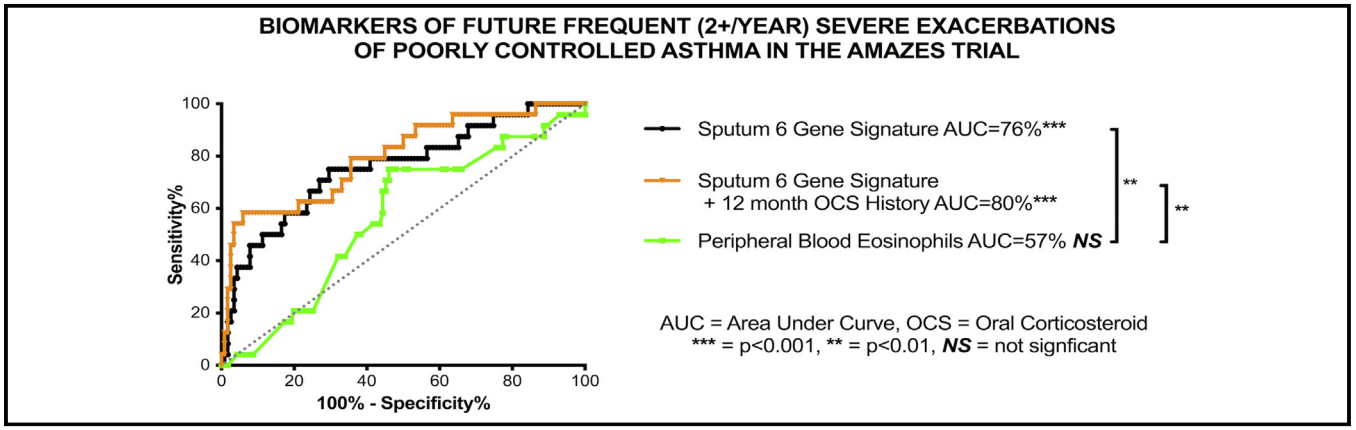
A sputum 6-gene signature predicts future exacerbations of poorly controlled asthma

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GRAPHICAL ABSTRACT



Background: Improved diagnostic tools for predicting future exacerbation frequency in asthmatic patients are required. A sputum gene expression signature of 6 biomarkers (6-gene signature [6GS], including Charcot-Leyden crystal galectin *[CLC]*; carboxypeptidase 3 *[CPA3]*; deoxyribonuclease 1-like 3 *[DNASE1L3]*; alkaline phosphatase, liver/bone/kidney *[ALPL]*; *CXCR2*; and *IL1B*) predicts inflammatory and treatment response phenotypes in patients with stable asthma. Recently, we

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demonstrated that azithromycin (AZM) add-on treatment in patients with uncontrolled moderate-to-severe asthma significantly reduced asthma exacerbations (AMAZES clinical trial).

Objectives: We sought to test whether the 6GS predicts future exacerbation and inflammatory phenotypes in a subpopulation of AMAZES and to test the effect of AZM therapy on 6GS expression and prognostic capacity.

Methods: One hundred forty-two patients (73 placebo-treated and 69 AZM-treated patients) had sputum stored for quantitative PCR of 6GS markers at baseline and after 48 weeks of treatment. Logistic regression and receiver operating characteristic and area under the curve (AUC) determination were performed on baseline measures, and in an exploratory analysis the predictive value of the 6GS was compared with conventional biomarkers for exacerbation and inflammatory phenotypes.

Results: The 6GS significantly predicted all future exacerbation phenotypes tested. Calculated AUCs for the 6GS were significantly greater than AUCs for peripheral blood eosinophil counts, sputum neutrophil counts, and combined sputum eosinophil and neutrophil counts. 6GS AUCs were also numerically but not significantly greater than those for fractional exhaled nitric oxide values and sputum eosinophil counts. AZM treatment altered neither 6GS expression nor the predictive capacity of the 6GS for future exacerbation phenotypes. The 6GS was a significant predictor of airway inflammatory phenotype in this population.

Conclusion: We demonstrate that a sputum gene signature can predict future exacerbation phenotypes of asthma, with the greatest biomarker performance in identifying those who would experience frequent severe exacerbations. AZM therapy did not modify 6GS expression or biomarker performance, suggesting the therapeutic action of AZM is independent of 6GS-related inflammatory pathways. (J Allergy Clin Immunol 2019;144:51-60.)

