

The effect of aspirin desensitization on novel biomarkers in aspirin-exacerbated respiratory diseases

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Background: Patients with aspirin-exacerbated respiratory disease have been shown to benefit clinically from aspirin desensitization followed by chronic high-dose aspirin therapy. However, the mechanism of this phenomenon is still unclear. **Objective:** The aim of this study was to characterize the airway inflammatory response to aspirin desensitization and after treatment with high-dose aspirin for 6 months.

Methods: Twenty-one adult patients with asthma, chronic polypoid sinusitis, and a convincing history of acute respiratory reaction to the ingestion of aspirin or nonsteroidal anti-inflammatory drugs were selected. These patients underwent an oral desensitization to aspirin over a 2-day period, followed by daily ingestion of aspirin 650 mg twice daily. Induced sputum samples and exhaled nitric oxide measurements were taken before the procedure, during the second day of the procedure, and after 6 months of treatment.

Results: There was a significant elevation in both the exhaled nitric oxide level ($P = .03$) and sputum tryptase level ($P = .05$) during the desensitization process. After 6 months of aspirin treatment, sputum IL-4 ($P = .0007$) and matrix metalloproteinase 9 (MMP-9; $P = .05$) decreased significantly compared with baseline. Predesensitization to postdesensitization changes in MMP-9 and tissue inhibitors of metalloproteinases 1 were highly correlated ($r = 0.79$; $P = .0003$). Immediately after the desensitization, MMP-9 and tryptase were correlated ($r = 0.82$; $P = .001$), whereas IL-4 was inversely related with FMS-like tyrosine kinase 3 ligand (FLT3-L) ($r = -0.79$; $P = .0008$). There was a significant decrease in the average symptom score at 6 months.

Conclusion: Consistent with previous reports, acute aspirin desensitization in patients with aspirin-exacerbated respiratory disease involves mast cell degranulation. In contrast, long-term treatment with aspirin involves suppression of IL-4 as well as downregulation of proinflammatory MMP-9 while T_H1 marker FLT3-L increases. (J Allergy Clin Immunol 2010;■■■:■■■-■■■.)

Key words: Aspirin-exacerbated respiratory disease, biomarkers, IL-4, MMP-9, FLT3 ligand

Abbreviations used

AERD: Aspirin-exacerbated respiratory disease
 CSS: Chronic Sinusitis Survey
 cysLT₁: Cysteinyl leukotriene receptor 1
 FeNO: Exhaled nitric oxide
 ICS: Inhaled corticosteroid
 LT: Leukotriene
 MMP-9: Matrix metalloproteinase 9
 NSAID: Nonsteroidal anti-inflammatory drug
 PAR-2: Protease-activated receptor
 SNOT-20: Sino-Nasal Outcome Test
 STAT6: Signal transducer and activator of transcription 6
 TIMP-1: Tissue inhibitors of metalloproteinases 1

The associations among asthma, nasal polyposis, and aspirin sensitivity have been observed for almost a century, but this syndrome gained more widespread recognition after publication of the work of Max Samter in 1968.¹ The precise pathogenesis of aspirin-exacerbated respiratory disease (AERD) has not been delineated; however, observations describing aberrations in the COX pathway, with overproduction of eicosanoids and hypothetical initiation of disease by an underlying viral illness, have been put forth. In addition, it is known that the ingestion of aspirin itself does not initiate the disease. In fact, before the first ingestion of aspirin/nonsteroidal anti-inflammatory drugs (NSAIDs), there is already ongoing mast cell and eosinophilic inflammation.²

The first double-blind placebo controlled study that evaluated aspirin therapy was performed by Stevenson et al.³ A group of 25 patients underwent aspirin treatment in a double-blind cross-over study. Patients reported improvement in nasal symptoms and reduced requirement for nasal steroid. The same group then reported the results of a retrospective analysis of 107 aspirin-sensitive patients, showing a reduction in the number of emergency department visits, hospitalizations, outpatient visits, upper respiratory tract infection (URI)/sinus-related antibiotic prescriptions, and sinus surgeries and improved sense of smell compared with the control group.⁴ Sixty-five aspirin-sensitive subjects were then followed prospectively after daily treatment with aspirin for 1 to 6 years.⁵ This cohort experienced significant reductions in the number of sinus infections, hospitalizations for asthma, and use of systemic steroids. These subjects also reported improved sense of smell, fewer sinus operations, and lower nasal corticosteroid requirement.

