

Effect of confounding cofactors on responses to pollens during natural season versus pollen challenge chamber exposure

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Background: The severity of allergic rhinoconjunctivitis (AR) symptomatology elicited after exposure to pollen in the absence versus the presence of confounding cofactors, such as in a pollen challenge chamber (PCC) and the natural pollinating season, respectively, might differ.

Objective: We sought to determine the correlation of AR severity in the natural season versus out-of-season PCC exposures.

Methods: Twenty-four Virginia live oak (VLO)–positive, 14 VLO-negative, 16 mountain cedar (MC)–positive, 8 MC-negative, and 26 ragweed-positive participants recorded AR symptoms (total symptom score [TSS]) during the VLO, MC, and ragweed pollinating seasons and during 2 consecutive PCC exposures of 3 hours each to these pollens separately.

Results: The TSSs recorded before the natural season were higher than the pre-PCC values. This prepriming was greater among VLO⁺ than MC⁺ participants, and it blunted further increases in TSSs during the VLO natural season. Nonatopic participants were nonreactive in the PCC. There was wide variation in the level of AR symptomatology after exposure to VLO, MC, or ragweed pollen in the PCC. Prepriming formed the basis for higher AR responses observed in the natural season than in the PCC, resulting in the identification of distinct PCC/natural season endophenotypes and a partial correlation between the TSSs recorded in the natural season versus those recorded in the PCC ($r = 0.34, 0.54, \text{ and } 0.65$ for VLO⁺, MC⁺, and ragweed-positive participants, respectively).

Conclusions: Prepriming in the natural pollinating season might obscure the true correlation between AR severity in the natural

season versus the PCC. By mitigating confounding cofactors, PCC exposures have utility for evaluation of novel AR therapeutics. (*J Allergy Clin Immunol* 2013;■■■:■■■-■■■.)

Key words: Allergy, pollen, challenge, confounding, season

Although allergic rhinoconjunctivitis (AR) after exposure to pollen is highly prevalent, afflicting up to 25% of North Americans,¹⁻³ curative therapies are lacking. Most clinical trials investigating novel AR therapies rely on studies conducted in the natural pollen season.^{4,5} However, the extent to which environmental or host factors might confound trial results is unknown. Environmental factors could include competing coseasonal pollens, perennial allergens, respiratory irritants, and the variable intensity of pollination, whereas host-specific factors include intersubject differences in the level of AR symptoms evoked. In the present study we determined the nature and extent of this confounding by comparing AR symptomatology evoked in atopic subjects during the natural pollinating seasons for Virginia live oak (VLO), mountain cedar (MC), and ragweed in San Antonio, Texas, versus out-of-season exposures to these pollens in a pollen challenge chamber (PCC) in which AR symptoms are elicited in a controlled environment.⁶⁻¹³

METHODS

Study participants

The studies were approved by IntegReview Institutional Review Board, Austin, Texas. All participants provided written informed consent and met the inclusion/exclusion criteria shown in [Table E1](#) in this article's Online Repository at www.jacionline.org. Twenty-four subjects who were skin test reactive to VLO (VLO⁺ subjects) and 14 nonreactive subjects (VLO⁻ subjects), as well as 16 MC⁺ and 8 MC⁻ subjects, were evaluated. One subject with positive results to VLO and MC and 5 participants with negative results to VLO and MC participated in both the VLO and MC studies. Additionally, 26 ragweed-positive subjects participated in a previous PCC study,¹³ and for comparative purposes, the data from the first 2 of the 4 PCC exposures were used in this study. Ragweed-negative participants were not studied.

Study design

An overview of the study design during the natural season and PCC phases is shown in [Fig 1, A](#).

Natural season phase

Visit 1 occurred before onset of the natural MC and VLO pollinating seasons. Medical/medication history and physical examination and urine pregnancy test results in female participants were obtained. Participants were skin tested to 16 aeroallergens by using the prick technique (see [Table E2](#) in this article's Online Repository at www.jacionline.org).¹⁴ Participants were dispensed a medical events calendar to record

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Abbreviations used

AR: Allergic rhinoconjunctivitis
 MC: Mountain cedar
 PCC: Pollen challenge chamber
 TSS: Total symptom score
 VLO: Virginia live oak

health status and instructed on the use of subject daily and symptoms evaluation diaries. Symptoms were self-scored twice daily on a Likert scale (see Table E1). A total symptom score (TSS) was derived by adding the total nasal symptom score and total ocular symptom score, yielding minimum and maximum scores of 0 and 21, respectively (see Table E1). Daily counts for pollen grains and mold spores were from the National Allergy Bureau. Medical events calendars and diaries were reviewed at visits 2 and 3 that were scheduled during the predicted peak and conclusion of the pollinating seasons, respectively. For ragweed-positive participants, their first visit was during the peak of the season, as described previously.¹³

Out-of-season PCC visits

The out-of-season period was late spring for MC, midsummer for VLO, and late fall for ragweed (Fig 1, B). Participants returned out of season for reassessment and 2 (for VLO and MC) or 4 (for ragweed) PCC 3-hour exposure visits. Participants were dispensed a 5-day run-in diary and recorded symptoms for 4 days (twice daily) before and the morning of the PCC exposure. In the PCC each subject recorded total nasal symptom and total ocular symptom scores at 30-minute intervals. Participants were free to withdraw from the study for any reason. Defatted VLO pollen (mean $\pm$ SD, 10,000 $\pm$ 2,000 grains/m³), defatted short ragweed pollen (3,500 $\pm$ 500 grains/m³), and nondefatted MC pollen (12,500 $\pm$ 2,500 grains/m³) were delivered by a computer-controlled feeder, as described previously (see Table E3 in this article's Online Repository at www.jacionline.org).¹⁵ Methods for collecting and counting pollen in the PCC, as well as selecting the dose of pollens used, are as described previously^{13,15} and outlined in the Methods section in this article's Online Repository at www.jacionline.org. The source for the pollen was Greer Laboratories (Lenoir, NC).

