

Variation of the group 5 grass pollen allergen content of airborne pollen in relation to geographic location and time in season

The HIALINE working group: Jeroen Buters, PhD,^{a,b} Marje Prank, MSc,^c Mikhail Sofiev, PhD,^c Gudrun Pusch, Dipl Ing,^{a,b} Roberto Albertini, PhD,^d Isabella Annesi-Maesano, MD, DSc, PhD,^{e,f} Celia Antunes, PhD,^{g,h} Heidrun Behrendt, MD,^{a,b} Uwe Berger, MBA,ⁱ Rui Brandao, PhD,^{g,†} Sevcan Celenk, PhD,^j Carmen Galan, PhD,^k Łukasz Grewling, PhD,^l Bogdan Jackowiak, PhD,^l Roy Kennedy, PhD,^m Auli Rantio-Lehtimäki, PhD,ⁿ Gerald Reese, PhD,^o Ingrida Sauliene, PhD,^p Matt Smith, PhD,ⁱ Michel Thibaudon, Pharm,^q Bernhard Weber, PhD,^o and Lorenzo Cecchi, MD^r *Munich and Reinbek, Germany, Helsinki and Turku, Finland, Parma and Florence, Italy, Paris and Brussieu, France, Évora and Coimbra, Portugal, Vienna, Austria, Gorukle-Bursa, Turkey, Cordoba, Spain, Poznan, Poland, Worcester, United Kingdom, and Siauliai, Lithuania*

Background: Allergies to grass pollen are the number one cause of outdoor hay fever. The human immune system reacts with symptoms to allergen from pollen.

Objective: We investigated the natural variability in release of the major group 5 allergen from grass pollen across Europe.

Methods: Airborne pollen and allergens were simultaneously collected daily with a volumetric spore trap and a high-volume cascade impactor at 10 sites across Europe for 3 consecutive years. Group 5 allergen levels were determined with a Phl p 5-specific ELISA in 2 fractions of ambient air: particulate matter

of greater than 10 µm in diameter and particulate matter greater than 2.5 µm and less than 10 µm in diameter. Mediator release by ambient air was determined in FcεRI-humanized basophils. The origin of pollen was modeled and condensed to pollen potency maps.

Results: On average, grass pollen released 2.3 pg of Phl p 5 per pollen. Allergen release per pollen (potency) varied substantially, ranging from less than 1 to 9 pg of Phl p 5 per pollen (5% to 95% percentile). The main variation was locally day to day. Average potency maps across Europe varied between years. Mediator release from basophilic granulocytes correlated better with allergen levels per cubic meter ($r^2 = 0.80$, $P < .001$) than with pollen grains per cubic meter ($r^2 = 0.61$, $P < .001$). In addition, pollen released different amounts of allergen in the non-pollen-bearing fraction of ambient air, depending on humidity.

Conclusion: Across Europe, the same amount of pollen released substantially different amounts of group 5 grass pollen allergen. This variation in allergen release is in addition to variations in pollen counts. Molecular aerobiology (ie, determining allergen in ambient air) might be a valuable addition to pollen counting. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: Allergen, grass, Phl p 5, Europe, PM10, PM2.5, exposure, allergy, basophil, modeling, SILAM, HIALINE

From ^aZAUM—Center of Allergy & Environment, Helmholtz Center Munich/Technische Universität München, Member of the German Center for Lung Research (DZL), Munich; ^bthe Kühne Foundation, Christine Kühne—Center for Allergy Research and Education (CK-CARE), Munich; ^cthe Finnish Meteorological Institute, Erik Palmenin Aukio 1, Helsinki; ^dthe Laboratory of Allergology, Department of Clinical and Experimental Medicine, University of Parma, U.O. Medical Immunology, University Hospital of Parma; ^ethe EPAR Department, INSERM, UMR_S1136, Institut Pierre Louis d'Epidémiologie et de Santé Publique, France; ^fthe EPAR Department, Sorbonne Universités, UPMC Univ Paris 06, UMR_S 1136, Institut Pierre Louis d'Epidémiologie et de Santé Publique, Paris; ^gICAAM—Institute of Mediterranean Crop and Environmental Sciences, University of Évora; ^hthe Center for Neuroscience and Cell Biology (CNC), University of Coimbra; ⁱthe Medical University of Vienna, Department of Oto-Rhino-Laryngology, Research Unit Aerobiology and Pollen Information, Vienna; ^jthe Aerobiology Laboratory, Biology Department, Science and Arts Faculty, Uludağ University, Gorukle-Bursa; ^kthe Department of Botany, Ecology and Plant Physiology, University of Córdoba, International Campus of Excellence on Agrifood (ceiA3), Córdoba; ^lthe Laboratory of Aeropalynology, Faculty of Biology, Adam Mickiewicz University, Poznań; ^mthe National Pollen and Aerobiology Research Unit, University of Worcester; ⁿthe University of Turku, CERUT, Aerobiology Unit, Turku; ^oAllergopharma GmbH & Co KG, Reinbek; ^pthe Department of Environmental Research, Siauliai University, Siauliai; ^qRNSA (Réseau National de Surveillance Aérobiologique), Brussieu; and ^rthe Interdepartmental Centre of Bioclimatology, University of Florence.

†Deceased.

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Corresponding author: Jeroen Buters, PhD, ZAUM—Center of Allergy & Environment, Helmholtz Center Munich/Technische Universität München, Biedersteinerstrasse 29, 80802 Munich, Germany. E-mail: buters@tum.de.

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Grass pollen is the major cause of allergic sensitizations to outdoor allergens all over Europe¹ and ranks second to house dust mite as a cause of respiratory allergy in most countries.²⁻⁵ The role of grass pollen in respiratory allergy was recently confirmed in a large epidemiologic study from the European Community Respiratory Health Study.⁶

Grass pollen originates from many species of the Poaceae family and release several proteins and glycoproteins,⁷ some of which are identified as allergens. These allergens are classified in 10 groups on the basis of their high intragroup cross-reactivity (see World Health Organization/International Union of Immunological Societies Allergen Nomenclature Sub-Committee at <http://www.allergen.org/>; Allergome at <http://www.allergome.org/>). *Phleum pratense* (timothy grass) is a major source of grass pollen in temperate areas, and because of the cross-reactivity mentioned above, its extracts and single allergen components are widely used for the *in vitro* and *in vivo* diagnosis of grass pollen allergy and specific immunotherapy.⁸ Although sensitizations against Phl p 1, 2, 4, 5, 6, 7, 11, 12, and

Abbreviations used

HIALINE:	European Union Project Health Impacts of Airborne Allergen Information Network
10>PM>2.5:	Particulate matter larger than 2.5 μm and less than 10 μm in diameter
PM>10:	Particulate matter larger than 10 μm in diameter

13 are described, Phl p 1, 2, 5, and 6 are markers of grass pollen sensitization because they are present exclusively in grass pollen.^{8,9} Phl p 1, 4, and 5 show the highest rates of sensitization (>50%) in patients with grass pollen-induced allergy.^{10,11} However, recent findings have shown that the prevalence of sensitization and clinical importance of Phl p 4 might be overestimated.^{12,13} Other than for the clinical relevance, Phl p 5 is important for its high allergenic activity and good characterization.¹⁴ For these reasons, the variant Phl p 5.0102 (Phl p 5a) was adopted as reference material for the standardization of extracts for immunotherapy and skin prick tests, as indicated by the Development of Certified Reference Materials for Allergenic Products and Validation of Methods for Their Quantification (CREATE) project's findings.¹⁵ Different species of the Poaceae family express homologues of Phl p 5^{16,17} that show similar immunologic responses.¹⁸

