtained from ELISA and immunogold scanning electron microscopy directly applied on filters that are used in air quality monitoring sites. By adaptation of the used protocols, it should be possible to obtain respective information about further allergens. (J Allergy Clin Immunol Global 2022;1:265-72.)

Key words: *Amb a 1 allergen, ELISA, immunogold scanning electron microscopy, fine dust filters, air quality monitoring, seasonal trend*

Different examinations of aerosols, apart from pollen itself, have shown that they can also contain allergen active material.[1-4] In this context, previous studies verified that airborne particles can contain allergens within a wide range of particle sizes.[5-7] The determination of the allergy potential of airborne particles is especially important for the respirable fraction of sizes smaller than approximately 3 μm,[8] because they can have a bigger effect on people's health, as they penetrate deeper into the human respiratory tract.[9,10]

There are numerous plants that people have an allergic reaction to. One of the allergen species that has become gradually more prominent in Europe is the invasive weed plant ragweed.[11] This species can produce about 1 billion pollen grains per plant,[12] and the pollen concentration seems to be independent of the land use (urban or rural),[13] especially because their remaining allergen activity can be shown even after long distance transport.[14] There are various studies that used different methods to ascertain a deeper insight into the dynamics, distribution, and impact on human health of ragweed pollen. Sophisticated methods to determine the presence of specific allergens or proteins in general are ELISA and immunogold electron microscopy.[15] With the first method, it is possible to obtain the concentration of specific allergens quantitatively. The second method enables finding the exact position of the allergens by marking them with gold nanoparticles. Both methods are used in this study to obtain information about the major ragweed pollen allergen *Ambrosia artemisiifolia* 1 (Amb a 1) in the air of Vienna during the high pollination season from August to September of 2017.

To enable this analysis, samples had to be collected during an appropriate period of time and if different measuring sites were used they had to be regionally climatic related. There are different sampling methods available for gathering airborne particles: cascade impactor,[16] cyclone,[17] electrostatic precipitator,[18] absolute filter,[19] or water-based condensation[20] to name just a few. One of the commonly used methods is the cascade impactor, which enables the collection of aerosols in different particle size fractions for a subsequent analysis.[21-25] Especially for immunogold electron microscopy, airborne particles are required to be examined in their natural state as much as possible. Thus, the alteration of the particles due to sampling should be kept to a minimum. An additional requirement for the present study was a good sample density in respect to its time and spatial resolution. To fulfill these requirements, absolute filters (AFs) were investigated, which are generally used in air quality monitoring

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Abbreviations used
AF: Absolute filter
Amb a 1: *Ambrosia artemisiifolia 1*
ESEM: Environmental scanning electron microscope
PM: Particulate matter
PSD: Particle size distribution
$PM_{2.5}$: Particulate matter 2.5: particle fraction with an aerodynamic diameter of <2.5 μm
PM_{10}: Particulate matter 10: particle fraction with an aerodynamic diameter of <10 μm

sites. In the examined city Vienna, several sites are available, from the city center to the more rural outer regions; thus, a good lateral and temporal resolution is available without any additional effort, such as installing new collection equipment. Another benefit of using AFs is that 2 major particle size fractions are simultaneously collected, $PM_{2.5}$ (particulate matter 2.5: particle fraction with an aerodynamic diameter of < 2.5 μm) and PM_{10} (particulate matter 10: particle fraction with an aerodynamic diameter of <10 μm). At the used measuring sites, cyclones are installed in front of the AFs, which preselect and separate off the maximum particle diameter that can reach the filter. Because the maximum particle size of the bigger particle fraction PM_{10} is 10 μm, no intact pollen grain of a typical volume equivalent diameter of 20 μm[26] should be present at the subsequent investigations. The drawback of using AFs is that both investigation methods used in this study have to be adapted to the used filter material. Miguel et al[27] showed that it is possible to obtain information about the allergen potential of PM_{10} using filters from air quality monitoring sites at a monthly average basis.[27] For the presented analysis, it was the aim to illustrate a seasonal trend; thus, a much smaller pooling time of 1 week had to be achieved. In addition, because the airborne allergen level usually varies largely in time and place,[25] the study was initialized to obtain information about the ragweed aeroallergens at high season in and around Vienna.

METHODS

Biological material

For examining the characteristics with focus on the allergen potential of both fresh and commercially available ragweed pollen, fresh pollen were collected during high season at the end of August 2017 in the south of Styria/Austria. The commercially available pollen was purchased from Bonapol (*Ambrosia elatior* [*artemisiifolia*]). Because whole ragweed pollen grains have a typical volume equivalent diameter of 20 μm, the collected pollen grains were purified by sieving for separation of foreign and bigger particles. Before data acquisition, both pollen types were stored at 4°C.