Statistical analysis

The primary outcome was the TSS. For each subject, the mean $\pm$ SD of all TSSs recorded per patient was estimated, and the maximum TSS was defined as the maximum TSS recorded in the PCC or natural season that was within 2 SDs of the mean TSS. A Poisson model was used to compare the extent of skin test reactivity among the VLO⁺, MC⁺, and ragweed-positive study participants. The Pearson correlation coefficient was calculated to compare maximum TSSs in the natural season versus those in the PCC. Linear regression analyses were conducted, where appropriate. Unsupervised hierarchical clustering with Euclidean distances and average linkage were conducted, as described previously,¹³ to investigate (1) the heterogeneity in responses in the PCC by using the TSSs recorded at each of the time points in the 2 PCC exposures and (2) the relationship between symptoms recorded in the natural season versus in the PCC by using the maximum TSS estimated in the natural season and PCC phases (see the Methods section in this article's Online Repository). In this analysis, to determine the clusters, a horizontal line was drawn across the dendrogram, and where that line crossed a vertical line, it represented a new cluster.

RESULTS**Natural seasons in South Texas**

In South Texas there are 4 seasons of pollination (Fig 1, B).¹⁶ The winter season (late December to early February) has a single pollen, MC, that has the most intense of the pollinating seasons. The spring season can be divided into 3 subseasons of early,

calendar, and late spring. In the early spring (February-March), there is a continuation of the MC with the onset of ash and elm tree pollination. The calendar spring season (March to early April) is dominated by VLO, with strong contributions from hackberry, willow, and grass pollination. The late spring season (April to early May) involves primarily grass combined with onset of pecan pollination. The summer (June-August) has no significant pollen because of the heat and lack of rainfall. Grass, normally a summer pollinator, is biphasic, with spring and fall periods of pollination. The fall season (September-November) has 2 subseasons, with the early period beginning in the late summer, during which the fall elm and grass pollinates. Ragweed and other weeds pollinate in the calendar fall season. There is a null-pollen period, usually from the beginning of November until MC pollinates in mid to late December. The historical peak pollen counts for VLO, MC, and ragweed recorded in San Antonio, Texas, during the calendar years 2006 to 2012 are shown in Table E3. Perennial allergens, including mites, mold spores, and animal danders, are present throughout the year (Fig 1, B).¹⁷

Study participants

Demographic characteristics of the VLO⁺, VLO⁻, MC⁺, MC⁻, and ragweed-positive participants are shown in Table E4 in this article's Online Repository at www.jacionline.org. In each participant group there were more female than male subjects, except for MC⁻ subjects, which had equal proportions of male and female subjects. Age ranged between 20 to 72 years. Differences in the proportion of European, African, and Hispanic American subjects among the VLO, ragweed, and MC participant groups did not reach statistical significance (see Table E4). Atopic participants reacted to multiple aeroallergens (see Table E5 in this article's Online Repository at www.jacionline.org). The median numbers of positive skin test results (of 16 aeroallergen tested) for VLO⁺, ragweed-positive, and MC⁺ participants were 14.5 (interquartile range, 11.50-15.25), 12.0 (interquartile range, 9.00-14.00), and 9.0 (interquartile range, 6.00-12.00), respectively. VLO⁺ and ragweed-positive participants, respectively, were allergic to 1.54 and 1.35 times more pollens than MC⁺ participants ($P < .001$ and $P = .006$, respectively; see Table E5). There was a trend for VLO⁺ participants reacting to a higher proportion of aeroallergens than ragweed-positive participants ($P = .114$).

Natural season phase

For VLO⁺ participants, the mean TSS at the beginning of the natural season and the maximum mean TSS recorded during the observation period in the VLO season were 12.75 (SD, 4.61) and 16.32 (SD, 3.42), respectively (Fig 1, B, and Fig 2, A); the corresponding values for the VLO⁻ participants were 1.14 (SD, 2.55) and 1.14 (SD, 2.55), respectively. For MC⁺ participants, the mean TSS at the beginning of the natural season and the maximum mean TSS recorded during the observation period in the MC season were 6.63 (SD, 4.66) and 14.88 (SD, 4.72), respectively (Fig 1, B, and Fig 2, B); the corresponding values for the MC⁻ participants were 0.25 (SD, 0.87) and 2.00 (SD, 5.08), respectively. The maximum mean TSS for ragweed-positive participants during the observation period in the ragweed season was