More recently, short-term benefits of aspirin desensitization have been shown. In 1 study, within 6 months, subjects reported

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fewer sinus infections and prednisone bursts, improved sense of smell, and overall improved nasal and asthma symptoms.⁶ In another study, within 4 weeks patients reported improved nasal and asthma symptoms, improved sense of smell, and reduced dose of oral prednisone.⁷

Although the clinical benefits of aspirin desensitization have been clearly shown, the mechanism by which this occurs is not clear. To date there has not been a unifying hypothesis explaining the immunologic aberration leading to disease activity. However, a host of immunologic observations have been identified in hopes of leading to a better understanding of disease pathogenesis. At baseline, even in the absence of aspirin/NSAID ingestion, patients with AERD have been shown to have increased levels of leukotrienes (LTs) as measured by urinary LTE₄, and these levels increase in proportion to the severity of the reaction during aspirin challenge.⁸⁻¹¹ Juergens et al¹² studied peripheral blood monocytes from patients with AERD and demonstrated a decrease in LTB₄ after aspirin desensitization. Other findings include a down-regulation of the cysteinyl LT receptor 1 (cysLT₁) on nasal submucosal cells and inhibition of T-cell IL-4 production after aspirin desensitization.^{13,14}

Currently there is not a biomarker that predicts disease activity, nor have there been any data in the cohort of patients with AERD evaluating mediators of airway remodeling or cytokine imbalances directly in the airway. The aim of this study is to characterize the airway inflammatory response after aspirin desensitization by assessing changes in novel noninvasive inflammatory markers in the airways of patients with AERD. We studied standard measures such as symptoms, exhaled nitric oxide (FeNO), and lung function as well as novel markers not previously studied in this cohort such as sputum tryptase, matrix metalloproteinase 9 (MMP-9), tissue inhibitors of metalloproteinases 1 (TIMP-1), FMS-like tyrosine kinase 3 ligand (FLT3-L), and IL-4.

METHODS

Subjects

Subjects were recruited from our outpatient clinic at National Jewish Health in Denver, Colo. Patients had a diagnosis of asthma, chronic hyperplastic sinus disease with nasal polyposis, and a convincing history of AERD. Twenty-one patients consented to undergo aspirin desensitization in our adult procedure unit and were discharged after the procedure on aspirin 650 mg bid.

Aspirin desensitization

Subjects underwent aspirin desensitization over a 2-day period. The morning of the procedure, patients were placed on continuous monitoring (pulse oximetry and cardiac monitor), and intravenous access was established. Aspirin desensitization protocols have been previously published, and our protocol is similar.¹⁵

Symptom score

At baseline and 6 months after treatment, subjects were asked to fill out the Sino-Nasal Outcome Test (SNOT-20) survey, a disease-specific, health-related quality-of-life measure for rhinosinusitis. This survey contains a total of 20 items, which include a variety of physical symptoms, functional limitations, and emotional consequences. Subjects were asked to score each item from 0 to 5. Higher scores indicated a higher burden of rhinosinusitis-related disease.¹⁶ Numerous rhinosinusitis control questionnaires exist, and each has various limitations. None of the disease-specific ones for sinusitis have been rigorously validated.¹⁷ Thus, we extracted the 5 symptom scores most related to nasal symptoms, "Need to blow nose," "Sneezing," "Runny nose," "Post nasal discharge," and "Thick nasal discharge," for analysis. In addition, because of limitations in the questionnaire, we chose to implement a second

one, the Chronic Sinusitis Survey (CSS), to capture changes in symptoms after desensitization completely.¹⁸

FeNO

All subjects had FeNO measured before and after aspirin desensitization as well as at 6 months in those willing to stay in the study. FeNO was measured by using the online NioX (Aerocrine, Solna, Sweden) analyzer with a 50 mL/s flow rate as recommended by the American and European Thoracic Societies.

Sputum collection

Subjects underwent induced sputum collection before desensitization. Sputum was collected and processed as previously described.¹⁹ Briefly, subjects were initially screened with spirometry and peak flow and only underwent induction if the FEV₁ was above 70% predicted. Subjects inhaled 3% saline in a nebulized form for up to 12 minutes. They were then asked to rinse their mouth before each attempt to produce sputum to limit contamination with saliva. Peak flow was measured every 2 to 3 minutes. If the peak flow dropped below 80% of the baseline value, the induction procedure was stopped, and patients were treated with nebulized albuterol until their FEV₁ returned to at least 90% of baseline. If the FEV₁ was below 70% predicted, induced sputum was not performed. Instead, the subject attempted spontaneous sputum production. Immediately after collection, the sputum was processed. Sputum plugs were extracted and weighed. Dithiothreitol 0.1% was added at a ratio of 4 mL dithiothreitol:1 g sputum. The solution was vortexed and then rocked at 4°C for 15 minutes. 1xPBS was added in the same volume as the 0.1% dithiothreitol, vortexed, and then rocked at 4°C for an additional 15 minutes. The solution was then filtered through a mesh filter and centrifuged at 790g (1800 rpm) for 10 minutes at 4°C. Aliquots of supernatant were frozen at -70°C until analysis.