Monitoring sites/fine dust filters

For analyzing fine dust filters in Vienna, AFs capturing PM_{10} and $PM_{2.5}$ were provided by "Umweltschutzabteilung (environmental protection department) – MA22" Vienna. The analyzed AFs were gathered daily in August and September 2017 and originating from 2 air quality monitoring sites in Vienna, Lobau and Taborstraße. These glass fiber AFs (60 g/m^2, Type: Qual.227/1/60 150 mm) were purchased from Munktell and are normally used for measuring the fine dust concentration at different sites of Vienna. According to the manufacturer information, the filter efficiency of particles between 0.12 μm and 0.30 μm is 99.30% and that of particles bigger than 0.30 μm is 99.96%.

The 2 locations are marked in Fig 1. One of the 2 sites is located in the city center (Taborstrasse), from where the PM_{10} and $PM_{2.5}$ filters were investigated. The second one is located at the outer more "rural" region (Lobau), from where the $PM_{2.5}$ filters were investigated. For the pollen concentration in the air (number of pollen/m^3), the official data provided by the "Pollenwarndienst (Austrian pollen warning service)" were obtained from the measurement station located at the roof of the Medical University of Vienna close to the city center (see Fig 1).

ELISA: Determination of allergen activity of Amb a 1 on fine dust filters

Quantification of the ragweed pollen allergen Amb a 1 was done via an allergen-specific ELISA. The ELISA kit was purchased from Indoor Biotechnologies, Inc (EL-AM1; Charlottesville, Va) including the protocol that was used. For the determination of the allergen activity measured in Units (U, typically mU), filters collected within 1 week were pooled (4 weeks in August 2017 and 2 weeks in September 2017). Pooled filters were extracted with PBS solution (10 mM, 0.05% Tween 20, pH 7.4) in proper tubes while shaking with 200 rpm (orbital shaker, Varioshake VS8 OE; Lauda/Bartelt, Graz, Austria; shaking amplitude = 10 mm) at 22°C ± 2°C. After extraction, samples were diluted properly in PBS containing 1% BSA for a possible quantification of the allergen within the detection range. The detection range is defined using an Amb a 1 allergen standard applied within the ELISA kit ranging from 0.5 to 250.0 mU/mL. For ELISA, all incubations were done at 22°C ± 2°C in 96-well plates (Nunc, MaxiSorpTM, ThermoFisher Scientific, Waltham, Mass), which were coated with polyclonal rabbit anti–Amb a 1 capture antibody using carbonate buffer (50 mM, pH 9.6) and incubated overnight at 4°C to 8°C. After washing the plates 3 times using PBS containing 0.05% vol/vol Tween 20, wells were filled with 1%-BSA-PBS-Tween 20 buffer to block potentially unspecific proteins and incubated for approximately 30 minutes. After repeating the washing steps using PBS-Tween 20, samples including appropriate dilutions as well as the Amb a 1 standard were added to the wells. After incubation for 1 hour, plates were washed as before and biotinylated polyclonal rabbit antibodies, which are specific to the Amb a 1 allergen, were added. Sequentially, after 1-hour incubation, plates were washed again 3 times and Streptavidin-peroxidase (Sigma Aldrich, St Louis, Mo) was added and incubated for 30 minutes. As substrate, 1 mM 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt, Sigma Aldrich) in combination with 30% H_2O_2 in citrate buffer (70 mM, pH 4.2) was added after washing the plates 3 times. After color development, resulting absorption of each well was measured photometrically at a wavelength of 405 nm via multilabel plate reader (Victor^3; Perkin Elmer, Waltham, Mass).

Preparation for electron microscopy

Because standardized AFs are used in air quality monitoring, the filters under investigation are predetermined. Usually, particles are extracted from air or a solution onto specific filters as substrates to obtain reliable results concerning their morphology and composition.[28,29] Here, often different methods are used to prevent agglomerations or overlay of different particles. However, studies have shown that solvent extraction or ultrasonication can alter the characteristics of the collected particle matter.[30,31] Immunogold electron microscopy is used to find out on which particles the allergen is present, to obtain information about their morphologies. Therefore, to prevent a possible particle alteration, the sample manipulation was kept to a minimum; thus, no particle extraction was performed in the investigation presented. In addition, to exclude an influence of adding an electrically conductive coating,[32] direct imaging of samples was performed in the low-vacuum mode of an environmental scanning electron microscope (ESEM). This versatile mode, in which water vapor was used as imaging gas, enables the investigation of electrically nonconductive specimens of material science and life science either statically, dynamically, or even in 3 dimensions.[33-35] The used AFs have a normal diameter of 150 mm. To obtain a suitable sample size for the immunogold labeling procedure, an in-house fabricated sharp round metal stamp with a diameter of 2 mm was used. Instead of hammering out, soft pressing was sufficient for extracting the samples from the original AF. After the labeling process, the finished samples were fixed by double-adhesive carbon tape onto aluminum scanning electron microscope stubs and subsequently investigated by the use of an ESEM Quanta600 FEG (FEI, Oregon). For imaging the backscattered electron, contrast was used, because the image gray values are a