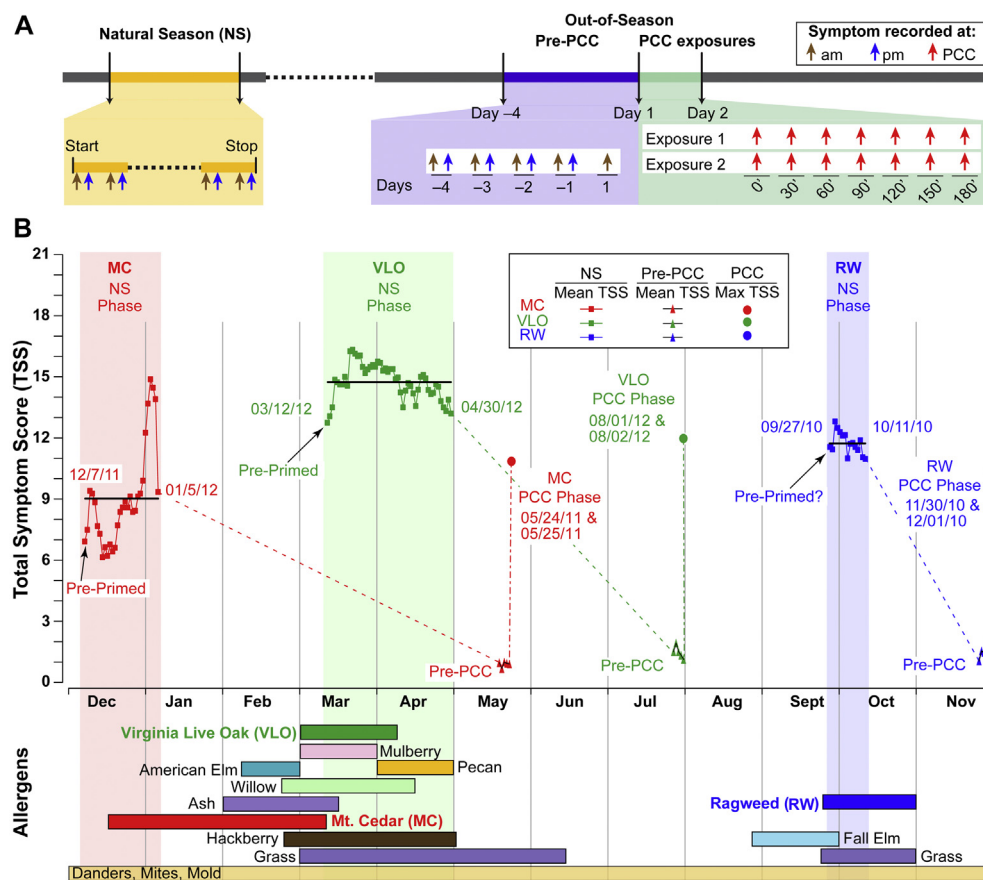


FIG 1. Study design and TSSs in the natural season (NS) and in the PCC. **A**, Study design. **B**, TSSs in the NS and PCC phases of the study. Color-coded boxes at the bottom indicate the intervals of the predicted pollen seasons and perennial factors. The vertical shaded boxes are the observation periods in the NS. Mean TSSs recorded on each day during the NS are indicated by using color-coded symbols, whereas the horizontal black line is the mean TSS in the NS. The mean of the maximum TSS of the study participants in the PCC phase is shown. Pre-Primed?, Evidence for prepriming is lacking because TSSs were not recorded before the ragweed NS. RW, Ragweed.

12.81 (SD, 5.32; Fig 1, B, and Fig 2, C); TSSs before the ragweed season were not recorded. These data indicated that VLO⁺ and MC⁺ participants were preprimed before entry into the VLO and MC natural seasons, respectively, and that the level of pre-priming was greater for VLO⁺ than MC⁺ participants (preseason TSS of approximately 13 vs approximately 7, respectively; Fig 1, B, and Fig 2, A and B).

Coincident with the spike in VLO or MC counts during the natural season, the increases in mean TSSs from preseason to maximum levels recorded during the observation period in the natural season phase of the study were 28% and 124% in VLO⁺ and MC⁺ participants, respectively (Fig 2, A and B). Regression analyses demonstrated that the higher the preseason TSS of either VLO⁺ or MC⁺ participants, the smaller the increase in TSS during their respective natural seasons (Fig 2, A and B, insets). These data suggested that higher preseason TSSs associated negatively with subsequent increases in TSSs during the natural season.

PCC phase

TSSs of atopic participants before entry into the PCC were much lower than those recorded before or during the natural season (compare time 0 in Fig 3 vs day 1 in observation period in

Fig 2). Pre-PCC TSSs of VLO⁺ and MC⁺ participants were similar to those recorded in nonatopic participants (Fig 3, A and B). During the priming (day 1) exposure in the PCC, atopic but not VLO⁻ and MC⁻ participants were responsive to pollen exposure (Fig 3, A and B). A similar response pattern but with slightly higher TSSs was observed during the second challenge day for each of the pollens tested, and 88%, 75%, and 73% of the VLO⁺, MC⁺, and ragweed-positive participants, respectively, achieved TSSs of 7 or greater (Fig 3).

Clustering analyses of TSSs recorded during the PCC exposures (Fig 4, A-C) and the corresponding trajectories of the TSSs of the subgroups defined by these clusters (Fig 4, D-F) demonstrated that interindividual differences in responses to pollen exposure in the PCC were highest, intermediate, and least among VLO⁺, MC⁺, and ragweed-positive participants, respectively. At the conclusion of the PCC exposures, the MC⁺ and ragweed-positive participants broadly classified into those with higher versus lower responsiveness, whereas the VLO⁺ participants categorized into those with lower, intermediate, and higher responsiveness (Fig 4, D-F).