Key words: *Asthma, sputum, biomarker, inflammation, exacerbation, macrolide, azithromycin, eosinophil, gene signature, clinical trial*

Abbreviations used

ACQ: Asthma Control Questionnaire
ALPL: Alkaline phosphatase, liver/bone/kidney
AUC: Area under the curve
AZM: Azithromycin
CLC: Charcot-Leyden crystal galectin
CPA3: Carboxypeptidase 3
ΔCt: Change in cycle threshold
DNASE1L3: Deoxyribonuclease 1-like 3
EA: Eosinophilic asthma
FENO: Fractional exhaled nitric oxide
6GS: Six-gene signature
ICS: Inhaled corticosteroid
NA: Neutrophilic asthma
NEA: Noneosinophilic asthma
OCS: Oral corticosteroid
PBE: Peripheral blood eosinophil
PGA: Paucigranulocytic asthma
qPCR: Quantitative PCR
ROC: Receiver operating characteristic

Asthma is a chronic respiratory disease characterized by variable or reversible airflow obstruction, often featuring airway inflammation. Analysis of induced sputum through quantification of relative abundance of eosinophils and neutrophils allows classification of asthma into inflammatory phenotypes.[1-3] Airway inflammometry can help guide the choice of conventional and emerging treatments for asthmatic patients.[4,5] Eosinophilic airway inflammation, in contrast to neutrophilic inflammation, is corticosteroid sensitive, and tailoring of inhaled corticosteroid (ICS) therapy guided by sputum eosinophil quantification showed greater benefit in clinical trials compared with conventional management.[6-8]

Sputum induction, processing, and analysis are technically demanding and therefore limited to specialist clinical research laboratories. Thus recent research has centered on identification of biomarkers of airway inflammation, which can be easily accessed and measured. Peripheral blood eosinophils (PBEs) and fractional exhaled nitric oxide (FENO) have demonstrated some value as biomarkers for selection of patients responsive to novel biological therapies targeting type 2 inflammation[4] but at best show modest correlation with airway inflammatory phenotype and have not proved accurate in predicting responsiveness to corticosteroids. Therefore improved biomarkers are needed.

Recent transcriptomic and proteomic studies have extended the assessment of sputum inflammation.[9-12] Previously, we reported a sputum gene expression signature comprised of 6 transcripts (Charcot-Leyden crystal galectin *[CLC]*; carboxypeptidase 3 *[CPA3]*; deoxyribonuclease 1-like 3 *[DNASE1L3]*; alkaline phosphatase, liver/bone/kidney *[ALPL]*; *CXCR2*; and *IL1B*) that distinguished airway inflammatory phenotypes of asthma with high specificity and sensitivity.[13] *CLC*, *CPA3*, and *DNASE1L3* expression are increased in patients with eosinophilic asthma (EA). Expression of *ALPL*, *CXCR2*, and *IL1B* is increased in neutrophilic asthma (NA) and mark innate inflammatory signaling pathways relating to TNF-α, CXCL1, and IL-1β, respectively. This 6-gene signature (6GS) also predicts responsiveness to ICS[13] and oral corticosteroid (OCS) treatment,[14] which suppress *CLC*, *CPA3*, and *DNASE1L3* expression. Development of sputum gene signatures can increase the feasibility of use of sputum-based measures in the clinic because sample processing (RNA extraction, cDNA synthesis, and quantitative PCR [qPCR]) can be automated, and the markers have high specificity.

Recently, we published findings from a clinical trial (AMAZES) that demonstrated that treatment of moderate-to-severe uncontrolled asthma with the macrolide azithromycin (AZM) reduced exacerbation frequency and improved quality of life over a 48-week period.[15] In this study none of the inflammatory or clinical features examined at baseline identified an AZM-responsive subpopulation. The mechanism of action through which AZM reduces asthma exacerbations remains unclear and could be related to its anti-inflammatory, antibacterial, or antiviral properties.

In the present study we evaluate the ability of the sputum 6GS to predict asthma exacerbation frequency and differentiate the airway inflammatory phenotype in a subpopulation of the AMAZES trial. The effect of AZM treatment on 6GS expression and prognostic potential was tested. The prognostic potential of the 6GS was compared with that of sputum cell, PBE, and FENO measurement. We hypothesized that the 6GS would provide superior prediction of exacerbation and inflammatory phenotype compared with other biomarkers.