It is not possible to distinguish grass pollen grains from different grass species by using light microscopy, but the identity of allergens and the amount of allergen released from pollen varies between species.^{8,17} Natural variation of allergen from biological sources was reported for birch pollen,¹⁹ olive pollen,²⁰ horses,²¹ and cats.²² Allergen release also varies during the lifespan of pollen.²³

Monitoring allergens in ambient air enables the detection of non-pollen-bound allergens, as encountered during special weather conditions, such as thunderstorms.^{7,24} In addition, the determination of thresholds for allergic symptoms based on pollen counts has produced variable results^{25,26} and might be improved by adding airborne allergen monitoring.²⁷

In the framework of the European Union Project Health Impacts of Airborne Allergen Information Network (HIALINE; www.hialine.eu), we monitored daily amounts of grass pollen and their major group 5 allergens simultaneously across Europe. We showed that levels of Phl p 5 released from grass pollen was variable and emphasized the necessity to consider molecular aerobiology in addition to counting pollen for the assessment of exposure to airborne allergens, especially in clinical trials and epidemiologic studies.

METHODS**Pollen in ambient air**

Airborne pollen concentrations were collected at 10 sites in Europe (Table I and see Table E1 and Fig E1 in this article's Online Repository at www.jacionline.org) by using Hirst-type volumetric spore traps, according to the requirements of the European Aeroallergen Network.²⁸ Quality of counts was controlled, as described previously.¹⁹

Allergen in ambient air

Air was simultaneously sampled at each site with ChemVol (Butraco, Son, The Netherlands) high-volume (800 L/min) cascade impactors equipped with dry polyurethane impacting substrates (that were prewashed with 0.1 mol/L

ammonium bicarbonate and water) using the stages for particulate matter greater than 10 μm (PM>10) and particulate matter greater than 2.5 μm but less than 10 μm in diameter (10>PM>2.5), as described previously.²⁹ Allergen was extracted in 0.1 mol/L ammonium bicarbonate containing 0.1% BSA in an end-over-end rotator at 100 rpm for 4 hours, lyophilized, and redissolved in one tenth of the original volume in PBS, according to standard procedures.^{23,30} Phl p 5 allergen was determined by means of ELISA with the mAbs 1D11 and BO 1 (Allergopharma, Reinbek, Germany)³¹ calibrated against recombinant Phl p 5.0102.¹⁵ The assay recognizes group 5 allergens of Poideae (Phl p 5.0102 and 5.0201); has no cross-reactivity with groups 1, 2, 3, 4, 6, and 13; and has a limit of detection of 1.0 ng of Phl p 5/mL, equaling about 2 pollen grains/m³ (1 mL of extract = 256 m³ of air). Response factors for the different group 5 allergens vary less than 2-fold (data not shown; Allergopharma, Reinbek, Germany). The assay recognizes C-terminal group 5 fragments,³² and ELISA correlated with mass spectrometric quantification (data not shown, Allergopharma). Quality control of ELISA data was performed according to the rules developed in HIALINE.¹⁹ Values less than the detection limit were reported as zero.

Basophil degranulation assay

An immune cell response independent of ELISA was determined based on induction of mediator release by ambient air extracts from an Fc ϵ RI-humanized rat basophil cell line (α -, β -, and γ -chain).³³ The cells were passively sensitized with human serum of a patient with grass pollen-induced symptoms, a positive SPT response, and a RAST score of greater than 3. The patient was sensitized to Phl p 1, 2/3, 4, 5b, 6, and 11 and Cyn d 1 but not Phl p 7 and 12. Levels of β -hexosaminidase as a proxy for histamine release was measured, as described previously.¹⁹ Each environmental sample was diluted sequentially, and only data from the linear part of the degranulation curve were reported.

Statistics

Pollen potency was determined several ways: (1) as the slope of the linear regression curve of pollen per cubic meter versus allergen per cubic meter with the intercept forced through zero and the strength of the relation expressed by the coefficient of correlation (r^2); (2) as the mode of the histogram of daily potencies; (3) as the median potency calculated for the whole data set and for subranges of daily pollen counts; and (4) as the 5th to 95th percentile range. The analysis excluded pollen counts of less than 10 grains/m³.^{19,34}

The station- and year-wise variability of potency was assessed from histograms for the individual sites. Differences were analyzed by using the Student t test, unless stated otherwise.³⁵ A P value of less than .05 was considered statistically significant. Values beyond 3 SDs of the mean were considered outliers.

The explanation factors for the potency variations included meteorological variables that were either monitored at the ChemVol locations or obtained from the nearest meteorological station.

Modeling

The System for Integrated Modelling of Atmospheric Composition (SILAM)^{36,37} was used to evaluate the flowering period and pollen atmospheric transport and also to compute the footprints of the observations, as described previously.¹⁹ The SILAM model was run with a time step of 15 minutes, and the footprints were calculated 60 hours backward in time for each daily observation at each site.

Daily observed potency was mapped to the origin of pollen through the footprints. The lowest values that make up 1% of the footprint integral were cut off to reflect the limited transport distance of grass pollen. The potency value was attributed to all areas covered by the footprint that flowered at the time of the footprint passage. The attributed potency values were then averaged over the season, finally resulting in potency maps that show the mean potency of pollen released from the area during a specific year.

All simulations used the same configuration that included 8 vertical layers up to approximately 6 km above the ground. The horizontal grid cell size was

TABLE I. Average yearly pollen count (pollen index) and allergen release per pollen for the stations in the different countries

		1999-2008*	2009		2010		2011		2009-2011, average ± SD (SD %)
		Pollen index, Σ Grains/m ³ /y	Pollen index, Σ Grains/m ³ /y	Potency, Phl p 5/grain (pg)	Pollen index, Σ Grains/m ³ /y	Potency, Phl p 5/grain (pg)	Pollen index, Σ Grains/m ³ /y	Potency, Phl p 5/grain (pg)	Potency, Phl p 5/grain (pg)
Austria	Vienna	3,024 ± 606†	NA	NA	2,604	3.546	2,411	2.941	3.244 ± 0.428 (13)
Finland	Turku	740 ± 267	654	1.592	738	1.992	782	1.535	1.706 ± 0.249 (15)
France	Varies‡	9,671 ± 3,808	10,420	2.405	7,130	2.403	6,708	2.937	2.582 ± 0.308 (12)
Germany	Munich	2,033 ± 604	2,041	3.042	1,821	2.127	2,184	2.133	2.434 ± 0.527 (22)
Italy	Parma	7,521 ± 2,973	4,135	1.508	4,362	2.871	5,409	0.821	1.733 ± 1.043 (60)
Poland	Poznan	4,697 ± 1,472§	6,078	2.703	7,898	3.398	4,482	1.996	2.699 ± 0.701 (26)
Portugal	Evora	6,617 ± 8,836	5,725	2.144	17,113	1.499	22,815	1.545	1.729 ± 0.360 (21)
Spain	Cordoba	5,609 ± 2,777	4,014	2.268	8,693	4.149	5,888	2.423	2.947 ± 1.044 (35)
Turkey	Bursa	1,242 ± 490¶	1,881	NA	2,892	3.126	3,812	NA	3.126
United Kingdom	Worcester	6,715 ± 2,223	4,885	5.906	5,141	3.570	3,531	4.178	4.551 ± 1.212 (27)
Average			2.696 ± 1.395		2.868 ± 0.840		2.279 ± 0.989		
potency ± SD									
SD (%)			52		29		43		

Potency (release of allergen per pollen) was determined by the slope of the linear regression (see the [Methods](#) section). Means ± SDs are presented. Exact location of the stations is given in [Table E1](#).