In general, atopic participants who manifested higher versus lower TSSs during the first PCC exposure exhibited higher versus lower TSSs on the second PCC exposure (Fig 4, G-I). In these

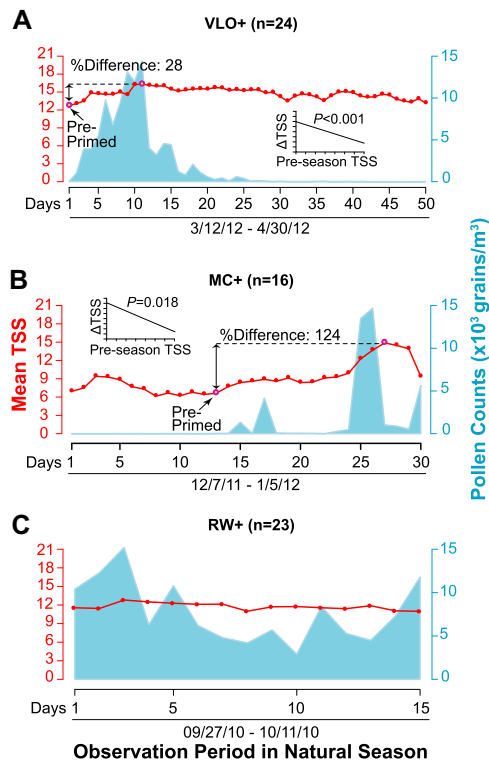


FIG 2. Pollen counts and mean TSSs of atopic participants during natural pollinating seasons. TSS data from VLO⁺ (A), MC⁺ (B), and ragweed-positive (C) participants. The average TSS for each day is shown. Δ TSS, Difference between mean TSSs recorded before the season and maximum TSSs recorded during the observation period in the natural season. Association of TSSs recorded before the season and subsequent changes in TSSs during the observation period in the natural season are shown as inserts in Fig 2, A and B (determined by using regression analyses). RW, Ragweed.

analyses the median TSS from all participants recorded on days 1 and 2 separately was estimated (population-level median), and whether a subject manifested a TSS that was greater than versus less than this population-level median on day 1 and 2 challenges was determined (Fig 4, G-I). For example, among VLO⁺ participants, subjects with a higher TSS during the first PCC exposure had a 13 times higher likelihood (odds ratio, 12.93; $P = .012$) of also having a higher TSS during the second PCC challenge (Fig 4, G).

Correlation of TSSs in the PCC versus the natural season

The overall correlation between the maximum TSS recorded in the natural season versus in the PCC was lower for VLO⁺ ($r = 0.34$, $P = .09$) than for MC⁺ ($r = 0.54$, $P = .03$) and ragweed-positive ($r = 0.65$, $P < .01$) participants (Fig 5, A-C). Clustering patterns (Fig 5, D-F) and corresponding scatter plots (Fig 5, A-C) of the maximum TSSs recorded in the PCC and during the observation period in the natural season phase of the study indicated that the VLO⁺, MC⁺, and ragweed-positive participants classified into distinct PCC/natural season AR endophenotypes. There was a wider range of endophenotypes among the VLO⁺ participants (Fig 5, D-F). None of the endophenotypes observed had the feature of symptom scores that were higher in the PCC than in the natural season (Fig 5, D-F). The PCC/natural season endophenotype categories and their frequencies (in parentheses)

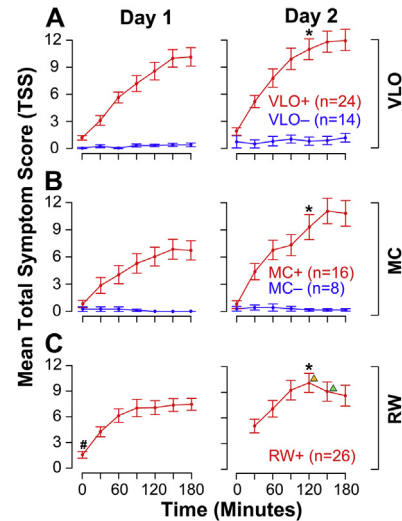


FIG 3. Trajectory of mean TSSs (with SEs) during PCC exposures. TSS data after exposure to VLO (A), MC (B), and ragweed (C) pollens. * $P < .05$ for differences in mean TSSs at day 2 versus day 1 at 120 minutes. Triangles, At day 2, 2 ragweed-positive participants exited at 120 minutes and 1 subject exited at 150 minutes because of high TSSs. #TSS values recorded during the run-in period before the PCC. Ragweed-negative participants were not available for analysis.

were low-high (33%), moderate-high (46%), and high-high (21%) for the VLO⁺ participants; low-low (50%) and high-high (50%) for the MC⁺ participants; and low-low (23%), low-moderate (8%), moderate-high (23%), and high-high (46%) for the ragweed-positive participants (Fig 5, D-F).

Eosinophil counts in the PCC versus the natural season

Eosinophil counts were available from MC⁺ participants and were used to evaluate changes in eosinophil counts after PCC exposures (before vs after the PCC; Fig 6, A) and during the MC natural season (before vs during the natural season; Fig 6, B) and the influence of prepriming on eosinophil counts (before the PCC vs before the natural season; Fig 6, C). We observed that although eosinophil counts increased in the PCC (Fig 6, A), there were minimal changes in eosinophil counts before and during the natural season (Fig 6, B). Furthermore, eosinophil counts before the PCC were lower than those before the natural season (Fig 6, C), suggesting that eosinophil counts were increased before onset of the MC natural season. Consistent with this finding, eosinophil counts before onset of the natural season were similar to those recorded after the PCC (Fig 6, A and B).

DISCUSSION

This study has 4 major findings, and our inferences are derived by the sum of the results summarized in Table E6 in this article's Online Repository at www.jacionline.org.

