

## Letter to the Editor

### Syk expression and IgE-mediated histamine release in basophils as biomarkers for predicting the clinical efficacy of omalizumab

To the Editor:

Omalizumab is a treatment for asthma that has a success rate of approximately 40% to 50%.<sup>1</sup> Recent mechanistic studies have suggested that basophil behavior may explain some of the variability in response to therapy. First, the biological changes that occur during treatment with omalizumab have suggested that changes in basophil IgE-mediated reactivity occur faster than changes in the mast cell response.<sup>2,3</sup> Other studies have shown that the IgE-mediated response of basophils results largely from limitations in the expression of the early required tyrosine kinase syk.<sup>4</sup> During treatment with omalizumab, syk expression increases in peripheral blood basophils, offsetting the functional effects mediated by the drug-induced reduction in cell surface density of FcεRI and its bound IgE. In a study of peanut allergy, it appeared that the syk effect on basophil function influenced the subsequent efficacy of omalizumab to suppress the oral food challenge.<sup>5</sup> It has been suggested that because this alteration in syk expression occurs during treatment of patients with asthma, it may blunt the success of treatment in this disease as well. Intrinsic to this suggestion is that changes in the basophil syk (and therefore, IgE-mediated histamine release [HR]) may provide a predictor for successful treatment with omalizumab. Furthermore, because the change that occurs during treatment is tightly correlated with the starting condition of the basophils, either initial syk expression or IgE-mediated HR (because syk expression predicts HR) might predict a successful treatment by omalizumab in patients with asthma. This study examined this question directly.

The study design is shown in Fig E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org). Briefly, patients with moderate-to-severe allergic asthma were monitored for 10 weeks before the administration of omalizumab (at standard dosing). The physician's assessment score<sup>1,6</sup> (PAS, scores of 1-5) was the primary metric of the patients' clinical condition (this metric is based on 6 evaluations: patient interview, biweekly disease activity, medical notes, spirometry, once daily digital symptom diary, and medication use; see this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). To compensate for a potential placebo effect, at week 4 of the 10-week run-in, patients were given a placebo injection and if clinical metrics, notably mobile app diary entries, decreased by 25% or more, the patients were dropped from the study. Basic metrics of basophil behavior, syk expression, and response to an optimal concentration of anti-IgE antibody were obtained at 4 time points during the study (weeks -6, 0, 8, and 16; where 0 is the day of omalizumab treatment; for details, see this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). During the 16 weeks of treatment with omalizumab, patients were monitored biweekly for changes in clinical metrics of their disease and twice for basophil metrics. The recruitment goal was to analyze those with moderate-to-severe asthma (a PAS of 4 or 5). The 2 groups performing clinical or basophil metrics were distinct and results brought together at the time of analysis. For details, see this article's Online Repository at [www.jacionline.org](http://www.jacionline.org).

Seventeen patients were enrolled in the study, with 10 meeting the required predrug PAS of 4 or 5 and completing the study. This article's Online Repository at [www.jacionline.org](http://www.jacionline.org) summarizes the general demographic characteristics for the study group (Table E1), the clinical asthma metrics before and after treatment (Table E2), and the basophil metrics for the 10 patients evaluated (Table E3). Fig E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org) shows that syk expression was both distributed and correlated with HR ( $R = 0.70$ ) as previously found and that syk upregulation during treatment occurred and correlated with an increase in HR ( $R = 0.75$ ), as expected. Fig 1, A, shows a statistically significant inverse correlation ( $R = -0.72$ ;  $P = .017$ ) between deltaPAS and basophil baseline HR (the most accessible basophil metric) and syk ratio versus deltaPAS ( $R = 0.73$ ;  $P = .014$ ). To assess the quality of the possible basophil metrics in predicting treatment success, receiver operator characteristics (ROC) analysis was performed using 2 binary clinical outcome metrics, either success or failure defined by the patient and clinician at the end of the study after review of the clinical metrics or a change in PAS ( $\leq -2$ ). Fig E3 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org) illustrates the differences in success or failure using the asthma index score, and Table E4 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org) summarizes the ability of basophil metrics to predict the clinical outcome. The analysis for "success" showed statistically significant chi-square values for all 4 metrics of the basophil (syk & HR ratio, starting syk or HR), with ROC values (areas in the ROC curves ranging from 0.79 to 1.0) in the excellent range as predictors (4 patients were considered successful, 6 unsuccessful; for further detail, see this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). As a further measure of success, 3 of the 4 successfully treated patients chose to continue treatment.