NA, Not applicable.

*A complete preceding decade was not available for all stations, in which case the maximum period available was taken.

†Vienna 1999-2008: 2 sites: (A) 1999-2002 ATWIAK; (B) 2003-2008 ATWIEN. The trap was moved from ATWIAK (AKH-Allgemeines Krankenhaus) to ATWIEN (ZAMG-Zentralanstalt für Meteorologie und Geodynamik).

‡In 1999-2008, the station was in Lyon; in 2010, it was in Saint-Genis l'Argentière; and in 2011, it was in Brussieu.

§From 2005-2008.

||From 2001-2008.

¶From 1999-2000, 2003-2005, and 2008; no data from 2001-2002 and 2006-2007.

25 km. Input meteorological data were taken from the operational archive of the European Centre for Medium-Range Weather Forecast.

RESULTS

Pollen in ambient air

Airborne grass pollen is ubiquitous in Europe, but large variations in pollen counts exist between countries. A map of the stations is presented in [Fig E1](#). The Finnish station in Turku (seaside) showed the lowest pollen counts, whereas the Portuguese station in Evora (rural site) recorded the highest counts ([Table I](#)). It should be noted that the years studied for each station were within the normal range of the preceding years for all stations (season timing and total pollen counts were within 1 SD from the long-term mean values, data not shown). The stations were distributed over Europe to obtain the most representative picture for the continent.

With 3 Hirst-type pollen traps operated simultaneously within 5 m of each other at about 9 m in height for a 3-week period during the grass pollen season (0-890 pollen grains/m³), we determined the variability between the traps to be about 5 pollen grains/m³ plus 20% (data not shown).

Allergen

In addition to variations in airborne pollen counts, pollen differed in allergen release per pollen (potency, [Fig 1](#)). Determined by means of linear regression (see the [Methods](#) section), the average group 5 allergen release per pollen was 2.3 pg of Phl p 5 per pollen ($r^2 = 0.497$, $P < .001$, $n = 1629$; see [Fig E2](#) in this article's Online Repository at [www.jacionline.org](#)). This number agrees well with the histogram

mode (2-2.5 pg of Phl p 5 per pollen, see the histogram in [Fig E3](#) in this article's Online Repository at [www.jacionline.org](#)). Median potency is somewhat greater, 2.6 pg of Phl p 5 per pollen, because of the asymmetric tails of the histogram. The pollen potencies for each station are given in [Table I](#), and histograms are shown in [Fig E4](#) in this article's Online Repository at [www.jacionline.org](#). Potency distribution can have quite a wide spread that is different for different regions. Interannual variability of the histograms at the same station is smaller than spatial differences. Taking the extremes in pollen counts (Finland vs Portugal), allergen release per pollen was not different (not significant). Potency values over Europe were correlated with pollen counts ([Fig 2](#)).

We detected many days when pollen did not release any Phl p 5 at all. These pollen grains without group 5 allergens were especially noticeable at the beginning of the grass pollen seasons. This also explains why pollen potency distribution is not normally distributed (see [Fig E3](#)). Using the 5th to 95th percentile, pollen potency ranged between less than 1 and 9 pg of Phl p 5 per pollen (excluding low pollen counts [ie, <10 pollen grains/m³]).

Mediator release from basophils

For one station (Munich, Germany), we determined the daily mediator release by basophils passively sensitized to grass pollen allergens. In Munich no overlapping pollen season was measured, except for nonallergenic *Pinus* species (pine) and *Picea* species (spruce) pollen. The degranulation was standardized as if 1 m³ of air was delivered to the cells, which could result in calculated degranulation rates of greater than 100% ([Fig 3](#)). The serum used showed sensitization against several major and minor grass pollen allergens (Phl p 1, 2/3, 4, 5b, 6, and 11). Although these specific