First, on separate exposures to VLO, MC, or ragweed pollen in the PCC, atopic subjects responded to these pollens by mounting symptoms consistent with AR. Responses were greater during the challenge exposure (day 2) compared with the priming exposure (day 1) in the PCC. Highlighting the specificity of these findings, the majority of the nonatopic participants did not respond to pollen exposures in the PCC. Clustering analyses revealed that there are wide interindividual differences in responsiveness to

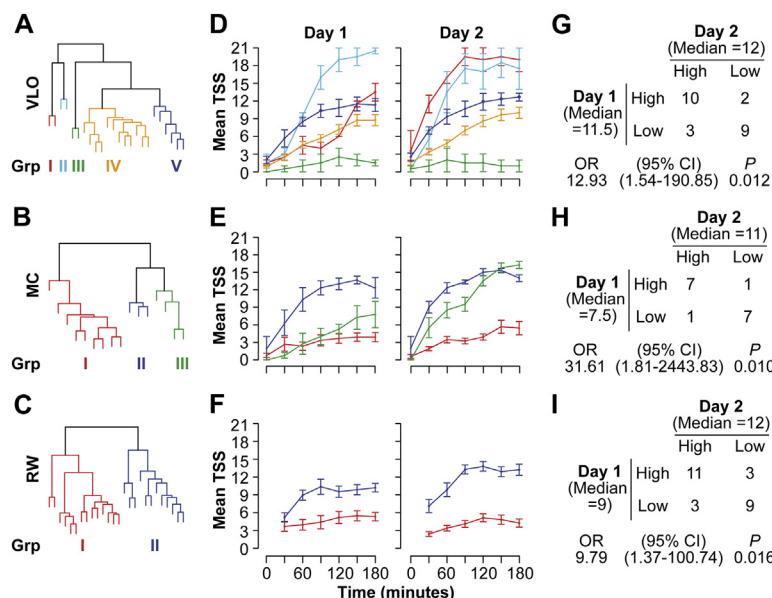


FIG 4. Interindividual differences in responsiveness to pollen exposure in the PCC. **A-C**, Hierarchical clustering was performed on the TSS measurements recorded by atopic subjects during each time point of the 2 PCC exposures. **D-F**, Mean TSSs with SEs were plotted for each 30-minute time point during the 2 PCC exposures for each of the subgroups identified by using hierarchical clustering, as shown in Fig 4, A to C. **G-I**, Association of higher versus lower maximum TSSs recorded on day 1 versus day 2. Higher versus lower was stratified according to the median of the maximum TSS recorded on day 1 and day 2 separately. OR, Odds ratio; RW, ragweed.

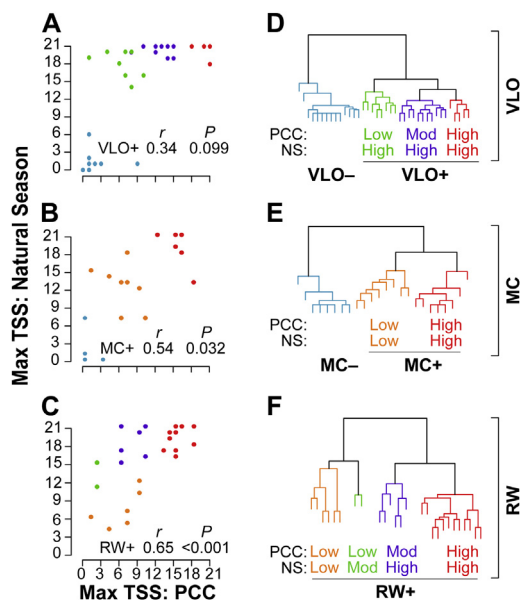


FIG 5. Correlation between maximum TSSs in the natural season (NS) and in the PCC. Scatter plots (**A-C**) and unsupervised hierarchical clustering (**D-F**) of maximum TSSs in the NS and in the PCC. Data for ragweed-negative participants were unavailable. Mod, Moderate; RW, ragweed. r (rho) and P values are for comparisons of the TSSs of atopic subjects in the NS versus the PCC.

pollen exposures in the PCC, with greater heterogeneity among VLO⁺ participants. However, the level of responsiveness during the priming and challenge exposure in the PCC were highly concordant, suggesting that responses to pollen in the PCC are not stochastic.

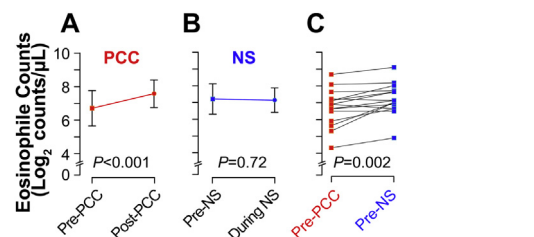


FIG 6. Changes in eosinophil counts in the PCC and in the natural season (NS) in MC⁺ participants. Peripheral blood eosinophil counts of 16 MC⁺ subjects who participated in both the NS and PCC phases of the study were compared before the PCC versus after the PCC (**A**), before the NS versus at the end of the observation period of the MC NS (**B**), and before the PCC versus before the NS (**C**). P values are from paired t tests.

Second, evaluation of atopic participants for whom natural season data were available (VLO⁺ and MC⁺ subjects) indicated that subjects entered the natural season preprimed. The pre-PCC symptom scores of atopic subjects were less than symptom scores before the start of the natural season. Biological data supported this prepriming effect. In MC⁺ participants exposure to MC in the PCC was associated with an increase in eosinophil counts to levels that were observed before the start of the MC natural season. Thus eosinophil counts before the start of the natural season were greater than those present before entry into the PCC, providing evidence for an effect of prepriming on eosinophil counts. By contrast, eosinophil counts were similar before and during the MC natural season. These contrasting changes in eosinophil counts in the PCC versus the natural season highlight the use of the PCC for elucidation of AR pathogenesis in the absence of seasonal cofactors that might confound biological responses directly attributable to exposure of specific pollens.

Third, we demonstrate that prepriming before the onset of the natural season might obscure or alter the level of subsequent responsiveness to pollen during the natural season. We found that higher levels of prepriming (as reflected by higher preseason TSSs) correlated negatively with the extent to which AR symptomatology increased further during the natural season. The lower levels of prepriming in MC⁺ compared with VLO⁺ participants (preseason TSSs of approximately 7 vs approximately 13, respectively) might provide a basis for why the TSSs increased more prominently in MC⁺ than VLO⁺ participants during the MC and VLO natural seasons, respectively (approximately 124% vs approximately 28%, respectively).