A second sputum collection was performed during the second day of desensitization. A third sputum collection was performed after 6 months of aspirin therapy in those willing to repeat the procedure. In addition, all subjects had spirometry performed according to the American Thoracic Society standards.

Tryptase

Sputum tryptase was measured by using the ImmunoCAP (Phadia, Portage, MI) system. Briefly, 40 μ L thawed sputum supernatant was run according to the manufacturer's instructions on the Unicap 100 instrument. Initial experiments with known quantities of tryptase were performed to insure proper recovery of the analyte. Sensitivity of the assay is down to 1 ng/mL.

IL-4

Frozen supernatants were thawed and analyzed for IL-4 by using the human cytokine LINCOplex premixed kit per the manufacturer's instructions (LINCO Research, Inc, St Charles, Mo). Briefly, antibodies specific to IL-4 were incubated with 25- μ L standards, controls, and samples overnight at 4°C, washed, then incubated with detection antibody, followed by streptavidin-phycoerythrin. After a final wash step, results were analyzed by using Bio-Rad Bio-Plex software (Hercules, CA) to determine the concentration of each cytokine in the samples. The sensitivity of the assay is 3.2 pg/mL.

Measurement of MMP-9

Measurement of total MMP-9 was performed by using a commercially available ELISA (R&D Systems, Minneapolis, Minn). Sputum supernatant were diluted 1:100 in assay buffer and added in duplicate. The optical densities for the standard curve values were log-transformed and plotted against the log of the expected concentrations. The resulting value was multiplied by 100 to account for the dilution factor.

Validation was performed on the R&D Systems ELISA kit used to measure MMP-9 in sputum. Assay linearity was determined with each run to ensure the standards were at the correct concentration and that the ELISA was performing optimally. The stability of samples was analyzed by evaluating expression of MMP-9 after several thaws and storage in varying conditions. The precision of

TABLE I. Demographics

Variable	Female (n = 14)	Male (n = 7)
Age (y)	57.1 (9.9)	54.3 (12.7)
Height (cm)	164.2 (5.4)	179.1 (7.9)
Weight (kg)	75.2 (17.1)	95.6 (11.0)
FEV ₁ (L)	2.1 (0.5)	3.6 (0.6)
FEV ₁ % of predicted	77.3 (15.8)	90.5 (14.6)

Table entries are mean (SD).

each assay was assessed to confirm that the same amount of each sample was measured consistently in the assay. The percent recovery of spiked samples was determined to confirm that the addition of a known amount of standard to sputum samples would be measured adequately.

For percent recovery, we assayed 6 samples after varying conditions, and the average percent recoveries are as follows: 1 freeze/thaw, 100 % recovery; 2 freeze/thaws, 98% recovery; 3 freeze/thaws, 94% recovery; 1 day 4°C, 107% recovery; 3 days 4°C, 105% recovery; and 7 days -70°C, 104% recovery. The percent recovery of spike samples ranged from 97% to 107%. The sensitivity of the assay was less than 0.156 ng/mL.

Measurement of TIMP-1

Measurement of total TIMP-1 was performed by using a commercially available ELISA (R&D Systems, Minneapolis, Minn). Sputum supernatant was diluted 1:25 in assay buffer and added in duplicate. The optical densities for the standard curve values were log-transformed and plotted against the log of the expected concentrations. The resulting value was multiplied by 25 to account for the dilution factor. The validation process was performed on the R&D Systems ELISA kit similar to MMP-9 described in the Measurement of MMP-9 subsection. The sensitivity of the assay was less than 0.08 ng/mL.

Measurement of FLT3 ligand

Measurements of total FLT3 ligand (FLT3-L) were performed by using a commercially available Lumines xMap (Millipore, Billerica, Mass). Sputum supernatant was added in duplicate. The median fluorescent intensity was determined within 30 minutes by using a Lumines 100. The median fluorescent intensities for the standard curve values were log-transformed and plotted against the log of the expected concentrations. The data were then calculated by using a 5-parameter logistic fit with BioPlex software from Bio-Rad. The sensitivity of the assay was less than 2.4 pg/mL.