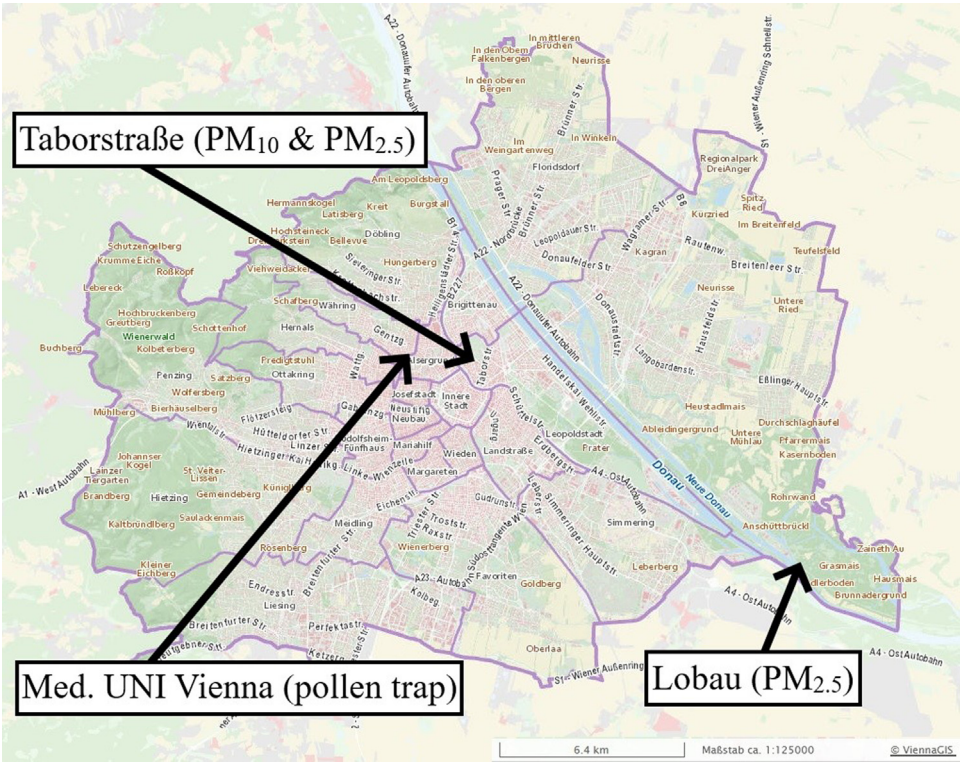

FIG 1. Vienna city map with marked positions of the pollen trap and the 2 air quality monitoring sites (© ViennaGIS).

monotonous function of the mean atomic number[36] of the investigated material. Thus, the gold nanoparticles used for marking the allergen have typically a high gray value compared with those of their surrounding materials and can easily be located.

Antibodies and gold-conjugation

To enable a specific labeling of the protein under investigation, Amb a1, a monoclonal primary antibody was used (MA-5F6 anti–Amb a 1; Indoor Biotechnologies). This antibody was chosen because of its high specificity to a single epitope, which is reflected in a low cross-reaction.[37] In usual labeling procedures, a "2-step" protocol is used to conjugate the gold nanoparticles to the primary antibody.[38-40] Here, in the first step, the primary antibody gets bound to the protein under investigation. Subsequently, a secondary antibody, already conjugated with gold nanoparticles, is bound to the primary antibody in a second step. The high contrast of the gold nanoparticles to the background in different imaging modes enables the detection of the protein under investigation in a transmission electron microscope or an ESEM. As mentioned in the next subsection, transferring steps should be kept to a minimum to prevent a possible particle loss. To avoid the second preparation step, a different approach was used. Within this method, the primary antibody gets directly conjugated with the gold nanoparticle. A nanoparticle conjugation kit was used (InnovaCoat GOLD, formerly Expedeon, Heidelberg, Germany) to covalently bind the used primary antibody to the gold nanoparticle. Therewith, a "single-step" labeling procedure could be used (see next subsection).