The mobile diary provided a rich data source of time-stamped information that suggests that the kinetics of effects were, in general, relatively rapid, occurring in the first 8 weeks. The diary included a question about upper respiratory tract infections (URTIs), and it was apparent that daily symptom scores tracked closely with days with URTI reporting, but only during treatment. There were 6 reported URTIs in the run-in period but only 1 of these appeared to coincide with increased symptom scores. In contrast, there were 7 URTIs reported in the treatment period, 6 of which coincided with increased symptoms and 1 for which the increase in symptoms lagged the URTI by several days. The chi-square for this pattern being associated with treatment was 9.5 ( $P = .002$ ). Fig E4 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org) shows an example of the association during the treatment period.

A previous food allergy study suggested that the specific-to-total IgE ratio might be an important consideration for predicting successful treatment.<sup>5</sup> For a study of asthma, it is not immediately obvious which allergen-specific IgE should be considered in such a predictive analysis. Three approaches to examining this issue are to (1) sum the specific-to-total ratios for all perennial allergens or (2) consider only the largest specific-to-total ratio and (3) consider the overall number of perennial and seasonal allergens for which the patient shows

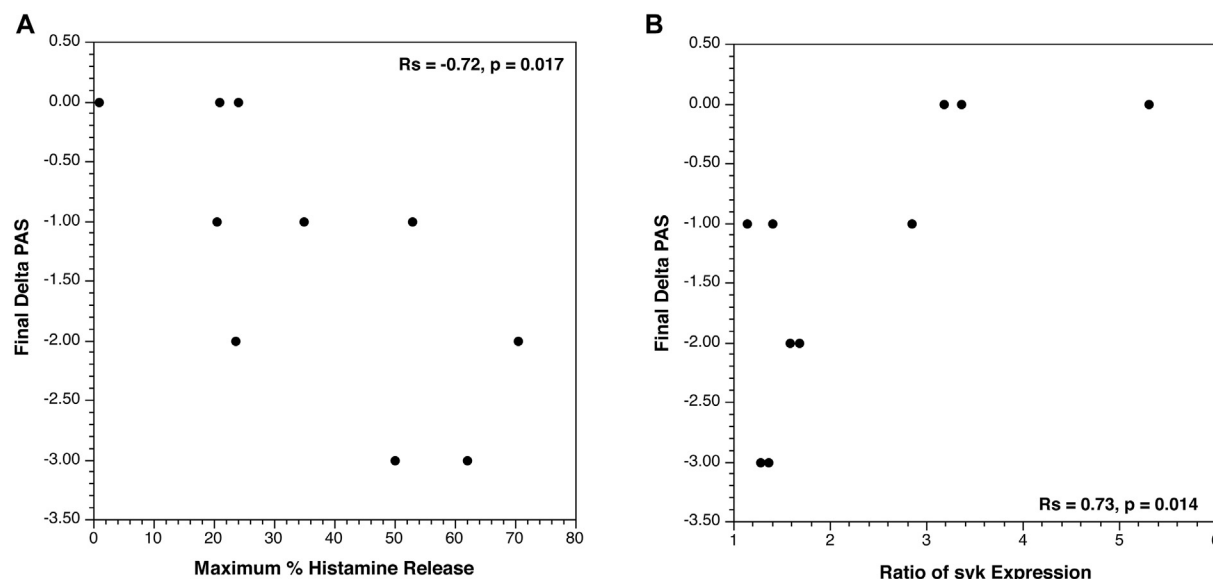


**FIG 1.** A, Relationship between the starting HR and change in PAS after treatment for those with moderate-to-severe asthma ( $PAS \geq 4$ ). B, Relationship between the syk ratio (treatment/baseline) and the change in PAS after treatment for those with moderate-to-severe asthma ( $PAS \geq 4$ ).

positive skin test results. For this study, none of the 3 metrics was found to alter the predictions based on basophil metrics. No other patient metrics were found to relate to the outcomes.