Statistics

Pearson correlations (*r*) were used to determine the strength of association between pre desensitization and postdesensitization changes between pairs of the following variables: MMP-9, TIMP-1, FeNO, sputum tryptase, IL-4, and FLT3-L. Correlations between these variables strictly at the postdesensitization time point were also examined. Variables with strong right skewed distributions were natural log-transformed before analysis (for change scores, differences of log scores were used for all but FeNO and FLT3; for postdesensitization data, all but TIMP-1 were log-transformed). Because of the high number of correlations examined, Benjamini-Hochberg and Benjamini-Liu false discovery rate procedures were applied using a false discovery rate of 0.10 within each set of correlations (15 correlations for each set).

Linear mixed models were used to examine changes in outcome variables over time (baseline, postbaseline, 6 months) for FeNO, tryptase, IL-4, and MMP-9. Time was modeled as a class variable, and the unstructured covariance structure was used to model repeated measures over time. Parameters were estimated by using maximum likelihood estimation. Medication variables (inhaled corticosteroids [ICSs], Montelukast, Prednisone) were initially tested as covariates and included in final models if they improved model fit on the basis of the Akaike's Information Criterion (AIC) statistic.²⁰ Outcome variables were natural log-transformed before analysis, but resulting estimates were inverted back for presentation and thus are geometric means.

TABLE II. Changes in sinonasal symptoms reported by subjects from baseline to 6 months for n = 14 subjects

Symptom category	Fewer symptoms (n)	Same number of symptoms (n)	More symptoms (n)	<i>P</i> value*
Need to blow nose	9	4	1	.02
Sneezing	7	7	0	.02
Runny nose	7	4	3	.34
Postnasal discharge	7	5	2	.18
Thick nasal discharge	6	7	1	.13

*Comparing rate of those with fewer symptoms versus rate of those with more symptoms by using binomial proportion test.

The number of subjects with a decrease, no change, or increase in symptoms from baseline to 6 months was determined for each sinonasal symptom category. The Wilcoxon signed-rank test was used to determine whether the average change across all categories was significantly nonzero. In addition, the rate of those with a decrease and the rate of those with an increase in symptoms (disregarding ties) were compared by using binomial proportion tests for each sinonasal category.

All reported *P* values in the article are based on 2-sided tests. Each CI was constructed via the fitted linear mixed model by using t-distribution methodology.

RESULTS

Patient characteristics

Twenty-one patients were enrolled in this study. These patients had a history of asthma, chronic sinusitis with polyposis, and a convincing history of aspirin/NSAID sensitivity. These patients underwent the aspirin desensitization procedure over 2 days at the National Jewish Health Adult Procedure Unit. Baseline characteristics were recorded on these patients, which included age, sex, and FEV₁ (Table I). Sputum was successfully induced in 16 of the subjects. All subjects had a respiratory reaction, with 4 having a pure upper respiratory reaction and the remaining either a combined lower and upper or just lower respiratory reaction. Three additional subjects with asthma, chronic sinusitis, and nasal polyps without aspirin intolerance were empirically treated by their physician with 650 bid of aspirin even though they were tolerant. These subjects agreed to provide a sputum sample at baseline and 6 months. All subjects tolerated aspirin therapy at 650 bid for the 6-month period without side effects.

Patients experienced improved nasal symptoms after 6 months of treatment

Patient symptoms were recorded by using the validated questionnaires SNOT-20 and CSS. The patient symptom scores improved (*P* = .038, Wilcoxon signed-rank test for average change across categories) after desensitization. Two specific symptoms, "Need to blow nose" and "Sneezing," were significantly improved after 6 months of aspirin treatment (*P* = .02). The tendency for improvement was also observed for other categories, but not significantly (Table II). Two subjects did not complete questionnaires at the 6-month time point, so n = 14 subjects were available for this analysis. The average score of all the individual domains on the CSS also showed significance (*P* = .004), and the individual items all trended toward significance (.04 < *P* < .22).