Immunogold labeling protocol

During the establishment of the appropriate preparation technique for enabling the immunogold labeling of allergen-containing dust particles (aeroallergens) directly on fine dust filters, it was pointed out that transfer and washing steps have to be minimized. This was because with every step fine dust particles may be washed out from the AFs. Therefore, several labeling steps were combined and washing steps were minimized. All following solutions were freshly prepared before usage. The usual initial step of fixation, with, for example, glutaraldehyde was avoided, because no measurable difference could be ascertained. This could be because the sample under investigation is mostly composed of inorganic components. For each individual step, a 1-mL droplet of the respective solution was dropped on a hydrophobic film (Bemis, Parafilm, PM996, WI 54956) in a droplet array. For the subsequent labeling procedure, the disks were put upside down on top of the respective droplet and because of the hydrophobic nature of the AF, only the uppermost filter part got immersed in the solution. The first step was 30 minutes of incubation with PBS (15 mM, pH 7.2) mixed with 0.05% vol/vol Tween 20 and 2% BSA. To neutralize this solution, the specimens were washed with PBS 4 times for 5 minutes each. Subsequently, the primary antibody, already conjugated with gold nanoparticles, was diluted in a 1:500 PBS solution and was incubated between 22 $\pm$ 2 hours at 4°C. To remove this surplus solution, the specimens were washed with PBS 2 times and finally washed 2 times with double distilled water, 5 minutes each, and air dried. The final washing step was necessary, to avoid the forming of widespread salt crystals, which hinder an area-covering investigation. Because the low-vacuum mode of an ESEM was used for imaging, no further preparation step was necessary. As positive control ball milled ragweed pollen and as negative control ball milled grass pollen were dispersed on pristine AF. Fig 2 shows as an exemple 2 SEM images of the positive and negative control of milled pollen fixed on double-adhesive carbon tape labeled in the same way as stated in this subsection.

RESULTS

The weekly pooled fine dust filters were quantitatively analyzed by ELISA from the first week of August to the second week of September. In addition, the sum over the same weeks of the ragweed pollen concentration was used to obtain information about the general flowering season of Vienna (see Fig 3). It can be seen that already at the beginning of the pollen season with low

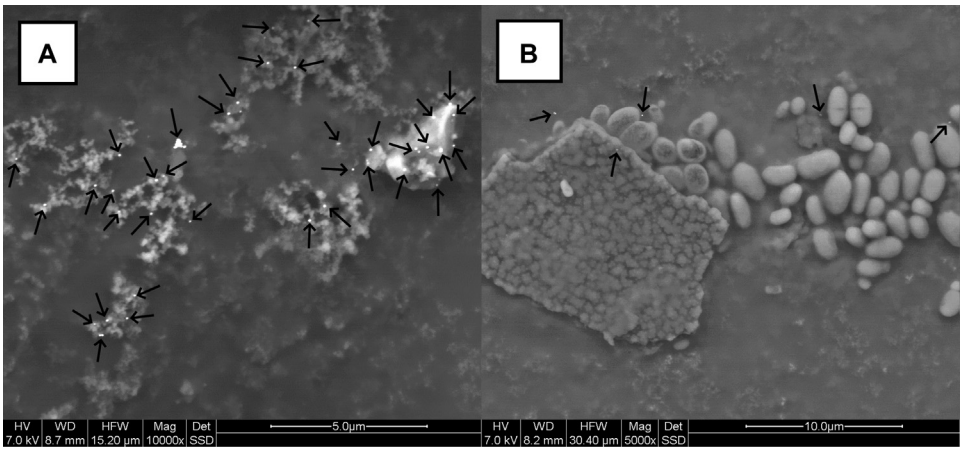

FIG 2. Backscattered electron images of immunogold labeled against Amb a 1 of ball milled (**A**) ragweed and (**B**) grass pollen fixed on carbon tape. Arrows mark the position of gold particles. *Note:* The area shown in *B* is 4 times bigger than in *A*.

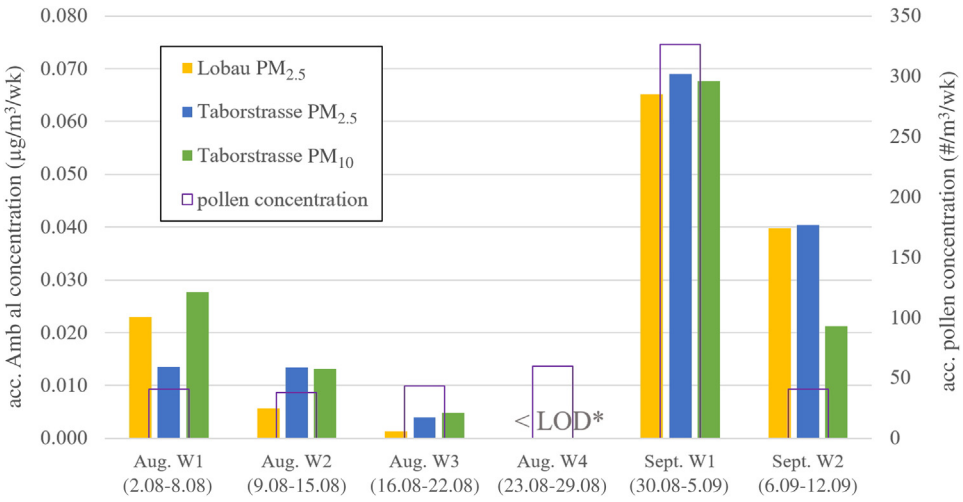

FIG 3. Amb a 1 concentration at weekly pooled fine dust filters from $PM_{2.5}$ (yellow bar) gathered at Lobau and $PM_{2.5}$ (blue bar) and PM_{10} (green bar) gathered at Taborstrasse. The purple line shows weekly pooled ragweed pollen concentrations measured at the medical University of Vienna. **LOD,* Limit of detection: in the fourth week of August, the pollen concentration was lower than the LOD.