At the inception of this study, it was the change in syk expression (ratio of syk, treatment/baseline) that was predicted to associate with the success of being treated with omalizumab. This was found to be true, but all 4 predictors produced excellent discrimination. The number of subjects is small and the study would need to be extended to a larger population to verify these findings. If the results are an indication that basophils could act as a biomarker, a simple pretreatment test examining basophil HR or CD63 expression (as a surrogate marker of HR) could be used to predict the success of omalizumab in a population with more severe asthma. It should be noted that these results suggest that basophil characteristics may be a biomarker of successful clinical response but do not necessarily indicate a causal relationship between basophil behavior and disease treatment. There are many factors that may contribute to this relationship. A practical observation is that if the basophil test suggests an ability to treat, treatment effects tend to occur rapidly.

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## METHODS

### Study design

The study was a single-site trial to evaluate the utility of baseline basophil biometrics to predict the efficacy of subcutaneously administered omalizumab as an add-on therapy for the treatment of adult 18- to 75-year-old patients who have been diagnosed with moderate-to-severe allergic asthma according to current approved guidelines. Patients were treated with omalizumab according to the standard dosing table for a period of 16 weeks.

The trial was composed of 2 distinct phases over a time period of 26 weeks, as shown in Fig E1. To assist in assessing the quality of treatment, there was a 4-week run-in period of standard care followed by a 6-week run-in period of standard care after a subcutaneous injection of placebo that reflected the dosing regimen for omalizumab dosing. Following the placebo run-in, patients were treated with omalizumab for 16 weeks before the final physician's assessment was made. This treatment period was based on previous studies indicating that maximum responses to treatment required at least 2 to 3 months. However, because the basophil response changes relatively rapidly, an additional assessment was made at 8 weeks.

The primary outcome metric for the basophil studies was the fold increase in syk expression (as determined by flow cytometry). The primary metric for the clinical outcome was the PAS.<sup>E1,E2</sup> The absolute change from baseline (week 4 to 10) in asthma symptoms was compared with week 24 to 26 (the last 2 weeks of the 16-week omalizumab treatment period). The physician's assessment (PAS) was based on 6 evaluations: (1) patient interview (a biweekly discussion with the patient on disease activity), (2) medical notes, (3) spirometry, (4) diary of symptoms (using an iPod/iPhone device and an "App" designed to accept patient-recorded information for a once-a-day diary entry consisting of a 4-point scale of 4 questions and 4 yes/no notations of important events), (5) rescue medication use, and (6) peak expiratory flow changes. A Juniper asthma quality-of-life assessment was taken at baseline and 16 weeks. PAS of 1 and 2 were considered control of symptoms and PAS of 3 to 5 degrees of poor control.

The statistical power analysis was based on the expectation that changes in PAS (1-5 scale) less than 2 would not be considered significant. Scores of 4 or 5 were considered moderate-to-severe asthma and expectations from the power analysis led to requiring a starting PAS of 4 or more. A related measure, the asthma index score, was also recorded. It was a composite score with numeric severity intensity ratings (0 = none to 3 = severe) for each of the following 4 areas: physical examination, review of the patient's diary for preceding 2 weeks, peak flow, and beta-agonist use records. This score was recorded at each visit.

### Patient diary

Subjects were supplied with a mobile device containing an application that maintained data both on the device and sent the data to a Web-based database. There were several features worth noting about the application. First, each entry was time-stamped. Second, the subject was prompted with reminders on the device to make an entry. Multiple entries were allowed although in the analysis, only the first each day was used. The application would tell the subjects how well they were maintaining the diary (both missed entries and repetitious entries). Third, there were 2 possible entries each day, the simple survey of disease activity and a peak expiratory flow entry.