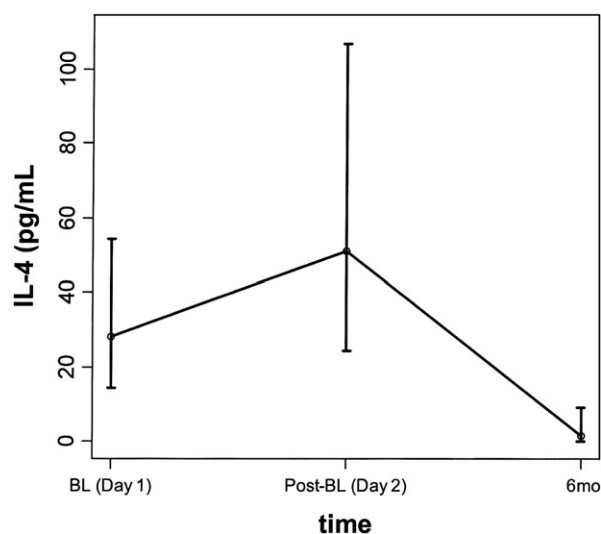


FIG 1. IL-4 means over time, with 95% CIs, based on the linear mixed model fit. IL-4 was modeled on the natural log scale, and estimates and CI endpoints were then inverted back for presentation, resulting in longer upper bars than lower bars. Note that CIs are relevant for fixed time points only and do not indicate variability of estimates for differences between time points because repeated-measures data were involved. BL, Baseline.

FeNO levels increase during aspirin desensitization, then return to baseline after 6 months of aspirin treatment

Mean FeNO levels increased by 20% during the aspirin desensitization process (baseline mean, 33.5 ppb; postbaseline mean, 40.3 ppb; 95% CI for relative increase, 3% to 41%; $P = .03$), then dropped slightly after 6 months of aspirin treatment to 38.1 ppb (14% increase relative to baseline; 95% CI, -17% to 55%; $P = .40$).

Sputum tryptase levels increased immediately after aspirin desensitization

Sputum tryptase was measurable and increased significantly during aspirin desensitization (baseline mean, 0.82 ng/mL; postbaseline mean, 1.44 ng/mL; 76% increase; 95% CI, 7% to 189%; $P = .03$). Sputum tryptase levels remained significantly elevated after 6 months of aspirin treatment (6-month mean, 1.25 ng/mL; 53% increase relative to baseline; 95% CI, 8% to 114%; $P = .02$).

Sputum IL-4 levels decrease dramatically 6 months after aspirin desensitization

Sputum IL-4 level was measurable at the baseline time point (baseline mean, 28.1 pg/mL), increased during desensitization (postbaseline mean, 51.1 pg/mL; 82% increase relative to baseline; 95% CI, -12% to 275%; $P = .10$), and decreased dramatically after 6 months of daily aspirin therapy (6-month mean, 1.5 pg/mL; 94.7% decrease relative to baseline; 95% CI, 67% to 99.2%; $P = .004$; Fig 1). Two of the 3 subjects who were aspirin-tolerant and treated with aspirin for 6 months showed an increase in IL-4, with the third showing no change. An indicator variable for ICS use was included as a covariate in the model because of an improved fit (on the basis of the AIC statistic).

FLT3-L, MMP-9, and TIMP-1

We analyzed FLT3-L, MMP-9, and TIMP-1 for correlation among themselves and with others (Table III). Strong positive correlations existed between MMP-9 and TIMP-1 for both predesensitization to postdesensitization changes ($r = 0.79$; $P = .0003$; Fig 2) and strictly at the postdesensitization time point ($r = 0.80$; $P = .0002$). Interestingly, there was an inverse correlation between IL-4 and FLT3-L at postdesensitization ($r = -0.71$; $P = .005$; Fig 3). A strong correlation between MMP-9 and tryptase at postdesensitization was also observed ($r = 0.82$; $P = .001$; Fig 4). There were several other marginally significant correlations that were not significant after applying the false discovery rate procedures.

There were no significant changes in mean MMP-9 levels between predesensitization and postdesensitization (baseline mean, 57.1 ng/mL; postbaseline mean, 56.6 ng/mL; $P = .98$ for relative change), but there was a marginally significant drop ($P = .05$) from postdesensitization to the 6-month time point (6-month mean, 24.7 ng/mL; 56% drop relative to postbaseline; 95% CI, 2% to 81%). We did not have enough sputum to run FLT3-L and TIMP-1 at the 6-month time point, so linear mixed models were not fit for these variables.