pollen concentrations in the air, high Amb a 1 concentrations are found in all measured fractions. Furthermore, it should be mentioned that in the period before August, only scattered pollen was detected (sum of third and fourth July weeks are 1 and 10 pollen, respectively). Subsequently to the pollen season start, a rather constant ragweed pollen concentration led to a falling allergen concentration in all PM fractions until week 4, when the concentration was lower than the limit of detection. During the peak season of the ragweed pollination at the first September week, expectedly highest allergen concentrations can be found at all measuring sites. Although the pollen concentration rapidly decreased in the second week of August, the allergen concentration stays comparatively high. Subsequently, the pooled pollen concentration of week 3 of September stayed on a moderate level of 56 and dropped to only 2 pollens in week 4. The ELISA results show that the Amb a 1 allergen is present on particles far smaller than a whole pollen grain of approximately 20 µm, because only particles of smaller than about 10 µm or 2.5 µm should be present on the investigated AF. Another indication that only particles/fractions are present on the AF is that during all SEM examinations no intact pollen grain was found on the microscopically investigated filters.

To obtain information about the exact position of the ragweed allergen quantified via ELISA, immunogold labeling was performed on the filters showing highest detected allergen concentration in the first week of September. For every of the 21 available AFs (2 $PM_{2.5}$ and 1 PM_{10}, 7 days each), at least 1 filter piece was prepared with the labeling protocol. Subsequently, only those filters were examined that showed a significantly stronger signal in the positive control than in the negative control. Altogether, 2 $PM_{2.5}$ (Lobau and Taborstrasse) and 2 PM_{10} (Taborstrasse) AF pieces were examined. Because AFs from 2 different air quality monitoring sites with 2 different nominal PM sizes were analyzed, the particle morphologies should also be different. Fig 4 representatively shows the labeling result of 1 of the PM_{10} filters from Taborstrasse. The first row shows (*A*) the