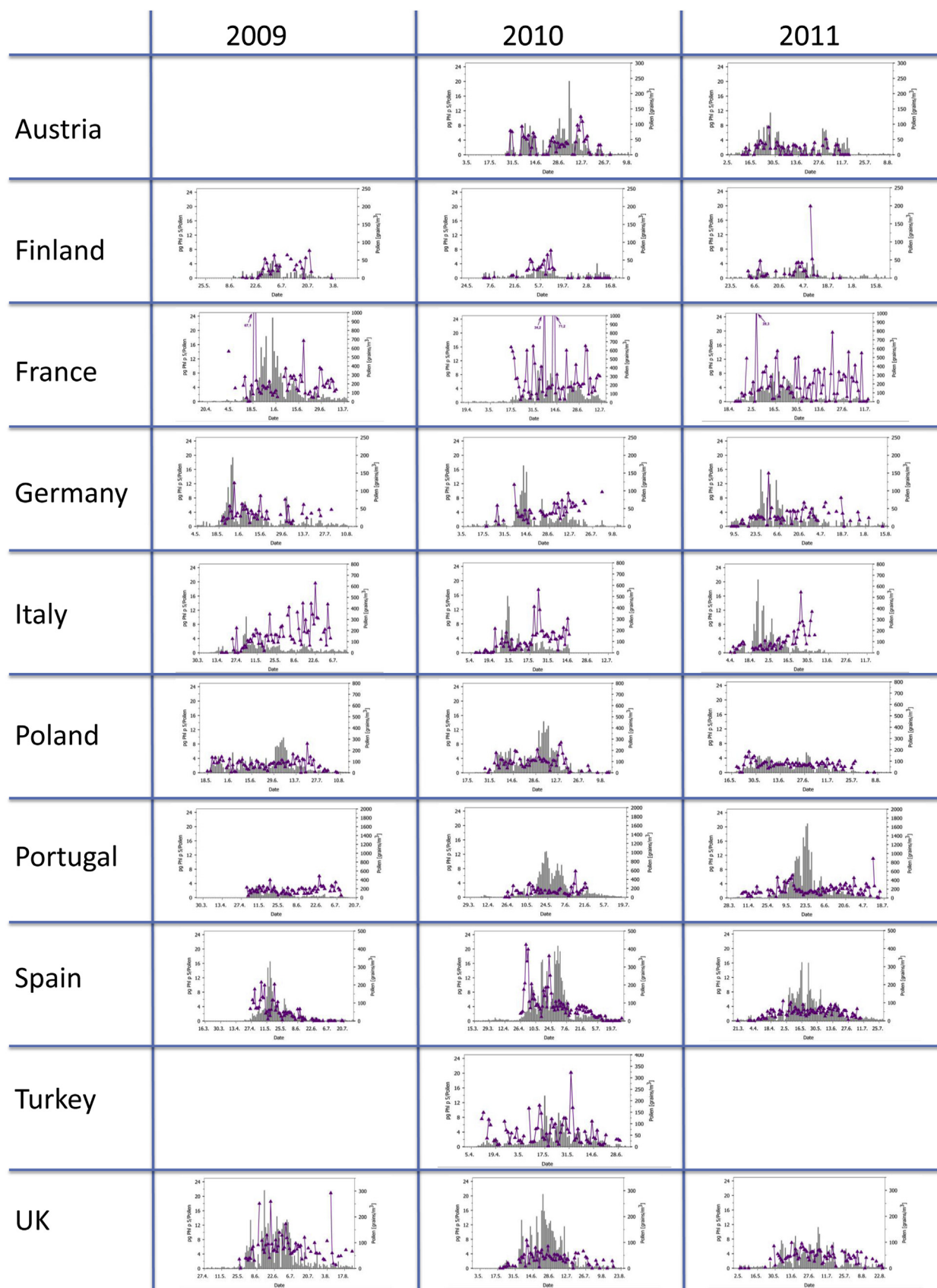


FIG 1. Pollen flight from all stations in 2009 until 2011 concomitant with pollen allergen release potency. Gray vertical bars represent pollen, and purple lines represent daily allergen released per pollen (daily pollen potency). UK, United Kingdom.

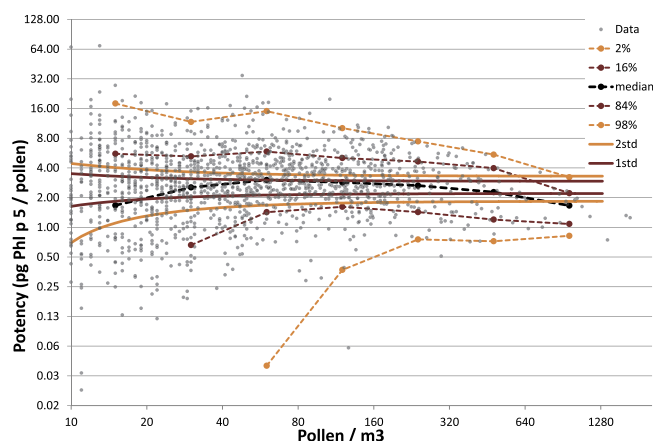


FIG 2. Experimental variability of grass pollen potency versus its estimated uncertainty. *Solid lines*, Potency uncertainty range calculated from assumed errors of pollen counts and ELISA. *Dashed lines*, Observed percentiles; *thick dots* indicate the centers of bins used for percentile estimation. The observed potency variability was much larger than can be explained by experimental error. Pollen counts of less than 10 pollen grains/m³ were excluded.

IgEs to several major or minor allergens could have mediated basophil degranulation, mediator release from basophilic granulocytes correlated well with Phl p 5 levels per cubic meter ($r^2 = 0.80$, $P < .001$) and with pollen levels per cubic meter ($r^2 = 0.61$, $P < .001$). Two outliers (probably because of the large dilution needed to achieve the linear part of the degranulation curve) were identified and indicated in Fig 3.

Potency maps

Potency maps compiled for each year (Fig 4, left column) showed a substantial variability over Europe. Sampling stations were located between 2 and 22 m (see Table E1). Fewer grass pollens were sampled at higher monitor locations because of the substantial sedimentation velocity of grass pollen. However, numeric simulations showed that the separation of different sizes along the height is negligible (<20% in the most extreme cases) at the used heights (data not shown). Thus the differences in sampling height in our study do not explain the differences in pollen potency.

Several regions, such as France and Germany, appeared to have quite high potency of the pollen released, reaching values greater than 5 pg of Phl p 5 per pollen. The lowest potency in all years was registered in Eastern Europe in 2011 at less than 2 pg of Phl p 5 per pollen. However, most regions appeared to vary from year to year.

A crude estimate of reliability of these maps can be obtained from the SD of potency (Fig 4, right column). The high potency estimated in France is usually accompanied by a large spread that is similar but less than the values themselves. The stripes visible on the maps are individual footprints corresponding to substantially different observed potencies. Their overlap results in high uncertainty of the final pattern. In other regions, however, the uncertainty was lower and independent of the actual potency value.

Relation of potency and pollen counts

The average pollen potency stayed almost constant throughout the observed pollen count range (Fig 2, thick black

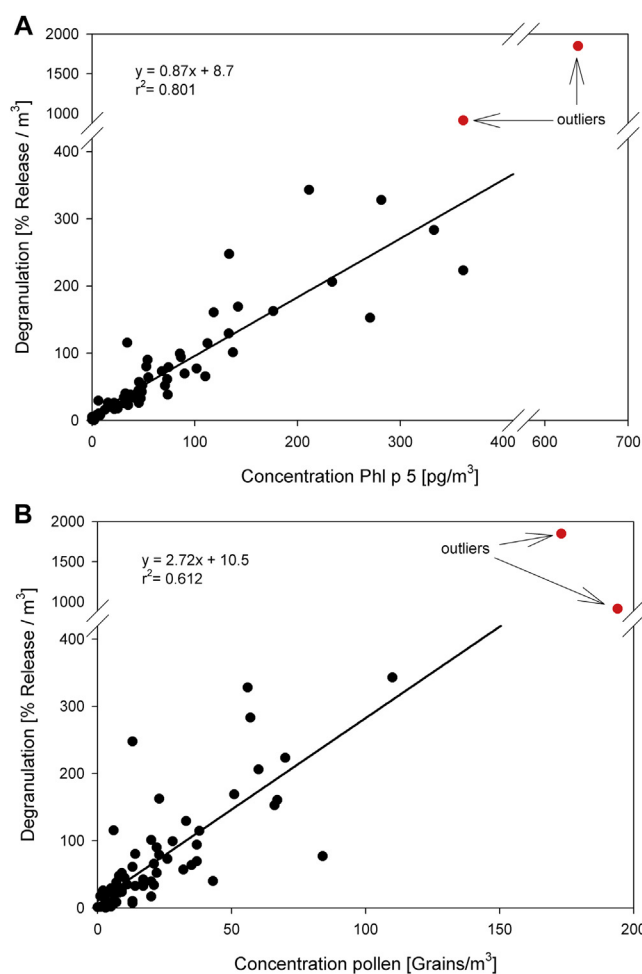


FIG 3. Degranulation of FcεRI-humanized rat basophils by extracts of ambient air. **A** and **B**, Degranulation caused by allergen of PM₁₀ (Fig 3, A) or degranulation caused by pollen (Fig 3, B). Two extremes indicated in the graph were excluded in both calculations of correlation (see the Methods section).

dashed line denotes its median). However, slight decreasing trends are noticeable for low and high counts. To the contrary, the spread clearly increases toward low counts. One can estimate what fraction of this variability is due to observational errors and what is to be attributed to natural potency variability. As stated above, the experimental error for pollen counts is about 5 pollen grains/m³ plus 20% of the observed value, and the error of ELISA is approximately 20%. Because potency distribution is non-Gaussian, robust estimates of the 1 and 2 SD ranges will be the differences between the potency values at 16% to 84% and 2% to 98%, respectively. As one can see, pollen varied more in potency than can be explained by the experimental errors.