METHODS

The AMAZES study[15] was a double-blind, placebo-controlled trial in which 420 adults with persistent symptomatic asthma despite current use of ICSs and long-acting bronchodilators were randomized to receive 500 mg of AZM 3 times per week or identical placebo for 48 weeks (see the Methods section in this article's Online Repository at www.jacionline.org). Induced sputum was collected before randomization and at 48 weeks. Asthma exacerbations were recorded as the primary study outcome.[15] The trial was approved by institutional ethics committees. All patients provided written informed consent.

Clinical methods

We performed the present analysis on a subset of AMAZES study participants[15] who were included if sputum was available for differential cell count and qPCR analysis from both the baseline and 48-week visits. Sputum induction and analysis were performed by using our previously described methods (see the Methods section in this article's Online Repository). Inflammatory phenotypes were defined as follows: EA (sputum eosinophil count $\geq 3\%$[7]), NA (sputum neutrophil count $\geq 61\%$[1]), mixed granulocytic asthma (sputum neutrophil count $\geq 61\%$ and eosinophil count $\geq 3\%$), and paucigranulocytic asthma (PGA; sputum neutrophil count $< 61\%$ and eosinophil count $< 3\%$). In the AMAZES trial exacerbation occurrence and type (severe or moderate) were determined in a structured interview. Decisions regarding treatment of trial participants during exacerbation were determined by the treating physician and were not part of the trial. Severe exacerbations were defined as worsening of asthma symptoms requiring 3 or more days of systemic corticosteroid treatment of 10 mg/d or greater or an asthma-specific hospitalization or emergency department visit requiring systemic corticosteroids. Moderate exacerbations were defined as any temporary increase in ICS and/or antibiotic use in conjunction with a deterioration in asthma symptoms (change in Asthma Control Questionnaire [ACQ-6] score of ≥0.5 or increased diary symptom score), any increase in β_2-agonist use for at least 2 days, or an emergency department visit not requiring systemic corticosteroids.

Gene expression analysis

Sputum gene expression of *CLC*, *CPA3*, *DNASE1L3*, *ALPL*, *CXCR2*, and *IL1B* was quantified, as previously described (see the Methods section in this article's Online Repository).[9] Statistical analysis of diagnostic ability was performed on the change in cycle threshold (ΔCt) between the target gene and the housekeeping gene β-actin. For relative gene expression levels, data were log-transformed ($2^{-\Delta Ct}$).

Statistical analysis

The risk of being an exacerbator, as opposed to a nonexacerbator, was modeled by using logistic regression (STATA 13; StataCorp, College Station, Tex) with a single marker (univariate) or a combination of markers (multiple logistic regression). Several alternative binary definitions of exacerbator status were used for the dependent variable according to both the frequency and severity of exacerbations. These included 1 or more versus none (any exacerbations) and 2 or more versus 1 or none (frequent exacerbations), where exacerbations included all exacerbations (total moderate and severe) or were limited to severe exacerbations.[15] Each model was adjusted for AZM treatment and conducted with and without interaction terms for treatment and individual gene expression to examine the potential effect of AZM treatment on the relationship between 6GS and future exacerbation. Models with and without interaction terms were then compared by using a log-likelihood ratio test, and if nonsignificant ($P < .05$), models with no interaction terms were used.

For each exacerbator status outcome and predictor set, each member of the study population was assigned a predicted value for the 6GS that was generated by means of input of the 6 genes as individual variables in a multiple logistic model according to exacerbator status outcome. Similarly, each member of the study population was assigned a predicted value for the other biomarkers tested by using the logistic model adjusted for AZM treatment.

Receiver operating characteristic (ROC) curves were generated for the 6GS and other biomarker-predicted values by exacerbator (outcome) status for each exacerbation model. The area under the curve (AUC) was estimated for each model as an indicator of the predictive accuracy of that model.

In an exploratory analysis ROC curves for the 6GS were compared with traditional biomarker ROC curves, including sputum eosinophil percentages, PBE counts, and FENO values. The predictive capacity of the 6GS (with and without adjustment for prior history of OCS use) and prior history of OCS use alone for severe exacerbations were also compared. Significance was accepted when the P value was less than .05.

Similar logistic or multiple logistic regression with ROC curve analysis was performed to test the ability of 6GS, PBE, and FENO values to predict airway inflammatory phenotype at baseline.