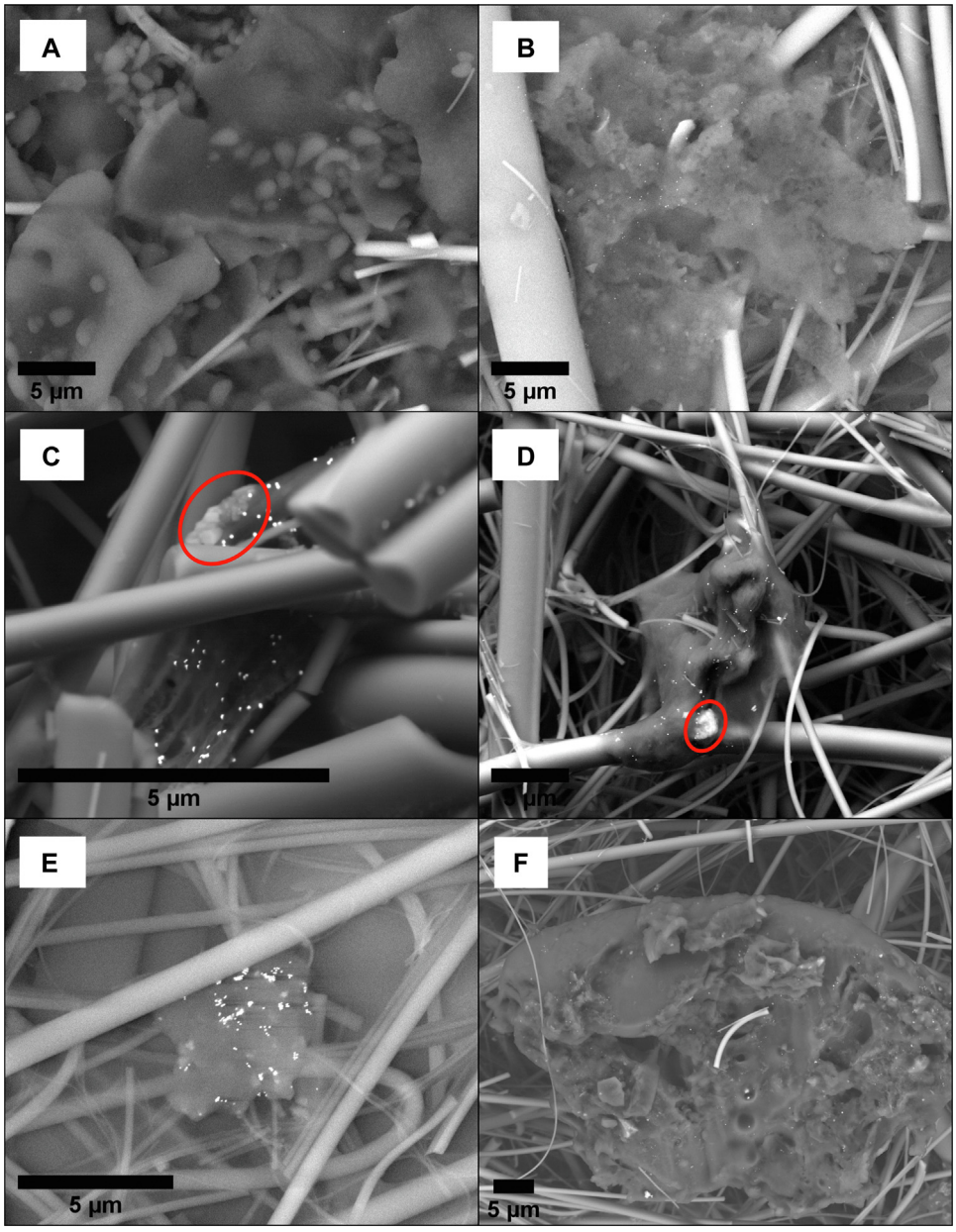

FIG 4. Immunogold labeled particles on AF of Taborstrasse: (**A**) negative control (milled grass pollen), (**B**) positive control (milled ragweed pollen), (**C-F**) distinct labeled particles at PM_{10} fine dust filter; red rings mark possible mineral inclusions in labeled particles (backscattered electron images, all scale bars: 5 µm).

negative control, which is significantly less labeled than the positive control in *B*. It should be mentioned that no significant labeling of the filter itself was observable. The images *C* to *F* show some of the labeled PM_{10} particles. Unsurprisingly, most labeled particles are of organic origin, but as can be seen in Fig 4, *C* and *D*, mineral inclusions can also be found, which was concluded because of their brighter and granular structure within darker matrices (see, eg, the red circle markings in the 2 images). It is noticeable that some of the observed particles are larger than 10 µm. This could be due to the circumstance that no strict cutoff at aerodynamic particle diameters of 10 µm is possible for stationary air quality monitoring.[41] Another explanation is that agglomerations of smaller particles are merged during the deposition process.

The same measurement as for the PM_{10} AF was performed for both $PM_{2.5}$ AFs of Taborstrasse and Lobau. Fig 5 representatively shows a labeled $PM_{2.5}$ AF from Lobau from the first week of September, where *A* shows the negative control and *B* the significantly higher labeled positive control. As data from the PM_{10} investigation have already shown, some particles were found that are bigger than the expected diameter of 2.5 µm (see, eg, Fig 5, *C*). The additional images in Fig 5, *D* to *F*, show that most of the labeled particles were smaller than the given aerodynamic diameter of 2.5 µm, as expected. The 2 green marks