Humidity

Allergen was collected in 2 fractions of ambient air. Within all of Europe, more than 89% ± 11% of the allergen was collected as PM₁₀. The geometric diameter of grass pollen is 20 to 40 μm,³⁸ exemplifying that allergen in ambient air coincides with pollen. However, on some days, allergen in 10>PM_{2.5},

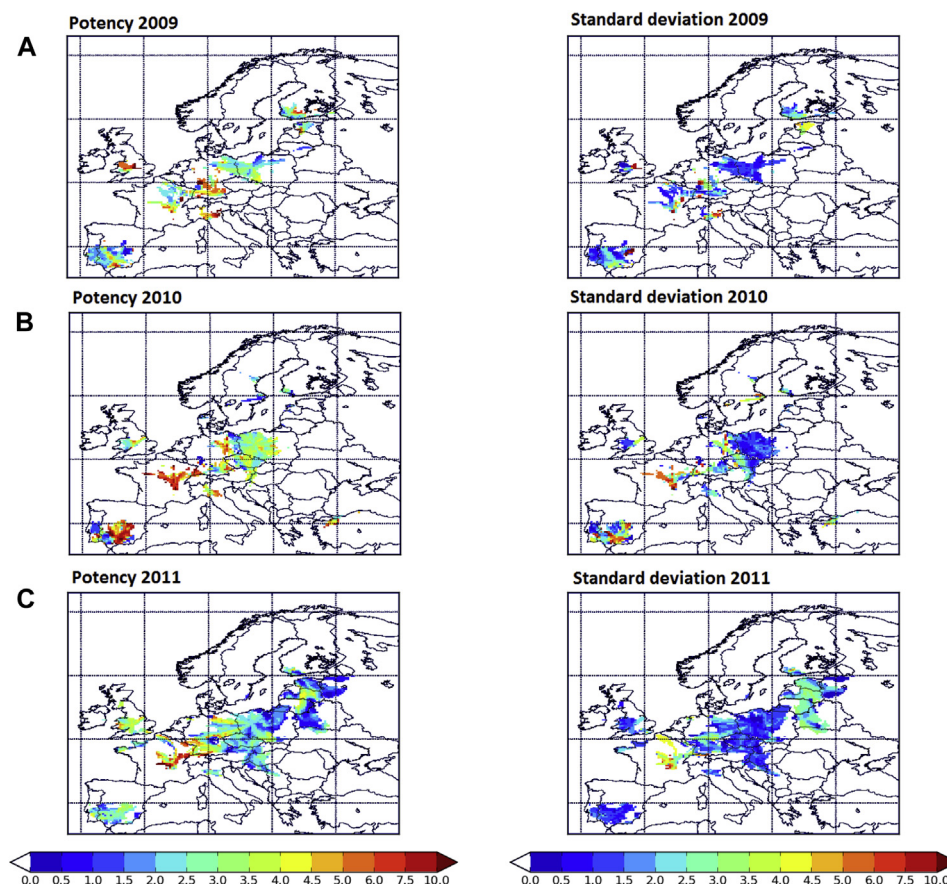


FIG 4. Map of Europe showing the potency of pollen to release allergen in 2009 (A), 2010 (B), and 2011 (C), depending on the pollen origin (source). Source attribution is needed because ripening of allergen occurs at the source. Concomitantly, the SD in the corresponding years is given. Pollen potency is not fixed to certain regions and changes between years.

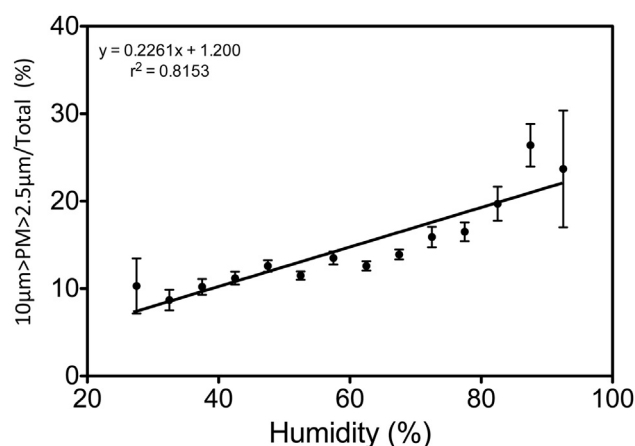


FIG 5. Phl p 5 as $10\mu\text{m}>\text{PM}>2.5\mu\text{m}$ versus total Phl p 5 in ambient air (total, ratio in percentages) for all stations for all years, depending on local relative humidity, showing allergen in the non-pollen-containing fraction of ambient air. Zero group 5 allergen values and values of less than 10 pollen grains/ m^3 were excluded. Means $\pm$ SDs are presented ($P < .001$, $n = 1252$).

where normally few pollen is detected, reached 20% of total airborne allergen. The allergen content of this fraction of air can contain smaller particles, which correlated with humidity ($P < .001$, Fig 5).

DISCUSSION

Pollen is the major outdoor cause for allergic rhinitis, and more than 350 pollen traps over Europe monitor pollen flight daily.³⁹ The immune system reacts with symptoms to allergens,⁴⁰ which are carried and released by pollen. The natural variability across Europe of how much allergen is carried by grass pollen was unknown.

This study shows that, on average, grass pollen in Europe released about 2.0 to 2.5 pg of group 5 allergens, the major grass pollen allergen, per pollen grain. This average value has a large variation because the amount of group 5 allergen released by the same amount of pollen ranged from less than 1 pg (detection limit) to 9 pg per pollen (5th to 95th percentile) and varied 7-fold between countries and years ($P < .01$).

Although pollen counts are indispensable for agriculture, phenology, climate change, and health, airborne allergen adds a new dimension to understanding allergic rhinitis and asthma. Indeed, depending on humidity, up to 20% of the allergen could be detected in the fraction of air containing smaller particles than pollen that could penetrate deeper into the lungs.

Pollen in ambient air correlated with allergic symptoms,^{26,40} also for grass pollen.⁴¹ However, the correlation is not clear because symptoms lag behind exposure and later in the season higher pollen counts do not evoke more symptoms. The human immune system reacts to allergens with symptoms, and allergen

release of pollen is not constant (Fig 1 and Table I). In addition, more events with high-potency pollen were observed when pollen counts were low (Fig 2). This could explain why even low levels of 2 to 9 pollen grains/m³ can result in allergic symptoms.⁴² Also, at high humidity, more allergen was detected in the fraction of air containing smaller particles that can penetrate deeper into the lungs⁴³ (Fig 5; ie, depending on humidity, a part of the allergen could penetrate deeper into the airways, approximately double at 100% relative humidity [rainy days] vs dry days with 30% relative humidity; $P < .001$). As a consequence, pollen concentration predicted allergen exposure but only with an r^2 correlation of 0.497 ($P < .001$, see Fig E2).