Fourth, prepriming also influenced the level of concordance in symptom scores in the natural season versus that in the PCC. On the basis of the AR symptomatology observed in the PCC versus the natural season, the atopic participants categorized into distinct PCC/natural season AR endophenotypes. In no instance did we identify a PCC/natural season AR endophenotype wherein symptom scores were less in the natural season compared with those in the PCC. VLO⁺ participants had higher levels of prepriming compared with MC⁺ participants, and the proportion of atopic subjects who manifested concordant (low-low or high-high) PCC/natural season endophenotypes was greater among the MC⁺ and ragweed-positive than VLO⁺ participants. The VLO⁺ participants were also distinctive in that the low-low and moderate-high PCC/natural season endophenotypes were absent and overrepresented, respectively, among these participants. These differences in the relative distribution of the endophenotypes might be the basis for the observation that the level of correlation between the TSSs recorded in the PCC and natural season for MC⁺ and ragweed-positive participants was higher, whereas it was much lower for VLO⁺ participants.

Collectively, these findings underscore that exposure to pollen in a PCC provides a unique modality to define the level of responsiveness of AR symptomatology that is attributable to pollen and mitigates the prepriming effects observed during the natural season. There are 3 major pollens in the San Antonio area that are associated with AR (MC, VLO, and ragweed).^{2,6} VLO tree pollen contributes to the spring pollen load in South Texas.¹⁸ Our VLO⁺ participants had more skin test reactivity to other pollens than the MC⁺ or ragweed-positive subjects. All of the VLO⁺ participants were also allergic to MC, and 65% or more were sensitive to other tree or grass pollens. For this reason and because the MC season precedes and partially overlaps with the VLO season, the VLO⁺ participants might have been preprimed by MC and other allergens, and this resulted in their commencing the VLO season with higher preseason TSSs. Hence seasonal cofactors that promote prepriming and the greater sensitivity of VLO⁺ participants to multiple allergens together might account for why these subjects maintained TSSs that were greater during the natural season than those recorded in the PCC and, consequently, a low correlation between TSSs recorded in the VLO natural season and the PCC.

Similar dynamics occur before the MC and ragweed seasons, although on a lesser scale. For example, although the MC season has no competing confounding pollen, mold spore counts are high in the late fall period because of increased rainfall and dead leaves on the ground. Hence prepriming from the latter confounding cofactors might have accounted for why MC⁺ participants entered the MC season preprimed with moderately high symptom scores. A limitation of our study is that we did not evaluate

ragweed-positive participants before the onset of the natural season and hence cannot fully describe the extent of prepriming that occurs in ragweed-positive participants.

Our results suggest some advantages for the conduct of a clinical trial for novel AR therapeutics in a PCC versus during the natural season. Envisage a clinical trial for VLO conducted in South Texas. Foremost, our data suggest that VLO⁺ subjects would enter the natural season preprimed (ie, with increased AR symptoms related to the preceding pollen season [MC] or other cofactors), a scenario that we show blunts further increases in AR symptoms related to VLO exposure. In part, this might mask the true efficacy of the therapeutic agent evaluated.¹⁹ Second, timing the onset of VLO pollination is difficult. Third, because pollination seasons overlap, this reduces the possibility of evaluating pollen-specific therapeutics in the natural season. In contrast, out-of-season PCC exposures mitigate this confounding, as well as other factors that might affect AR symptomatology (eg, mold, humidity, dust, and temperature).^{6-13,17,20} Fourth, the recording of symptoms measures over the entire natural season by trial participants might, because of trial fatigue or inattention, introduce errors. By contrast, the rapid induction of symptoms in a PCC offers the advantage of reducing the duration of clinical trials with potentially less error.

Finally, evaluation of atopic subjects from baseline (non-primed) levels in the PCC might provide a more accurate identification of subjects who exhibit greater versus lesser responsiveness to pollen exposure. Such categorization might be an important consideration because unequal representation of participants with higher versus lower symptoms in the placebo versus treatment arm of a double-blind randomized clinical trial might confound the analysis. Because most clinical trials for novel AR therapeutics are initially tested in relatively small sample sizes, randomization might not obviate the confounding if the trial arms are imbalanced for participants who are high versus low responders to pollen. For example, a greater proportion of high responders in the placebo arm compared with the therapy arm might artificially decrease the estimated efficacy of the therapeutic tested.

We are mindful of the limitations of conducting a clinical trial in a PCC. Foremost, our findings might not be generalizable to low-exposure settings in the PCC. Also, given the artificial nature of the exposure, a PCC does not mimic the complexity and length of exposure seen during the natural season.

Our results also have implications for evaluating the mechanisms that underpin AR pathogenesis. Typically, the study of AR pathogenesis is conducted by analyses of biological samples of atopic participants obtained during the natural season and comparing results with those obtained by using similar biological samples from nonatopic participants. However, such cross-sectional analyses do not account for the contribution of environmental factors unrelated to the pollen season (eg, mold) in modifying the experimental parameters evaluated (eg, transcriptional profiles or cytokine biomarkers). This confounding can be potentially mitigated by analyzing biological samples obtained before and after pollen challenge in a PCC, as illustrated by our findings related to changes in eosinophil counts in the natural season versus the PCC. On balance, our data suggest that the PCC offers unique advantages for the conduct of randomized clinical trials for AR therapeutics and the study of AR pathogenesis compared with similar studies in the natural pollinating seasons.