The daily survey was composed of 4 Likert-scale questions and 4 yes-no questions. The survey required approximately 30 seconds to complete. The Likert-scale questions used "sliders" and the survey would not allow completion (entry into the database) unless each question was answered. The application informed the subject when sliders were missed (the default step of the slider was "no entry made"). The Likert-scale questions were (1) severity of daytime asthma (scale of 1-4), (2) nighttime asthma (scale of 1-4), (3) use of inhaler (scale of 1-4), and (4) limitations on physical activity (scale of 1-4). The yes/no questions were (1) miss work or school, (2) visit the emergency department or hospital, (3) new medicines (of any kind), (4) cold (URTI) today.

Patients were removed from study during the run-in period if they were unable to maintain the diary or showed a total score of less than 32 in the first 4 weeks and/or 24 in the 6 weeks following the placebo injection.

### Inclusion and exclusion criteria

Initial inclusion and exclusion criteria followed the Food and Drug Administration–approved treatment guidelines for omalizumab. Inclusion was based solely on the standard clinical criteria for use of omalizumab without regard to the basophil metrics. The exclusionary criteria were as follows:

- Patients will be excluded if they have prior sensitivity to omalizumab, and acute respiratory tract infection before or during the run-in period, or a need for regular  $\beta$  agonist use.
- Treatment with an investigational agent within 30 days of screening.
- Previously treated with omalizumab within a year before screening.
- Treatment 1 month before screening with systemic or cutaneous corticosteroids, hydroxychloroquine, methotrexate, cyclosporine, cyclophosphamide, intravenous IgG, and plasmapheresis.
- Clinically relevant laboratory anomalies at screening including individuals with reduced hematocrit ( $<32\%$ ), white blood cell count ( $2400/\mu\text{L}$ ), platelet count ( $<75,000/\mu\text{L}$ ), and increased creatinine ( $>141.4$  micromolar/L), or aspartate aminotransferase ( $>100$  IU/L).
- Patients with current malignancy, history of malignancy, or currently under workup for suspected malignancy, or bleeding disorder.
- History of any medical condition that is unstable.
- Inability to comply with study and follow-up procedures.
- Patients may not take systemic corticosteroids or use cutaneous corticosteroid (prescription or over the counter) within 2 weeks before screening.
- Women of childbearing potential who are pregnant or nursing mothers, or who are of childbearing potential (postmenarche) and are not practicing an acceptable form of contraception (as determined by the site investigator).
- Individuals with body weight less than 30 kg or greater than 150 kg.
- Mental illness or history of drug or alcohol abuse that, in the opinion of the investigator, would interfere with the participant's ability to comply with study requirements.
- Inability or unwillingness of a participant to give written informed consent or comply with study protocol.
- Contraindications to omalizumab including patients with a previous hypersensitivity to omalizumab.

Past or current medical problems or findings from physical examination or laboratory testing that are not listed above, which, in the opinion of the investigator, may pose additional risks from participation in the study, may interfere with the participant's ability to comply with study requirements, or may compromise the quality of the data obtained from the study.

To prevent influence between the clinical metrics and the basophil metrics, 2 databases were maintained so that the clinical team and the *in vitro* measures team were blinded to each other's results. Following the screening of 57 subjects and 17 enrolled in the study, the 2 databases were merged for analysis after the decision to hold the study at 10 completed subjects that met the study requirements. Dropouts occurred for various non-drug-related reasons, most often failure to meet the standards for analysis such as acceptable diary maintenance, insufficiently high scores, or failure to appear for evaluation.

### Basophil and serology metrics

A number of metrics of basophil function were measured at the end of 4 weeks of run-in, the day of first omalizumab injection, and 8 weeks and 16 weeks after the start of omalizumab. These included basophil anti-IgE-mediated HR and syk expression, basophil Fc $\epsilon$ RI densities, and HR response to the non-IgE stimulus, fmet-leu-phe tripeptide. Other serology such as allergen-specific (using the standard 6 perennial-allergen panel) and total IgE levels were assessed.