Allergen in ambient air

For birch pollen, a 10-fold natural variation in allergen release per pollen was published; for olive pollen, this was 12-fold.^{19,20} For the sake of comparing the allergen release variability for grass with those results, we also followed the approach of those studies and compared the mean potency of the top 10% of values with the lowest 10%. For grass, this additionally required excluding pollen without group 5 allergens and pollen with potencies of greater than 20 pg Phl p 5 per pollen. Also excluded were days with pollen counts of less than 10 pollen grains/m³. Then the variability in potency for grass pollen was estimated to be 17-fold. With all the ambiguity of such a comparison, the range of variations among the considered taxa appeared to be the largest for grass.

A weakness of this study is that no other grass pollen allergens, particularly group 1 allergens, were determined. Grass pollen exposure involves a succession of pollen from different grass species.⁴⁴ Grass pollen of different species release similar, cross-reacting group 5 allergens that are not identical (www.allergome.org, accessed August 2013). Thus *Dactylis glomerata* pollen releases Dac g 5, whereas *P pratense* releases Phl p 5. The response factor of each Phl p 5 analogue is different,¹⁸ as reported for other allergens, such as Phl p 1.¹⁷ We calibrated all samples against Phl p 5.0102, analogue to calibrating protein concentration against BSA,⁴⁵ because Phl p 5.0102 is the pharmacopeia standard for *Phleum* species preparations used in specific immunotherapy.³¹ For group 5 allergens, the difference in response factor between the early flowering major species *D glomerata* and the late blooming species *P pratense* is low, maximally about 2-fold, with Phl p 5 having the lower response factor. Differences in response factor are not sufficient to explain the observed difference in pollen potency (range, <1-9 pg of Phl p 5 per pollen [5th to 95th percentile]).^{17,18}

The flowering succession of grass species includes, among other minor species, *Alopecurus pratensis*, *Poa pratensis*, *Lolium perenne*, *D glomerata*, and *P pratense*.⁴⁴ *A pratensis* (beginning of the season) and *Phragmites* species (end of season) do not release Phl p 5 (and data not shown),^{8,18} and indeed, at the beginning of the season, several stations detected pollen without group 5 allergens (Fig 1 and see Figs E3 and E4). For instance, the lower potency of pollen in Munich at the beginning of the grass pollen season in 2011 (Fig 1) was not due to a lower response factor because *D glomerata* flowers before *P pratense* and has a higher response factor to Phl p 5.0102 but must be due to a lower release of total group 5 allergens per pollen.

Analogous to having pollen without group 5 allergens at the beginning of the grass pollen season, at the end of the grass pollen

season in Finland, where *Phragmites* species (common reed) are more abundant, also pollen with no Phl p 5 is detected. In other countries the contribution to pollen counts by this species is limited.^{18,46}

Mediator release

In the basophil degranulation bioassay β -hexosaminidase as a proxy for histamine release by the particle equivalent of 1 m³ of ambient air correlated well with allergen levels per cubic meter ($r^2 = 0.80$, $P < .001$) and pollen levels per cubic meter ($r^2 = 0.61$, $P < .001$, Fig 3). Pollen releases more allergy-modulating factors than allergens.⁴⁷ The degranulation assay also agrees with our hypothesis that pollen releases different amounts of Phl p 5, as reported previously for other pollen species.^{19,20} However, the basophil assay is not a substitute for allergy symptoms. It should also be taken into account that the reliability of the group 5 ELISA as a predictor of biological activity depends on the variability of the group 1/group 5 ratio. This highly significant correlation presumably indicates that the group 1/group 5 ratio in our air samples, which were all from the Munich area, is relatively constant and that the contribution of other allergens is small. We cannot exclude that pollen collected in Munich might have a different and/or more stable group 1/group 5 ratio than pollen collected in other parts of Europe.

Potency variations in time and space

Pollen grains varied in the amount of allergen they release. However, most data stem from single samples and might not be representative because of interday, intercountry, and interyear variability of the natural sources.^{17,18,48} In addition, allergen levels throughout the literature were quantified with different ELISAs, yielding variable results. Therefore we used the Development of Certified Reference Materials for Allergenic Products and Validation of Methods for Their Quantification proposed methods and reference materials,^{15,49} eliminating this variability.

Considering the potency maps and their SDs, one should keep in mind the peculiarity of grass pollen: in different regions and at different times, it is released by different species, which are likely to have different potencies. This is corroborated by substantial variability between the monthly potency maps for each season (data not shown). In Fig 4 this temporal variability contributes to the overall seasonal SD (Fig 4, right column).

At the source, variability in pollen potency can be the result of 2 ripening processes occurring simultaneously. First, pollens increase their allergen content rapidly on ripening inside the anthers just before pollination.²³ Second, when anthers have ripened sufficiently, they react to actual weather conditions, releasing pollen.⁵⁰ These parallel ripening processes mean that the amount of allergen in the released pollen depends on the actual weather before and during the flowering season, resulting in weather-dependent variations in potency at the monitoring site.

Pollens varied more in their potency than can be explained by experimental error (Fig 2). Potency has an uncertainty stemming from both uncertainty in pollen count and uncertainty in Phl p 5 determinations. For pollen counts, the inaccuracy is poorly

known,⁵¹ and we estimated it as 5 pollen grains/m³ plus 20% of the observed value, which is similar to the inaccuracy for birch pollen counts.¹⁹ For ELISA in this study, the inaccuracy was 20.4%, which is similar, as generally reported for ELISAs.^{19,52} Using both uncertainties, we showed that the observed potency variability was several-fold higher than what could be explained by the experimental error (Fig 2). Because we determined the amount of allergen in both fractions, PM₁₀ and 10>PM_{2.5} of ambient air, 2 explanation are possible. Either pollens with low pollen counts release more allergen or more allergen was already present as 10>PM_{2.5}, which could be free allergen.

Humidity

At higher relative humidity of ambient air during pollination, more allergen was detected as 10>PM_{2.5} (Fig 5). Pollen grains were detected in this fraction of air, despite their large size, because of incomplete separation by means of cascade impaction.^{29,53} At higher humidity, either more pollen was collected as 10>PM_{2.5} because of pollen grains changing their aerodynamic diameters or more allergen is present in the form of smaller particles. In contrast to other observations, which described extreme weather conditions, such as thunderstorms, when these effects appeared,^{24,54,55} we could show that by less extreme weather conditions, such as higher humidity, more allergen, either as changed pollen or as smaller particles, was available in ambient air that could penetrate deeper into the lung, evoking more severe symptoms. As grass pollen swells and increases its aerodynamic diameter on exposure to higher humidity (A. LeMoal, unpublished observation), we think the presence of more small Phl p 5-containing particles at higher humidity is the most likely explanation. This hypothesis was also suggested by others⁴¹ and even more closely related to the results of Schäppi et al⁴⁸ after light rainfall condition.

The results indicate that the pollen potency is not constant and, in addition, could be explained by a free fraction of allergen in ambient air that could depend on humidity and is more easily detected when less pollen is in the air (Figs 2 and 5).

Conclusion

Pollens from Europe varied in their natural capacity to release group 5 allergens. This is in addition to the natural variation in pollen counts. In a biological test system mediator release correlated better with ambient allergen concentrations than with pollen counts. No clear geographic pattern of pollen potency was detected. Although pollen is an excellent proxy for exposure, more can be learned from the actual monitoring of allergens in ambient air. Indeed, at higher humidity, more allergen seems to be present on smaller particles that could penetrate deeper into the airways.