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Clinical implications: Seasonal cofactors resulted in higher AR symptomatology than induced in the PCC. Seasonal cofactors might confound clinical trials conducted in the natural season setting.

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METHODS

Methods for determination of pollen counts delivered in the PCC

In a previous study we described the methods by which pollen counts delivered in the PCC were estimated.^{E1} Briefly, pollen was collected by using 2 methods: (1) Allergenco-D Posi-track cassettes (Environmental Monitoring Systems, Charleston, SC) powered by suction pumps at 15 L/m impacted the pollen grains onto a silicone-coated microscopic slide at 5 locations within the PCC (center and 4 corners), which were then counted microscopically at 30-minute intervals, and (2) an Allergenco MK-3 staged collector operated in concert with the cassettes, and values were determined at the end of the PCC exposure.

Considerations to choose the appropriate pollen counts in the chamber

Table E3 shows peak pollen counts for VLO, MC, and ragweed during the natural season over the past several years. These counts were extremely high (presumably for short time periods) on these days. Signs and symptoms seen in patients on these days can also be very intense, and patients respond to treatment at these levels. These levels in the natural season provided some guidance regarding the dose of pollen to use.

Table E3 shows that the range of MC pollen counts during the peak of the natural seasons for the years 2006 to 2012 were very wide: approximately 8,380 to 48,000 grains/m³, with mean counts of approximately 22,097 grains/m³. The optimal dose selected was based on our clinical experience, the pollen counts in San Antonio, and the duration of priming and number of daily challenge PCC runs. Because the exposure to pollen in the PCC is for a short duration of time (typically 3 hours), to mimic AR symptomatology observed in the natural season, we typically initiate our evaluation with higher pollen counts in the PCC compared with the average pollen concentration during the natural season and then optimize this dose (higher or lower) in trial PCC runs.

To estimate the optimal dose of MC pollen to be used in the PCC, we examined the responsiveness of various doses of this pollen. In our previous work^{E1} we have provided details regarding the graded approach we used to determine the level of MC pollen that causes approximately 60% of participants to respond with symptoms after exposure to pollen in the PCC (ie, development of TSSs ≥ 7). In trial runs we selected a dose of pollen that (1) elicited the total range of symptoms seen during natural exposures, (2) did not elicit any unbearable signs or symptoms, (3) did not cause nonatopic subjects any significant symptoms, and (4) did not maximize symptom scores in a significant number of sensitive participants. An identical approach was taken for the oak-sensitive population. The level for ragweed pollen was taken from the

existing literature.^{E2} Levels of ragweed pollen that we used in the PCC were greater than the pollen counts in San Antonio in the natural season.

These were the general principles we used while selecting the adequate pollen dose to be used in our PCC. Because the pollen doses we used were calibrated to our PCC and because the delivery systems of the pollen and the air-conditioning and filtration units are likely to differ from PCC to PCC, the dose required to elicit symptomatology might differ in other chambers.

Methods for cluster analysis

Unsupervised hierarchical clustering with Euclidean distances and average linkage were conducted, as described previously,^{E3} to investigate the following. In both analyses, to determine the clusters, a horizontal line is drawn across the dendrogram, and where that line crosses a vertical line, it represents a new cluster.

1. *Differences among subjects in the responses elicited in the PCC.* For these analyses we used all the TSS measurements at each time point recorded during each of the 2 PCC exposures (Fig 4). The clustering algorithm compares subjects at each time point and does not compare the time points. By including the TSSs recorded from the 2 PCC exposures in the clustering analyses, we differentiated those subjects whose responses on day 2 were different from their responses on day 1 and those subjects with concordant responses on days 1 and 2, while also stratifying subjects by magnitude of response.
2. *Relationship between symptoms recorded in the natural season versus the PCC by using the maximum TSS from the natural season and the maximum TSS from the PCC phases* (Fig 5). The maximum TSS in the natural season versus that in the PCC could be either concordant or discordant. For example, a person might be classified as a low responder in the PCC but a high responder in the natural season (ie, have the low-high PCC/natural season AR endophenotype).

REFERENCES

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TABLE E1. Inclusion/exclusion criteria for entry into the PCC phase of the study

Inclusion criteria for nonatopic participants
• Age, 18-65 years • No history of seasonal AR to VLO, MC, or ragweed • Negative response on VLO, MC, or ragweed skin tests
Inclusion criteria for atopic participants
• Age, 18-65 years • History of seasonal AR to VLO, MC, or ragweed • Positive response on VLO, MC, or ragweed skin test
Exclusion criteria
• Nasal or ocular symptoms from perennial allergy • Pregnant or lactating female subject • Significant medical illnesses or abnormalities that might interfere with the study • Respiratory tract infection, bacterial sinusitis, or both during the 2 weeks before the screening visit • Medical history of significant pulmonary disease, active asthma, or both requiring daily drug therapy • Participation in a clinical trial with an investigational drug in the last 30 days • History of rebound nasal congestion from extended use of topical decongestants • Initiation of immune therapy unless receiving a stable dose for ≥ 1 month before the screening visit • History of nasal malformations or history of alcohol and/or drug abuse
Symptom scores (self-scored)
• Likert symptom scale: 0, absent; 1, mild; 2, moderate; and 3, severe • TNSS: Sum of scores based on nasal congestion, sneezing, rhinorrhea, and itching • TOSS: Sum of scores based on ocular redness, tearing, and itching • TSS: Sum of the TNSS and TOSS

TNSS, Total nasal symptom score; TOSS, total ocular symptom score.