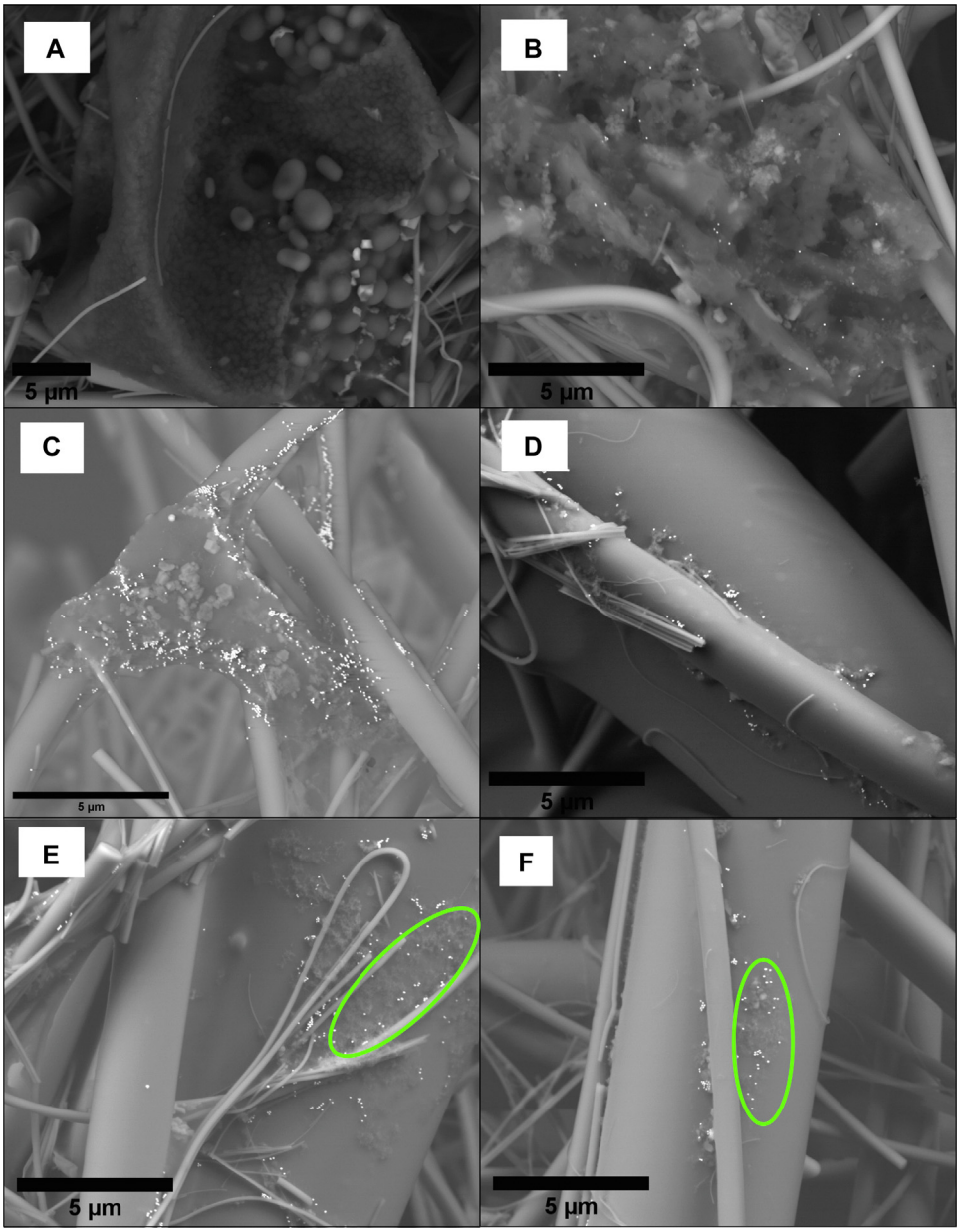

FIG 5. Immunogold labeled particles on AF of Lobau: (**A**) negative control (milled grass pollen), (**B**) positive control (milled ragweed pollen), (**C-F**) distinct labeled particles at $PM_{2.5}$ fine dust filter (backscattered electron images, all scale bars: 5 µm). Green marks: possible accumulation of individual particles.

accentuate regions of accumulations, where no distinction of individual particles could be determined.

Because images of the labeled particles were available, it is possible to measure particle morphologies, such as the longest diameter. Thus, a first assessment of the particle size distribution (PSD) of particles in the fraction PM_{10} and $PM_{2.5}$ can be obtained by manually searching and measuring the appropriate particles in all investigated areas. The biggest problem of the presented data was that most of the labeled particles were found as agglomerates (see, eg, the green marks in Fig 5, *E* and *F*). Here, the labeled regions seem to be clusters of small individual particles for which no clear differentiation regarding the individual size is possible. For the PSD, only distinct isolated particles, as in Fig 4, *D* and *E*, with no shadowing of the particle from the AF as in Fig 4, *C*, were counted. Therefore, only a limited number of individual available particles could be found. For the PM_{10} fraction, a total of 27 particles and for the $PM_{2.5}$ fraction, a total of 28 particles could be counted at all investigated AFs.

Fig 6 shows a diagram of the detected particles, which gives first insights into the most frequently detected particle sizes that were labeled against the ragweed allergen Amb a 1. At all AFs, most of the labeled particles are located at the lower end of the PSD, that is, in the smaller particle size fraction. This could be due to 2 possible circumstances: most particles deposited on the