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Key messages

- The same amount of pollen can release variable amounts of allergen ranging from less than 1 to 9 pg of Phl p 5 per pollen (5th to 95th percentile).
- This difference varies per location in Europe and during the pollen season.
- At higher humidity, the amount of group 5 allergen not associated with pollen increases.

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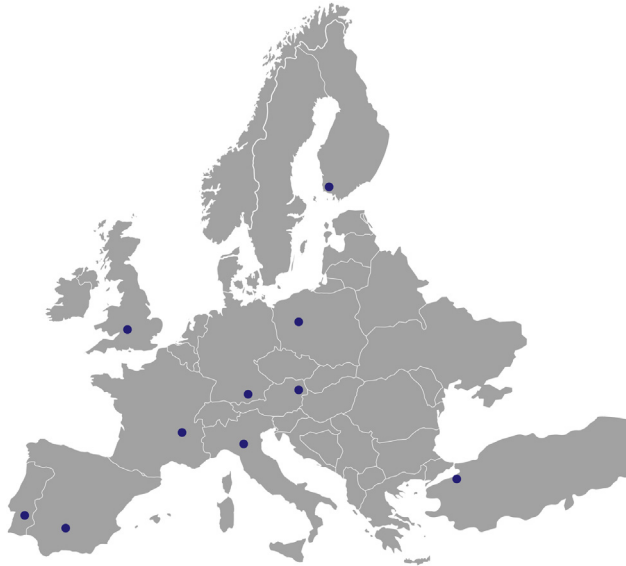


FIG E1. Map of station locations used in HIALINE.

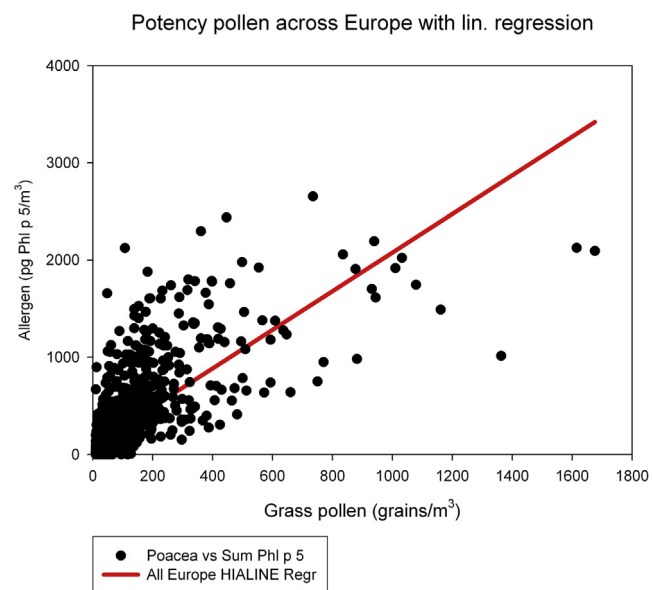


FIG E2. Pollen flight from all stations in 2009 until 2011 concomitant with airborne allergen concentration. Pollen values of less than 10 pollen grains/m³ were deleted for this correlation. The slope of the Pearson linear regression curve is given and represents allergen release per pollen (potency, 2.3 pg Phl p 5/pollen, $r^2=0.495$, $P < .001$, $n = 1629$).

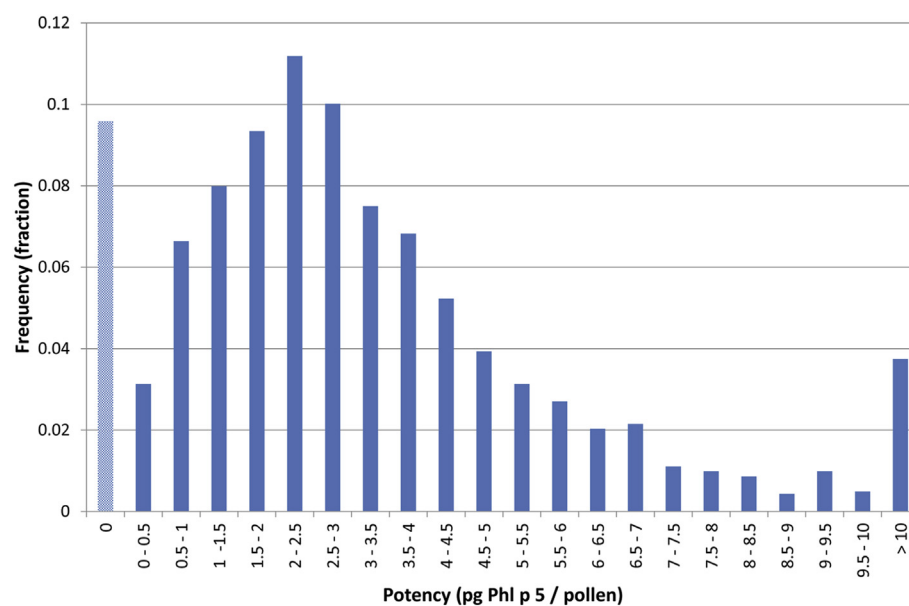


FIG E3. Histogram of grass pollen potency across Europe. The high frequency of low-potency pollen was dominated by pollen releasing no Phl p 5.