## Materials

The following were purchased: piperazine-N, N-bis-2-ethanesulfonic acid, BSA, ethylenediamine N, N, N', N'-tetraacetic acid, EDTA, D-glucose, fmet-leu-phe tripeptide (Sigma, St Louis, Mo); crystallized human serum albumin (HSA) (Miles Laboratories, Elkhart, Ind); Percoll (Pharmacia, Piscataway, NJ); anti-syk mAb 4D10 (Santa Cruz Biotechnology, Santa Cruz, Calif); monoclonal anti-human IgE antibody (6061P) (Hybridoma Labs, Baltimore, Md); monoclonals anti-FcεRI antibodies 22E7 and 15A5 (gift of former Hoffman-LaRoche) (measured total FcεRI and unoccupied FcεRI, respectively). Countercurrent elutriation and labeling with antibodies for flow cytometry was conducted in PIPES-albumin-glucose (PAG) containing 0.25% BSA in place of 0.003% HSA.

## Buffers

PAG buffer consisted of 25 mM PIPES, 110 mM NaCl, 5 mM KCl, 0.1% glucose, and 0.003% HSA. PAGCM was PAG supplemented with 1 mM CaCl<sub>2</sub> and 1 mM MgCl<sub>2</sub>. PAG-EDTA consisted of PAG supplemented with 4 mM EDTA.

## Basophil isolation, sensitization, and HR reaction

Blood was partially enriched for basophils using single-step Percoll gradients. After washing to obtain a mononuclear cell preparation, cells were resuspended in PAG (to prepare for fixation and flow cytometry) or PAGCM buffer (to assess HR). Total histamine content was obtained by lysis of the cell preparation with 1.6% perchloric acid.

## Histamine measurements

Histamine was measured by automated fluorimetry.<sup>E3</sup> The percentage of total HR was calculated after subtraction of spontaneous HR.

## Basophil cell surface IgE measurement

As described previously, calibration of the antibody (22E7) used to detect FcεRI by flow cytometry involved a comparison of flow cytometric results with explicit measurement of IgE eluted from a counted number of basophils.<sup>E4</sup> The flow cytometric results were then equated with this measured density of IgE/basophil. See MacGlashan<sup>E5</sup> for a more complete description of the calibration procedure.

## Analysis of syk protein expression by intracellular flow cytometry

All analysis was performed on a BD FACSCalibur flow cytometer. The measurement of syk protein expression by flow cytometry using the anti-syk antibody 4D10 (Santa Cruz Biotechnology) has been previously described and validated with respect to standard Western blotting.<sup>E6,E7</sup> This method has been used on both impure and purified cell fractions with equivalent results. Briefly, fixed mixed leukocytes were permeabilized (Fix and Perm Kit, Caltag,

Carlsbad, Calif) in the presence of 4D10 or IgG<sub>2a</sub> antibody, then labeled with anti-IL-3R-phycoerythrin (BD Biosciences, Franklin Lakes, NJ) and an anti-mIgG<sub>2a</sub>-alexa647 antibody (Molecular Probes, Eugene, Ore) to complete the labeling. Syk protein expression is reported as normalized net mean fluorescence intensity, the difference between 4D10 and isotype-labeled cells corrected for instrument variability using CaliBRITE APC calibration beads (BD Biosciences). As described previously, the flow cytometric method of determining syk was calibrated against a quantitative Western blot that used a recombinant syk protein as a standard.

## RESULTS USING ROC ANALYSIS

The regression results prompted a chi-square analysis to assess the ROC curves for the various basophil metrics to act as predictors of the success of treatment. Success was assessed at the end of treatment by the physician and patient and was based on the various clinical metrics, notably the PAS and its similar metric, the asthma index over the course of treatment. It is illustrative that success was accurately reflected in the trend lines of the asthma index scores for each patient. The asthma index score for treatment failures decreased, on average, by 3%, whereas the asthma index scores for treatment successes decreased, on average, by 82%. Fig E3 demonstrates the difference in the 2 groups using the asthma index. Parenthetically, for this study, the asthma index provided an excellent dichotomous outcome that reflected success and failure. Table E4 presents the results of the ROC analysis using 2 binary outcome metrics, success or failure (as described above), or deltaPAS less or equal to −2.