DISCUSSION

Although the primary purpose of this study was to evaluate the effect of aspirin therapy on novel biomarkers before and after aspirin desensitization, we did measure symptom changes over time as aspirin therapy was initiated. The symptom data in this study validated the previously known clinical benefit in nasal symptoms after performing aspirin desensitization in patients with AERD.^{4-6,21,22} Patients reported improvement in symptoms as captured by the SNOT-20 questionnaire. All 5 questions related to nasal symptom and function showed a decrease in symptoms, but 2 specific symptom scores, "Need to blow nose" and "Sneezing," showed a statistically significant decrease at the 6-month time point. Both of these specifically reflect the intranasal axis relative to the composite SNOT-20, which was validated in patients after sinus surgery. Because of the limitations of the SNOT-20, we also used the CSS, which is a broader tool capturing data regarding infections, congestion, use of antibiotics, and so forth. We again noted improvement in CSS symptom scores. The composite score, which was an average of the individual questions, showed significance as reported. In regard to the biomarkers, we noted that aspirin desensitization is, in the short term, associated with an increase in FeNO, which returns to baseline after 6 months of aspirin treatment. This elevation in FeNO after challenge is consistent with a previous report on the effect of lysine-aspirin bronchial challenge²³ that reported an increase in FeNO after challenge in subjects with aspirin-intolerant asthma but did not see a similar rise in the subjects with aspirin-tolerant asthma. The proposed mechanism for this increase in FeNO is most likely a result of a concurrent increase in airway eosinophils. Studies have reported correlations between increased sputum eosinophil counts and FeNO.^{24,25} After segmental bronchial challenge of subjects with asthma with lysine aspirin, a significant reduction in the numbers of tryptase-positive mast cells is seen in the bronchial mucosa, whereas there is a noted increase in the infiltration of eosinophils.²⁶ In addition, two reports from Szczeklik et al and Sladek et al have shown that aspirin challenge results in an increase in bronchoalveolar lavage eosinophil counts.^{27,28}

TABLE III. Pearson correlations between pairs of variables.

Variable	With variable	Postdesensitization			Predesensitization to postdesensitization change		
		<i>r</i>	<i>P</i> value	<i>n</i>	<i>r</i>	<i>P</i> value	<i>n</i>
MMP-9	TIMP-1	0.80*	.0002	16	0.79*	.0003	16
	FLT3-L	−0.07	.78	16	0.09	.73	16
	FeNO	0.01	.98	15	0.54	.04	15
	Tryptase	0.82*	.001	12	0.63	.03	12
TIMP-1	IL-4	0.13	.65	14	0.23	.44	14
	FLT3-L	−0.37	.15	16	−0.36	.17	16
	FeNO	0.23	.41	15	0.29	.29	15
	Tryptase	0.61	.03	12	0.67	.02	12
FLT3-L	IL-4	0.31	.28	14	0.50	.07	14
	FeNO	0.03	.93	15	−0.01	.98	15
	Tryptase	−0.31	.32	12	−0.26	.41	12
	IL-4	−0.71*	.005	14	−0.28	.33	14
FeNO	Tryptase	0.08	.78	14	0.26	.41	12
	IL-4	0.23	.41	15	0.05	.88	13
Tryptase	IL-4	0.48	.10	13	0.35	.30	11

Some variables were natural log-transformed before analysis (see Methods section).

*Significant with false discovery rate = 0.10 (both Benjamini-Hochberg and Benjamini-Liu procedures) applied to set of correlations with same time frame.

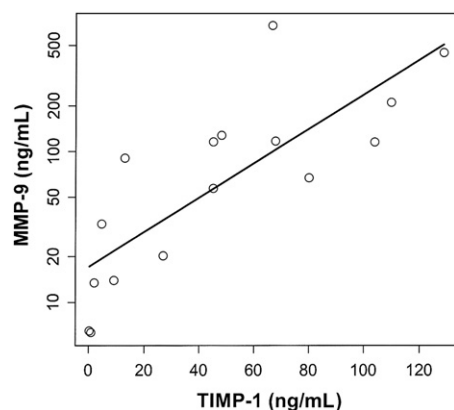


FIG 2. Scatterplot of MMP-9 versus TIMP-1 at the postdesensitization time point, with the least squares regression line. Pearson $r = 0.80$; $P = .0002$. The natural log scale was used for MMP-9. See the Statistics subsection for more analysis details.

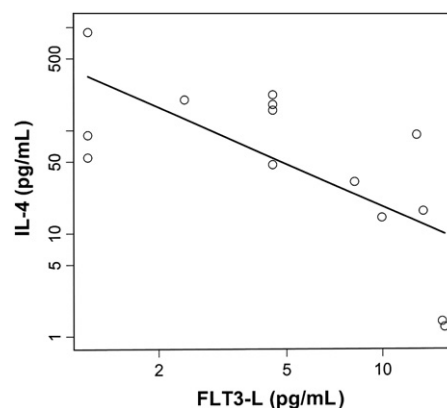


FIG 3. Scatterplot of IL-4 versus FLT3-L at the postdesensitization time point, with the least squares regression line. Pearson $r = -0.71$; $P = .005$. The natural log scale was used for both variables. See the Statistics subsection for more analysis details.