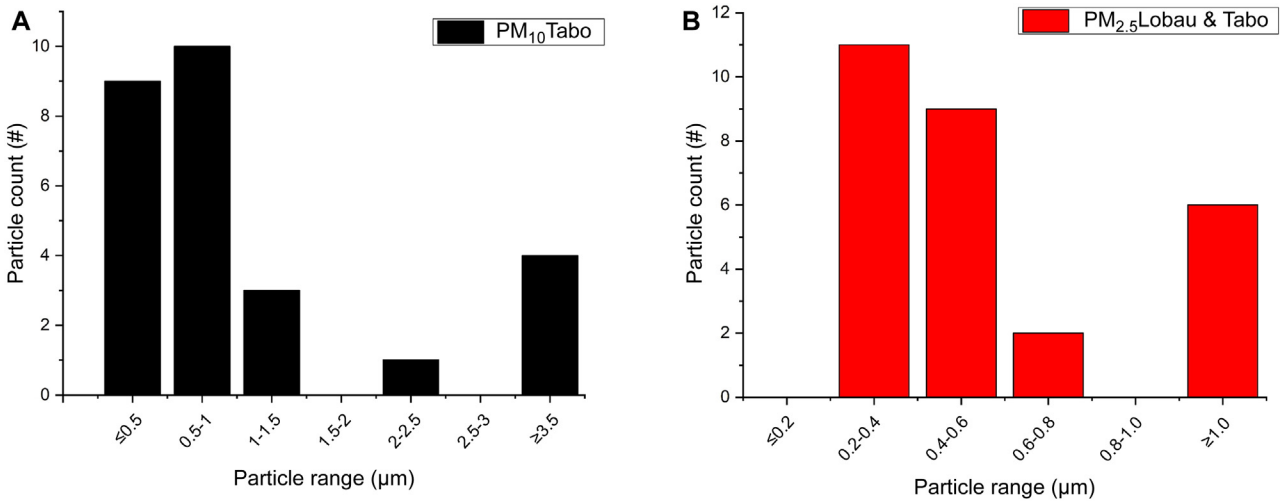

FIG 6. Assessment of the PSD of only those individual available particles that are labeled against Amb a 1, of both (**A**) the PM_{10} (Taborstrasse) and (**B**) the $PM_{2.5}$ (Lobau and Taborstrasse) particle fraction (semistatistical analysis).

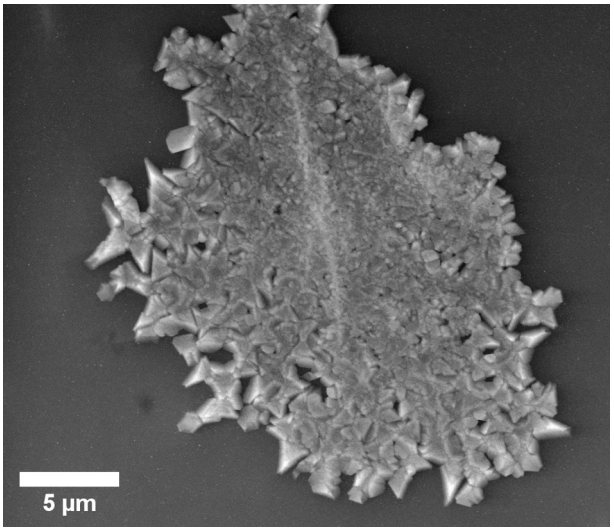

FIG 7. Backscattered electron image of residuals after drying of used labeling droplet on carbon tape.

filter can naturally be found in the lower PSD fraction or bigger particles are more susceptible than smaller ones to get washed out from the filter during the labeling procedure. Parts *D* to *F* of Fig 5 are examples of repeatedly found possible agglomerates of small particles that are highly labeled. This indicates that the found PSD corresponds to the actual distribution. To obtain information about the particles that got washed out from the AF during the labeling procedure, images in the low-vacuum mode of the ESEM of the residuals after the evaporation of the solution are made. Fig 7 shows a backscattered electron image of 1 of these residuals. Unfortunately, most particles are crystallized saline particles that probably overlay the washed-out particles. Thus, no distinct prediction can be made about the type and size of particles that got lost during sample preparation. So, the stated PSDs should only be seen as first indications of allergen-containing particle sizes, but no statistical evaluation can be made. Nevertheless, the shown procedure gives the possibility to investigate aeroallergens in a rather natural state. By using a more automated counting method, it should be possible to obtain more particle counts in a reasonable time, thus enabling a statistical evaluation.

Conclusions

ELISA measurements show a clear evidence of ascertainable allergen concentrations in PM_{10} as well as $PM_{2.5}$ fine dusts already at the ragweed pollen season start at both investigated locations in Vienna in 2017. The highest Amb a 1 allergen concentration was found during the related highest pollen concentration in the first week of September. Although the subsequent pollen concentration drops after this peak period to the level before, the allergen concentration stays comparatively high in all PM fractions and locations. Immunogold SEM investigation reveal that the Amb a 1 allergen can be found on organic as well as on agglomerates of organic and inorganic particles. Initial analysis of the particle sizes has shown that allergen carriers can be found in the smallest particle fraction investigated in this study of less than 1 µm. To obtain enough particle counts enabling a significant statistical analysis, further investigations are needed. Nevertheless, the presented study shows that both methods, ELISA and immunogold electron microscopy, can also be applied on filters normally used in air quality monitoring sites, which are set up comprehensively. Their usage could enable investigations of high temporal and local aeroallergen variations, without the effort of installing new aerosol measurement equipment.

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