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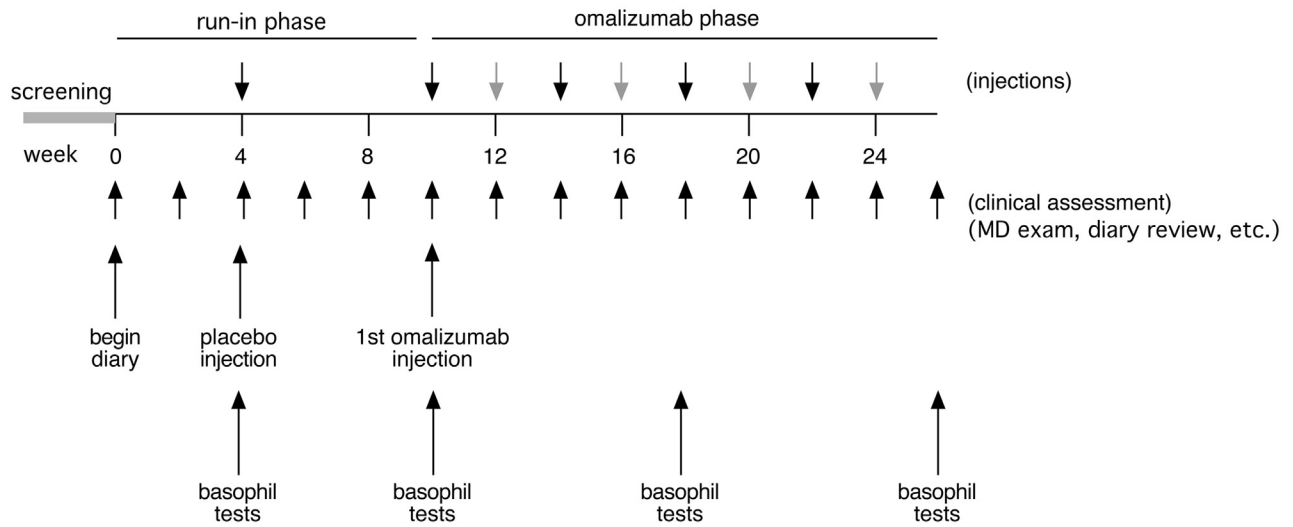


**FIG E1.** Study design timeline. *MD*, Doctor of Medicine.

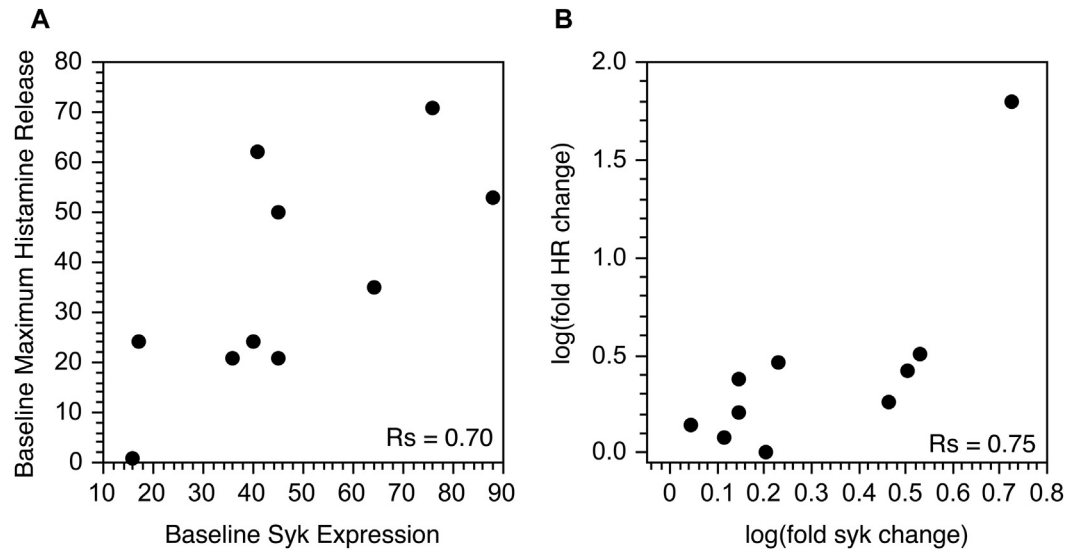


**FIG E2.** Relationship between **(A)** starting syk expression and starting maximum HR and **(B)** ratio of the change in syk and the ratio of the change in maximum HR.