For analysis of qPCR data, the Mann-Whitney test was used for comparison between inflammatory subtypes and comparison at visit 10 between treatments. For comparison of baseline with visit 10 data within each treatment group, the Wilcoxon paired test was performed.

RESULTS

Subjects' characteristics

Most patients were classified as Global Initiative for Asthma step 4 (85.9%), 48.6% were classified as having severe asthma (European Respiratory Society/American Thoracic Society guidelines),[16] and all had persistent symptomatic (ACQ-6 score ≥ 0.75) asthma despite ongoing treatment.[15] Major clinical and inflammatory characteristics were similar between participants randomized to the placebo and AZM arms of the trial, including age, sex, asthma control, asthma severity, spirometric results, and systemic and airway inflammatory measures (Table I). Of note, the primary outcome of reduced exacerbations in AZM-treated patients previously reported in the whole AMAZES cohort was recapitulated in this subpopulation (Table I).

6GS is significantly associated with future exacerbations independently of AZM treatment status

We first examined the relationship between 6GS measurement at baseline and exacerbations subsequently recorded during the 48-week AMAZES trial (moderate and severe or severe only). There was no significant interaction between AZM treatment and the relationship between 6GS values and future exacerbations and no significant difference between the models with or without interaction terms. A significant association was observed between the combined 6GS components and future moderate and severe exacerbations (model $P = .036$) and future frequent severe exacerbations (model $P = .022$).

6GS outperforms traditional biomarkers as a prognostic test for future exacerbation phenotypes

In a series of exploratory analyses, we performed logistic regression with ROC analysis using the 6GS and sputum eosinophil and/or neutrophil, PBE, and FENO measurements quantified at baseline to evaluate their relative prognostic value for various exacerbation phenotypes based on exacerbations recorded during the trial period. Because there was no interaction between AZM treatment and association of the 6GS with future exacerbations, we combined placebo- and AZM-treated patients in our initial analysis.

TABLE I. Subjects' characteristics

	All	Placebo group	AZM group
No.	142	73	69
Age (y)*	60.62 (49.79-69.14)	60.01 (48.78-67.80)	62.21 (53.21-69.19)
Sex (M/F)	65/77	33/40	32/37
Atopy†	110/139 (79.1%)	57/70 (81.4%)	53/69 (76.8%)
Ex-smoker†	50 (35.2%)	26 (35.6%)	24 (34.8%)
Pack years*	9.15 (1.30-24.0)	9.2 (1.4-25.0)	9.15 (1.3-22.0)
ACQ score*	1.58 (1.0-2.17)	1.67 (1.17-2.33)	1.33 (1.0-2.17)
GINA step 4†	120 (84.5%)	61 (83.6%)	59 (85.5%)
Severe asthma†	69 (48.6%)	34 (46.6%)	35 (50.7%)
Pre-b2 FEV_1 (%)‡	73.85 (18.84)	73.23 (18.93)	74.52 (18.85)
Pre-b2 FVC (%)‡	84.07 (14.62)	82.70 (14.57)	85.55 (14.64)
Pre-b2 FEV_1/FVC (%)‡	67.73 (11.19)	68.15 (10.92)	67.28 (11.54)
ICS dose (BDP μg/d)*	1000 (800-2000)	1000 (800-2000)	1280 (800-2000)
FENO (ppb)*	25.80 (15.58-47.45), n = 68	31.65 (18.30-53.0), n = 34	21.03 (14.30-34.70), n = 34
Blood eosinophils ($\times 10^9$/L)*	0.29 (0.2-0.4)	0.3 (0.2-0.4)	0.2 (0.12-0.4)
Sputum cell counts			
Sputum cell viability*	72.1 (55.4-84.0)	78.0 (61.0-86.1)	68.2 (53.3-82.7)
Total cell count ($\times 10^6$/mL)*	4.55 (2.61-7.56), n = 139	4.86 (2.70-9.27), n = 73	4.23 (2.25-6.75), n = 66
Neutrophils (%)*	32.5 (14.0-54.0)	33.5 (18.0-55.0)	31.75 (12.50-52.75)
Eosinophils (%)*	1.75 (0.50-9.50)	2.0 (0.50-6.25)	1.63 (0.25-11.50)
Macrophages (%)*	50.50 (31.60-69.0)	51.0 (31.75-68.75)	45.63 (31.60-69.0)
Lymphocytes (%)*	0.75 (0.25-1.75)	0.75 (0.25-1.50)	0.75 (0.25-2.0)
Columnar epithelial cells (%)*	2.50 (1.0-5.75)	2.50 (0.75-4.75)	2.38 (1.25-6.50)
C2R-stained eosinophils (%)*	2.25 (0.50-9.50)	2.50 (0.75-9.25)	2.0 (0.50-10.0)
Sputum phenotype			
Eosinophilic‡	58 (41.7%)	32 (43.8%)	26 (39.4%)
Neutrophilic‡	21 (15.1%)	13 (17.8%)	8 (12.1%)
Paucigranulocytic‡	55 (39.6%)	24 (32.9%)	31 (47.0%)
Mixed‡	5 (3.6%)	4 (5.5%)	1 (1.5%)
Exacerbations/person-year during AMAZES trial			
Total	1.61	2.11	1.07§
Severe	0.77	1.04	0.48§
Moderate	0.84	1.07	0.59§