TABLE E2. List of aeroallergens tested by using skin prick tests in study participants

Mountain cedar	Virginia live oak	Pecan	Fall elm
Mesquite	Arizona ash	Johnson grass	Bermuda grass
Mixed ragweed	Spiny pigweed	Mold mix I	Mold mix II
Dog dander	Cat dander	Dust mite mix	Cockroach

TABLE E3. Historically highest pollen counts during calendar years 2006 to 2012 and pollen counts delivered in the PCC

Year	2006	2007	2008	2009	2010	2011	2012	Average, 2006-2012 (grains/mm ³), mean ± SD	Pollen delivered in PCC (grains/mm ³), mean ± SD
VLO count 1	2,700	8,720	12,450	8,270	40,000	19,640	9,300	12,512 ± 9,739	10,000 ± 2,000
VLO count 2	1,830	7,350	10,150	7,840	21,700	16,790	8,430		
MC count 1	20,250	21,300	14,200	21,850	48,000	32,000	27,400	22,097 ± 9,634	12,500 ± 2,500
MC count 2	12,860	17,660	8,380	18,170	26,760	18,530	22,000		
Ragweed count 1	860	1,180	620	160	1,520	120	1,030	674 ± 460	3,500 ± 500
Ragweed count 2	520	750	550	90	1,230	90	720		

Shown are pollen counts recorded in a natural open area at ground level by calendar year and pollen counts delivered in the PCC. Counts 1 and 2 refer to the 2 highest counts recorded during natural peak seasons (courtesy of Paul H. Ratner, MD).

TABLE E4. Demographic characteristics of the study participants

Variables	VLO ⁺ (n = 24)*	VLO ⁻ (n = 14)	MC ⁺ (n = 16)*	MC ⁻ (n = 8)	Ragweed positive (n = 26)*
Age (y), mean (SD)	41.3 (13.5)	51.0 (9.9)	42.6 (11.1)	41.6 (12.2)	41.5 (9.5)
Male sex, no. (%)	10 (41.7)	5 (35.7)	3 (18.75)	4 (50)	10 (38.5)
Ethnicity, no. (%)					
White	15 (62.5)	4 (28.6)	6 (37.5)	4 (50)	15 (57.7)
Hispanic	8 (33.3)	8 (57.1)	10 (62.5)	4 (50)	10 (38.5)
African	1 (4.2)	2 (14.3)	0 (0)	0 (0)	1 (3.8)

*By using the Fisher exact test, the distribution of ethnicities was not different for comparisons between VLO⁺ versus MC⁺ ($P = .197$), ragweed-positive versus MC⁺ ($P = .341$), or VLO⁺ versus ragweed-positive ($P = .779$) participants.

TABLE E5. Skin test responses to different aeroallergens*

Aeroallergen	VLO ⁺ † (n = 24)	MC ⁺ † (n = 16)	Ragweed positive† (n = 26)
MC	24 (100%)	16 (100%)	23 (88.46%)
VLO	24 (100%)	12 (75%)	21 (80.77%)
Cedar elm	21 (87.5%)	6 (37.5%)	12 (46.15%)
Pecan	21 (87.5%)	8 (50%)	19 (73.08%)
Mesquite	21 (87.5%)	8 (50%)	18 (69.23%)
Arizona ash	18 (75%)	6 (37.5%)	13 (50%)
Mixed ragweed	21 (87.5%)	11 (68.75%)	26 (100%)
Spiny pigweed	16 (66.67%)	8 (50%)	18 (69.23%)
Bermuda grass	23 (95.83%)	6 (37.5%)	23 (88.46%)
Johnson grass	18 (75%)	6 (37.5%)	23 (88.46%)
Mold mix 1	16 (66.67%)	10 (62.5%)	22 (84.62%)
Mold mix 2	17 (70.83%)	4 (25%)	17 (65.38%)
Dog dander	17 (70.83%)	6 (37.5%)	11 (42.31%)
Cat dander	17 (70.83%)	9 (56.25%)	18 (69.23%)
Dust mites	18 (75%)	13 (81.25%)	21 (80.77%)
Cockroach mix	19 (79.17%)	11 (68.75%)	13 (50%)
Median (IQR), no. of positive aeroallergen test results per participant‡	14.50 (11.50-15.25)	9.00 (6.00-12.00)	12.00 (9.00-14.00)

*Shown is the number (percentage) of positive skin test results. Skin test results were considered positive if the induration was 3 mm or greater.

†Of the 24 VLO⁺ subjects, 13 of them have dogs, and 5 of them have cats. The pet ownership for MC⁺ and ragweed-positive subjects was unknown.

‡Of 16 aeroallergens tested.

TABLE E6. Summary of main findings pertinent to understanding the correlation of TSSs recorded in the natural season versus in the PCC

Factors	VLO	MC	Ragweed
Seasonal factors			
Natural season	Spring	Winter	Fall
Coseasonal pollens*	+++	—	+
Other environmental cofactors*	++++	++	+++
Atopic participants			
Reactive to other pollens by skin test*	++++	++	+++
Other pollens present during natural season*	++++	+	++
Preprimed by other pollens/allergens*	+++	+	+/-
TSS at entry into the natural season (preseason)	12.8	6.6	NA
Mean TSS during the natural season	14.7	9.0	11.8
Peak TSS during the natural season	16.3	14.9	12.8
Mean TSS pre-PCC	2.4	1.9	2.8
Mean of maximum TSSs in PCC	12.2	10.9	10.4
Correlation of TSS between natural season and PCC	34%	54%	65%
Nonatopic participants			
Mean TSS during the natural season	0.3	0.4	—
Mean TSS pre-PCC	0.2	0.0	—
Mean of maximum TSSs in PCC	1.3	0.4	—

*The presence versus absence of these parameters were denoted as "+" versus "-." Positive responses were categorized into 4 grades (+ to ++++).