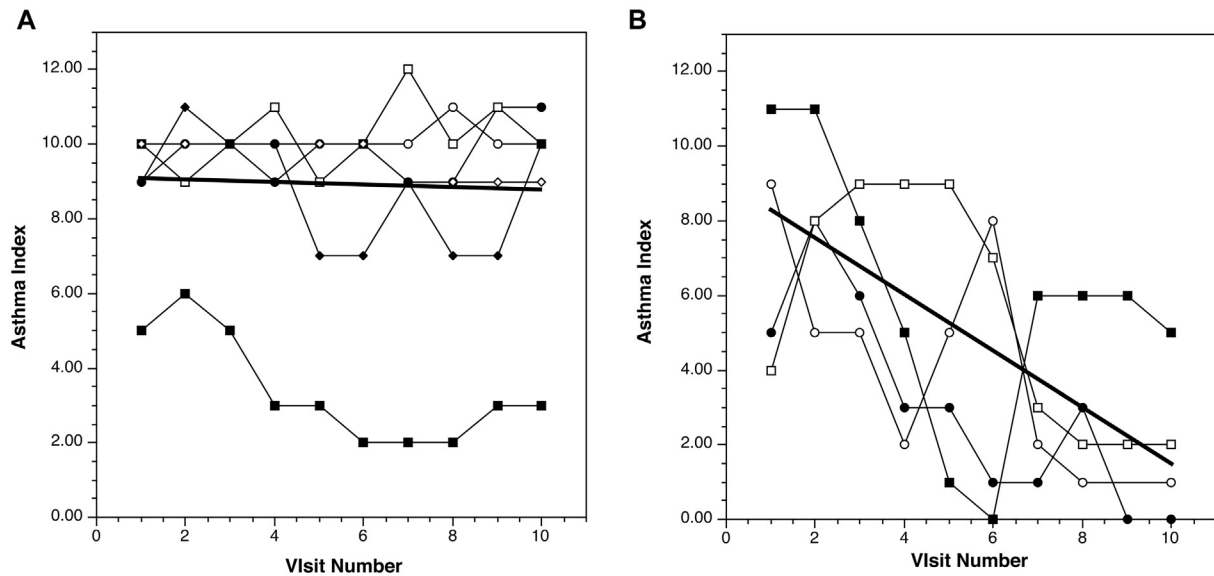


**FIG E3.** Asthma index scores for each visit for the 2 groups of patients, treatment failure (A) or success (B). The heavy lines represent the average regression fits for the groups.

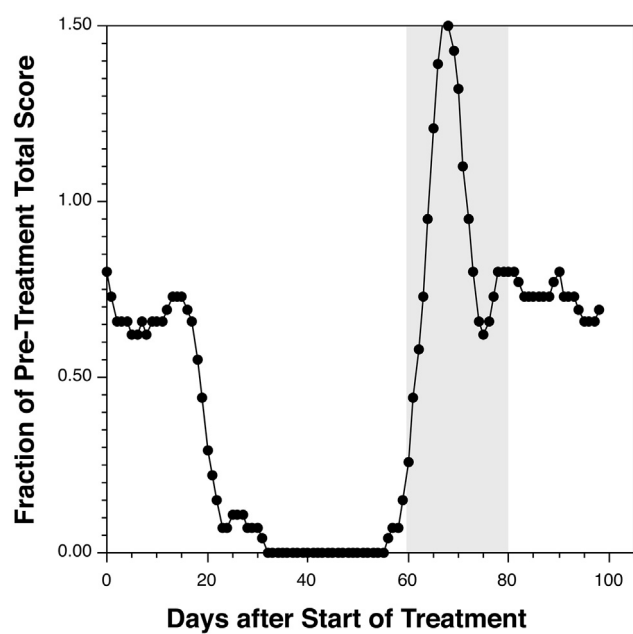


**FIG E4.** One example of the diary composite score during the 16 weeks of treatment. A moving window average of 7 days was used to smooth the daily scores. The gray zone marks the patient's report of a URTI in diary.