BDP, Beclomethasone dipropionate; *C2R*, chromotrope 2R; *FVC*, forced vital capacity; *GINA*, Global Initiative for Asthma; *Pre-b2*, pre-β2 agonist.
*Median (first-third quartiles).
†Number (percentage).
‡Mean (SD).
§Negative binomial regression: $P < .03$.

Sputum eosinophil, sputum neutrophil, and PBE counts and FENO values did not provide statistically significant discriminatory capacity for those patients who experienced at least 1 severe exacerbation (exacerbators) during the trial from those who experienced none (nonexacerbators; Fig 1, *A*, and Table II). In contrast, sputum 6GS values provided modest but significant prediction of severe exacerbators versus nonexacerbators (AUC = 68.1%, $P < .0001$; Table II and see Table E1 in this article's Online Repository at www.jacionline.org).

Sputum eosinophil counts, eosinophil and neutrophil counts combined, and 6GS values provided significant discriminatory capacity of patients who experienced frequent (≥2) versus infrequent (<2) severe exacerbations (6GS AUC = 76.1%, $P < .0001$; sputum eosinophil AUC = 70.3%, $P = .002$; sputum eosinophil and neutrophil AUC = 68.4%, $P = .012$; Fig 1, *B*, and Table II). In the subset of patients in whom FENO values were measured, both 6GS and FENO values provided significant prognostic capacity (6GS AUC = 83.7%, $P < .0001$; FENO AUC = 75.6%, $P < .0001$). Of all biomarkers examined, the sputum 6GS provided the highest AUC values and significantly outperformed sputum neutrophil counts, eosinophil and neutrophil counts combined, and PBE counts in predicting the frequent severe exacerbation phenotype (Table II and see Table E2 in this article's Online Repository at www.jacionline.org).

In the AMAZES study moderate exacerbations were also quantified.[15] We performed logistic regression analysis comparing exacerbators versus nonexacerbators and frequent versus infrequent exacerbator phenotypes for total (sum of moderate and severe) exacerbations. The 6GS value and sputum eosinophil and/or neutrophil counts significantly predicted exacerbators versus nonexacerbators (total; 6GS AUC = 69.6%, $P < .0001$; eosinophil AUC = 63%, $P = .008$; neutrophil AUC = 63.3%, $P = .005$; eosinophil and neutrophil AUC = 64.2%, $P = .003$; Fig 1, *C*; Table II; and see Table E3 in this article's Online Repository at www.jacionline.org). In this analysis the 6GS statistically outperformed sputum neutrophil and PBE measurement. 6GS and sputum eosinophil and/or neutrophil measurement significantly discriminated frequent (≥2) total exacerbators from infrequent exacerbators (6GS AUC = 66.4%, $P = .001$; eosinophil AUC = 60.6%, $P = .034$; neutrophil AUC = 60.8%, $P = .029$; eosinophil and neutrophil AUC = 62.1%, $P = .016$; Fig 1, *D*; Table II; and see Table E4 in this article's Online Repository at www.jacionline.org), and the 6GS significantly outperformed PBE measurement.