Therefore, tissue eosinophilia resulting from aspirin challenge may secondarily lead to an increase in FeNO, which helps to explain our findings. However, elevated FeNO after aspirin challenge was not seen with low-dose administration of aspirin in those with persistent asthma, although this study did not segregate subjects with aspirin-intolerant asthma and aspirin-tolerant asthma. Thus the precise mechanism for the noted increase in FeNO is not fully understood but suggests that the increase in FeNO after aspirin challenge may be dose-dependent. All patients in this study were on various pharmacotherapies at baseline. Medication and dosage changes were recorded at each visit. It is reasonable to assume that various medications, especially corticosteroids, could affect markers such as FeNO. However, our analysis suggested that such variables did not significantly account for changes in biomarker levels over time. ICS was included in the model for IL-4, but it in fact had little impact on changes in IL-4 estimates over time, because the majority of subjects did not change ICS dosages during the study.

Tryptase serves as a marker for mast cell degranulation. We noted a significant increase in sputum tryptase levels during the

desensitization process, and unlike the FeNO, tryptase remained elevated after 6 months of therapy. Serum tryptase has been previously shown to increase immediately after aspirin challenge.²⁹⁻³¹ However, to our knowledge, sputum tryptase levels have not been studied previously in patients after long-term treatment with aspirin. The ImmunoCap measures both α and β tryptase and reports a total value. Because the baseline tryptase value is a summation of both the α (inactive, or immature tryptase) and the β (active, mature tryptase), the elevated tryptase value during aspirin desensitization could be interpreted as primarily a result of increased β -tryptase. As such, daily aspirin exposure could be triggering ongoing mast cell degranulation, albeit at a low level. Bochenek et al³¹ showed that both at baseline and subsequent to aspirin challenge, there was a rise in mast cell products (serum prostaglandin D2 and its metabolite) and these levels in the short term continued to rise, suggesting chronic ongoing mast cell activation as well as direct activation by aspirin. These authors did not measure any sputum markers, but our findings in specimens obtained directly from the airway support the reported conclusions based on peripheral blood markers.

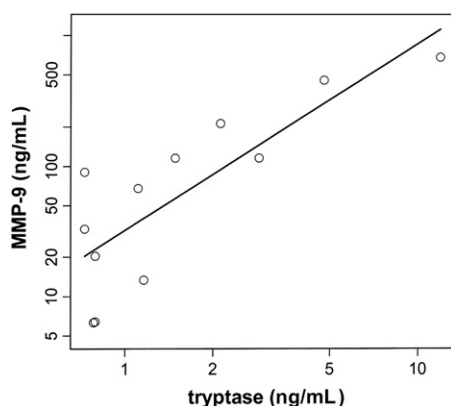


FIG 4. Scatterplot of MMP-9 versus tryptase at the postdesensitization time point, with the least squares regression line. Pearson $r = 0.82$; $P = .001$. The natural log scale was used for both variables. See the Statistics subsection for more analysis details.

Sputum IL-4 levels decreased dramatically after 6 months of aspirin treatment, whereas on day 2, there was a trend toward an increase in IL-4, but this did not reach significance. *In vitro* data with aspirin support this finding.¹⁴ We hypothesize that long-term aspirin therapy in tolerized individuals may be downregulating cysLT_1 receptors secondary to decreased IL-4 levels. Sousa et al¹³ have reported an increase in cysLT_1 receptors on leukocytes from the nasal mucosa at baseline and a significant reduction in these receptors after aspirin desensitization, but the mechanism for this decrease was not delineated. Previous data from Early et al have shown IL-4 induces cysteinyl LT receptors on both T and B cells.³² In addition, the same group recently reported in an *in vitro* model (monocyte culture) that aspirin inhibits IL-4 nuclear extracts engaging signal transducer and activator of transcription 6 (STAT6) binding sites, thus blocking the inducible expression of STAT6.³³ Therefore, the reported decrease in IL-4 in our study explains the observation in the article by Sousa et al¹³ in that the aspirin causes downregulation of IL-4, and subsequent downregulation of cysteinyl LT receptors on lymphocytes, ultimately leading to reduced $\text{T}_\text{H}2$ inflammation, which may be mediated through STAT6. The following question arises: Would a similar effect be seen in those aspirin-tolerant but expressing the phenotypic features of chronic sinusitis, nasal polyps, and asthma? In our study, each subject served as his or her own control, but we were fortunate to have 3 additional subjects who were aspirin/NSAID-tolerant but were empirically treated with aspirin. IL-4 increased in 2 and was unchanged in the third, suggesting that the reported effect in the desensitized cohort was unique to the intolerant group.