**TABLE E1.** Summary descriptors

| Descriptor                | Value       |
|---------------------------|-------------|
| Enrollment fraction       | 30%         |
| Completion fraction       | 59%         |
| Average age (y)           | 44          |
| Average weight (lb)       | 207         |
| Average serum IgE (IU/mL) | 306         |
| Females                   | 90%         |
| Smokers                   | 20%         |
| Immunotherapy             | 30%         |
| Seasonal allergens        | 90%         |
| Max sp/total IgE          | 0.12 ± 0.04 |
| Sum sp/total IgE          | 0.16 ± 0.04 |

Dropouts were due to non-treatment-related events (poor completion of diary or failure to show). Max sp/total IgE refers to the highest of the perennial allergen specific/total IgE ratios, and sum sp/total IgE refers to the summed fraction of all measured perennial allergens compared with total IgE. Immunotherapy could be past or current; seasonal allergens were reported.

**TABLE E2.** Asthma metrics not related to the PAS or diary entries

| Metric           | Baseline    | 16-wk treated | <i>P</i> value |
|------------------|-------------|---------------|----------------|
| FVC              | 2.62 ± 0.16 | 2.76 ± 0.17   | NS             |
| %FVC             | 77 ± 4      | 71 ± 7        | NS             |
| FEV <sub>1</sub> | 1.75 ± 0.16 | 1.97 ± 0.18   | NS             |
| PAS              | 4.3 ± 0.6   | 3.0 ± 0.4     | .006           |
| Asthma index*    | 8.5 ± 0.6   | 6.1 ± 1.4     | .027           |
| Inhaler use†     | 1.36 ± 0.27 | 1.07 ± 0.24   | NS             |
| Quality of life  | 3.8 ± 0.4   | 4.2 ± 0.4     | NS             |

FVC, Forced vital capacity; NS, not significant.

\*The asthma index measurement was the average of 2 baseline determinations vs the last 2 visit determinations.

†Inhaler use was derived from the diary entries.

**TABLE E3.** Basophil metrics

| Metric           | Baseline        | Ratio (final/bsl) | P value |
|------------------|-----------------|-------------------|---------|
| %maxHR           | 36 ± 7          | 2.69              | .025    |
| FMLP %HR         | 20 ± 5          | 1.14              | NS      |
| Syk (flow units) | 47 ± 7          | 2.03              | .002    |
| FceRI            | 37,000 ± 10,000 | 0.33              | <.0001  |

*bsl*, Baseline; *FceRI*, average expression (receptors/cell) from calibrated flow cytometric assay; *FMLP*, fmet-leu-phe tripeptide, a non-IgE- mediated response; % maxHR, maximum response to an optimal concentration of 6061P, anti-IgE antibody; syk flow units, net mean fluorescence intensity in the flow cytometric-based syk expression assay.

**TABLE E4.** Association between metrics of the basophil phenotype and the clinical outcome of treatment

| Group                    | Basophil metric | $\chi^2$ (Success)                 | P value | ROC area |
|--------------------------|-----------------|------------------------------------|---------|----------|
| PAS $\geq$ 4<br>(n = 10) | syk ratio       | 6.67                               | .0098   | 0.92     |
|                          | HR ratio        | 10.00                              | .0016   | 1.00     |
|                          | Starting syk    | 4.29                               | .0384   | 0.79     |
|                          | Starting HR     | 10.00                              | .0016   | 1.00     |
| Group                    | Basophil metric | $\chi^2$ ( $\Delta$ PAS $\leq$ -2) | P value | ROC area |
| PAS $\geq$ 4<br>(n = 10) | syk ratio       | 4.44                               | .0350   | 0.75     |
|                          | HR ratio        | 3.75                               | .0528   | NA       |
|                          | Starting syk    | 2.86                               | .0910   | NA       |
|                          | Starting HR     | 3.75                               | .0528   | NA       |

NA, Not available/applicable.

The  $\chi^2$  analysis refers to the best threshold based on the inflection point of the ROC analysis or the best for each possible threshold. The ROC area was not calculated for chi-square results for which  $P < .05$ .