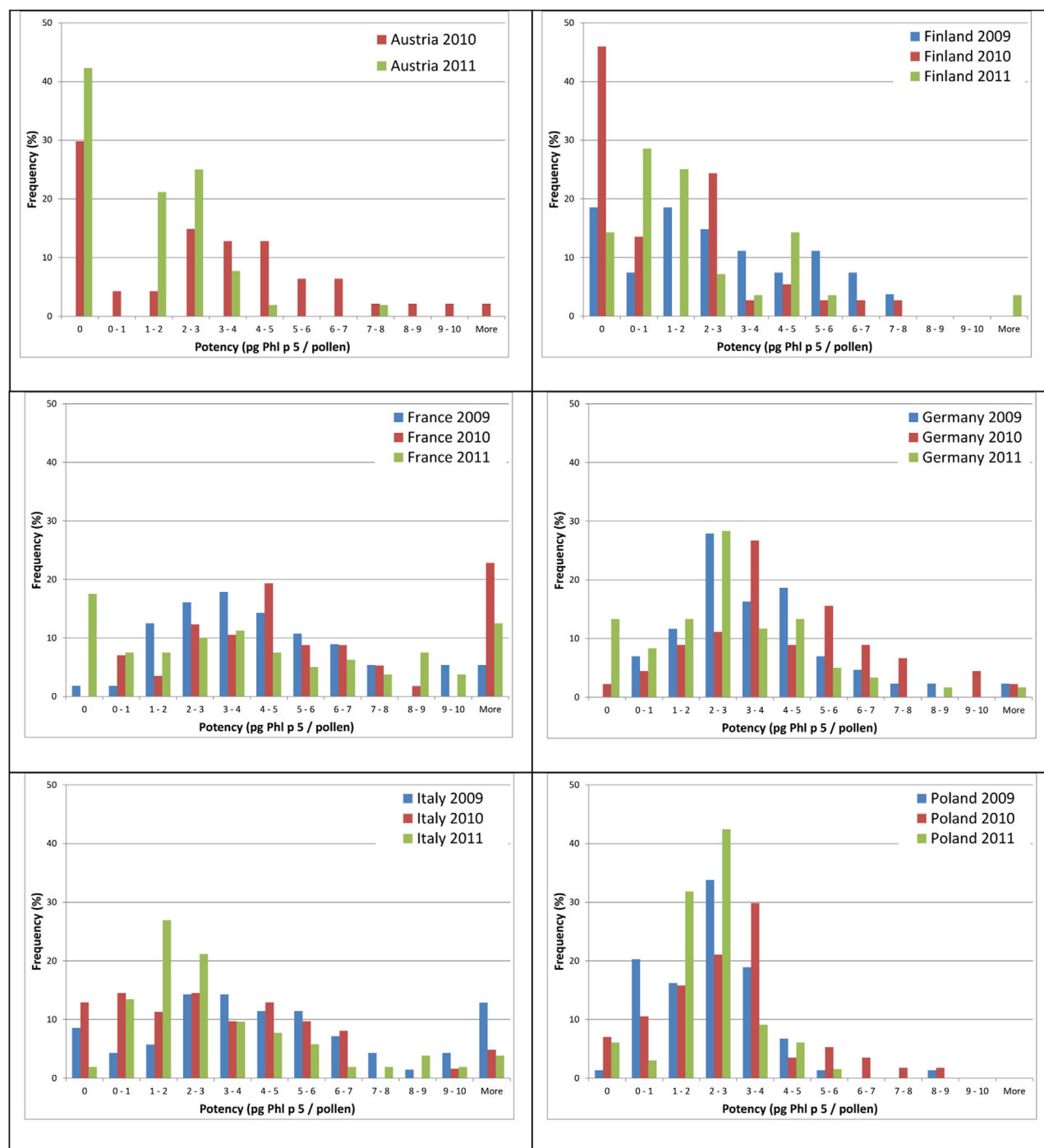


FIG E4. Histograms of grass pollen potency for each observation site and year.

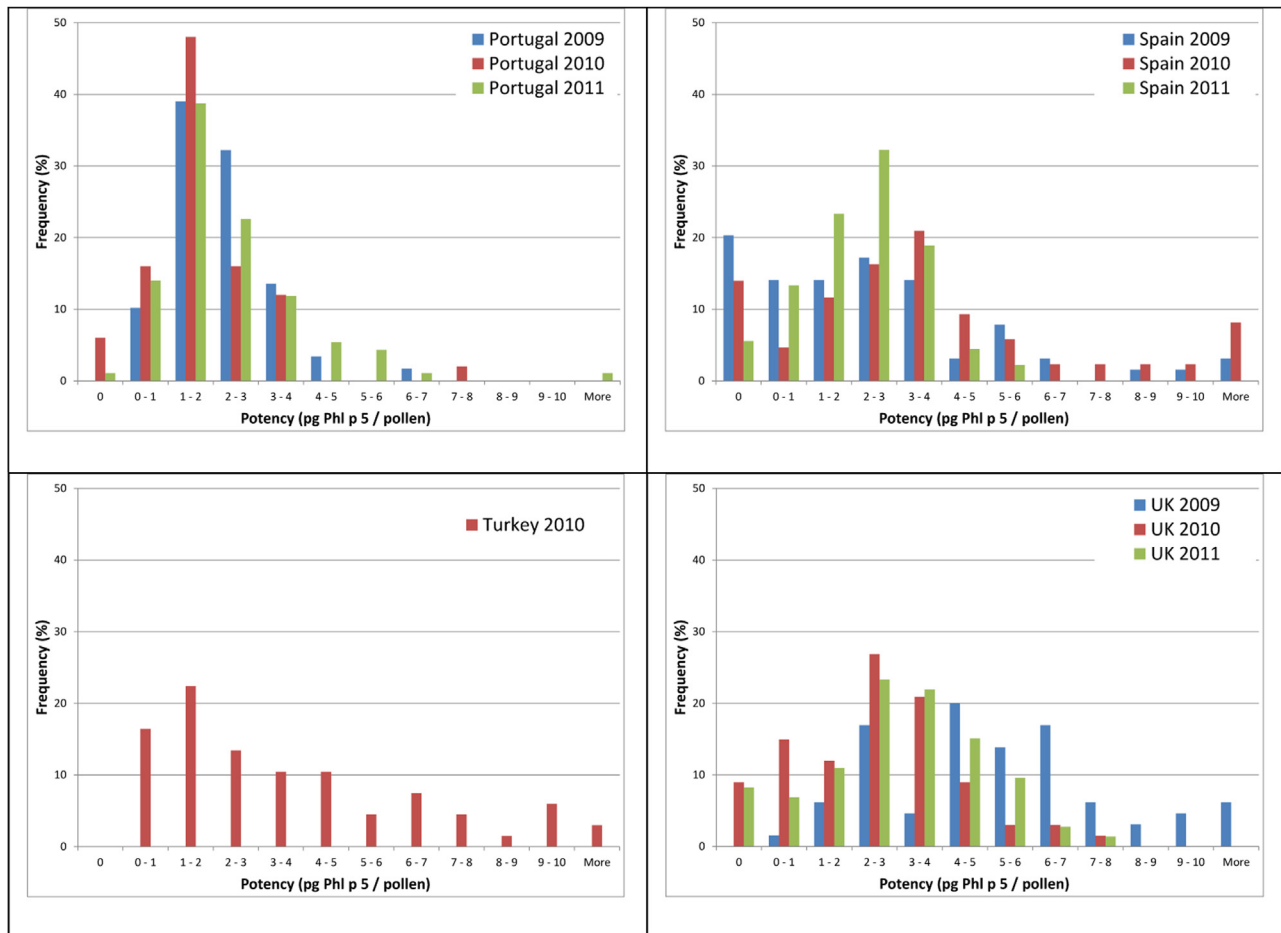


FIG E4. (Continued).

TABLE E1. Station descriptions

		Location	Above ground (m)	Above sea level (m)
Austria	Vienna	48°14'56.0" North, 16°21'22.0" East	9.0	209
Finland	Turku	60°27'18.34" North, 22°17'07.49" East	15	40
France	Lyon 2009-2010*	45°42'40.3" North, 4°29'34.6" East	2	478
	Lyon 2010†	45°45'09.5" North, 4°32'17.9" East	2	448
Germany	Munich	48°09'52.3" North, 11°35'35.4" East	1.8	520
Italy	Parma	44°48'16.2" North, 10°18'56.9" East	18	52
Poland	Poznan	52°28'01.8" North, 16°55'27.3" East	18	93
Portugal	Evora	38°34'07.2" North, 7°54'68.9" West	12	300
Spain	Cordoba	37°53'0.5" North, 4°46'45.0" West	22	123
Turkey	Bursa	40°13'21.2" North, 28°51'48.2" East	9	169
United Kingdom	Worcester	52°19'69.0" North, 2°24'20.0" West	10	43

ChemVol and Hirst-type pollen trap were located at the same location at heights within 5 m of each other.

*Saint-Genis l'Argentiére.

†Brussieu (8 km apart).