We evaluated several exploratory markers (FLT3-L, MMP-9, and TIMP-1) in our subjects undergoing aspirin desensitization. Interestingly, FLT3-L has not been studied in human asthma. In mouse studies, FLT3-L mediates anti-inflammatory effects through plasmacytoid dendritic cells and induction of $\text{T}_\text{H}1$ cells.^{34,35} Edwan and colleagues studied mouse models of allergic inflammation and demonstrated that FLT3-L reverses airway hyperresponsiveness in ovalbumin-sensitized mice.^{36,37} The authors hypothesized that the antiasthma effect of FLT3-L was modulated through lung dendritic cells. We found an inverse correlation of postdesensitization FLT3-L levels and IL-4 (Fig 3). Thus, the initial rise seen in IL-4 was counter to the decreased FLT3-L levels, suggesting a trend to antagonize the $\text{T}_\text{H}2$ stimulus (IL-4) as the

patient was tolerized. Unfortunately, we were unable to run 6-month FLT3-L levels as stated under section FLT3-L, MMP-9, and TIMP-1 to see whether the decrease in IL-4 noted at 6 months was associated with an increase in FLT3-L.

MMP-9 and its inhibitor TIMP-1 have been studied in human asthma and are felt to mediate both inflammation and airway remodeling.³⁸ MMP-9 production in asthma has been related to both recruited inflammatory cells (neutrophils and eosinophils) and airway structural cells,³⁸⁻⁴⁰ but these enzymes have not been studied in AERD. Of the myriad of MMPs, MMP-9 is likely to be important in asthma. MMP-9 knockout mice have shown an infiltration of inflammatory cells in the airway.⁴⁰ In addition, a significant correlation was found in immunohistochemistry and *in situ* hybridization between MMP-9 expression and eosinophil counts.⁴¹ Allergen challenge studies have shown that the levels of both MMP-9 and TIMP-1 increase after the challenge.^{42,43}

We similarly found a significant correlation between MMP-9 and TIMP-1 after aspirin desensitization not previously reported. However, we also noted a positive correlation between MMP-9 and tryptase after desensitization. This finding is explained by data from Vliagoftis et al⁴⁴ showing protease-activated receptor (PAR-2) expression on airway epithelial cells and the activation of PAR-2 by mast cell tryptase leading to increases in epithelial-derived MMP-9. We have reported an increase in sputum tryptase, which through PAR-2 receptors may have led to our finding of increased MMP-9 after desensitization. We noted a significant decrease at 6 months in IL-4, and a similar pattern was seen in MMP-9, again supporting the downregulation of proinflammatory stimuli after prolonged aspirin therapy.

As asthma is increasingly viewed as a heterogeneous disease with different phenotypes, we have shown changes in novel biomarkers that have not been previously studied in the aspirin-sensitive population. We have provided some missing links to previous observations in the AERD cohort. The absolute levels of these markers were heterogeneous in our subjects, but our contention is that the relationship of these markers after desensitization is more important because the character of the inflammatory response in asthma varies from individual to individual. What is now needed is a long-term study to see whether the levels of these markers correlate with clinical outcomes in those subjects who ultimately show a positive response to aspirin in traditional clinical parameters such as decreased prednisone use, decreased surgical intervention, and improvement in computed tomography imaging of the sinuses. One shortcoming of our study is that we are lacking 6-month data in all subjects because of dropouts or insufficient sputum quantity in those who remained. As a result we were unable to run all the markers at the 6-month time point. However, we report changes in biomarkers either not previously studied in the aspirin cohort or not evaluated in the human lung such as FLT3-L while providing some observations such as the decrease in IL-4, which shed light on earlier reports not clearly understood. We believe that these results will pave the way for future long-term studies to elucidate the underlying mechanisms conferring long-term clinical benefit from aspirin desensitization.

In conclusion, we have shown that aspirin desensitization in patients with AERD involves airway mast cell degranulation acutely, giving rise to markers such as sputum tryptase while inducing concurrent increase in MMP-9. Further, aspirin desensitization is associated with a decrease in IL-4 over a 6-month period and in the shorter term is inversely related to a $\text{T}_\text{H}1$ -inducing marker, FLT3-L.

Key messages

- This study shows a decrease in IL-4 after aspirin desensitization and 6 months of aspirin therapy in patients with AERD. This has not been previously demonstrated and adds to the potential mechanism of action of this therapy.
- This study evaluates the effect of aspirin desensitization on novel biomarkers in the AERD asthma cohort and markers not previously studied in human asthma (FLT3 ligand).
- The study evaluates the effect of aspirin therapy after desensitization on previously characterized biomarkers, FeNO and sputum tryptase, with latter not having been studied previously in the AERD cohort.
- The study validates the previous clinical benefit of aspirin desensitization.

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