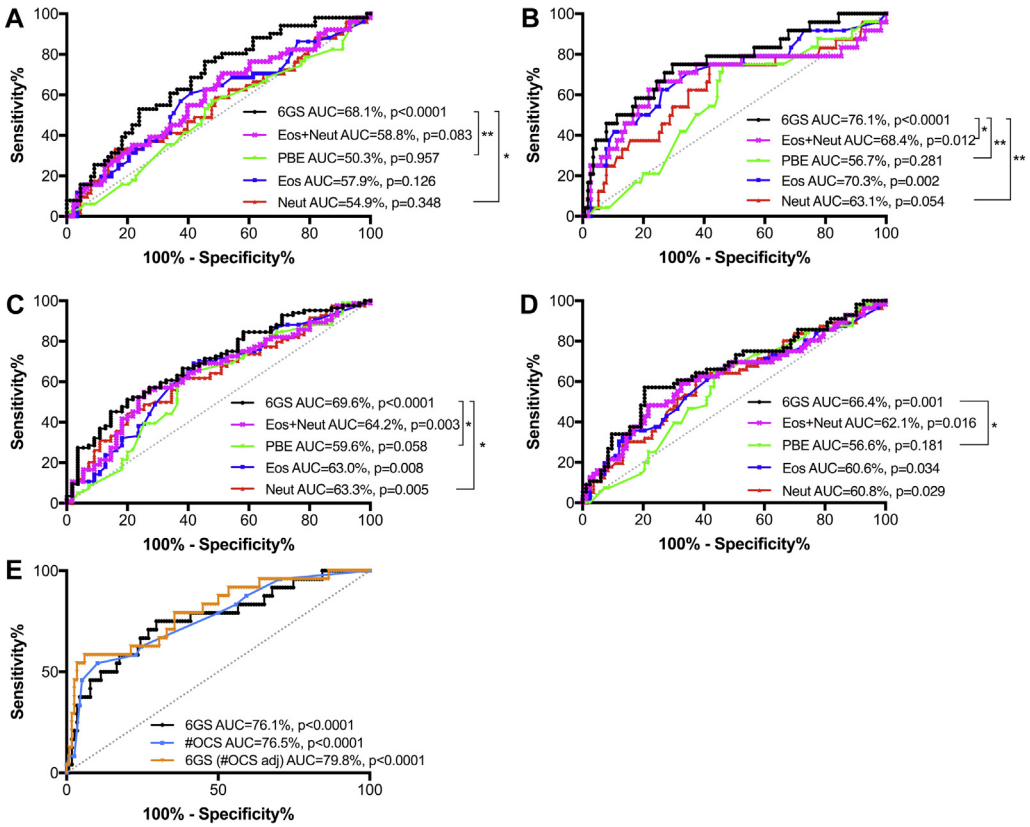


FIG 1. ROC analysis of diagnostic performance of 6GS values, sputum eosinophil *(Eos)* and/or neutrophil *(Neut)* counts, and PBE counts for predicting asthma exacerbation phenotypes. ROC curve comparison was performed in both placebo- and AZM-treated patients (combined) enrolled in the AMAZES trial. Biomarkers examined were as follows: *black line*, sputum 6GS; *pink line*, combined sputum eosinophils and neutrophils; *blue line*, sputum eosinophils; *red line*, sputum neutrophils; *green line*, PBEs. **A-D,** Comparisons shown are for nonexacerbators versus exacerbators (severe exacerbations only; Fig 1, *A*), infrequent exacerbators versus frequent exacerbators (severe exacerbations only; Fig 1, *B*), nonexacerbators versus exacerbators (sum of moderate and severe exacerbations; Fig 1, *C*), and infrequent exacerbators versus frequent exacerbators (sum of moderate and severe exacerbations; Fig 1, *D*). **E,** ROC analysis was also performed to compare the prognostic capacity of the sputum 6GS, OCS courses (prior 12 months), and the 6GS adjusted for prior OCS courses to identify frequent versus nonfrequent severe exacerbators. **P* < .05 and ***P* < .01.

History of OCS use for the 12-month period before the study baseline visit was recorded. Prior OCS history alone could significantly predict future severe exacerbation frequency with a similar AUC to the 6GS (prior OCS use AUC = 76.5%, $P < .0001$). When the 6GS was evaluated and data were adjusted for prior OCS history, the highest AUC for predicting the severe exacerbation phenotype was achieved (6GS adjusted for prior OCS use AUC = 79.8%, $P < .0001$; Fig 1, *E*).

AZM treatment does not alter 6GS expression or prediction of future exacerbation status

We evaluated the effect of 48 weeks of AZM treatment on 6GS transcript expression. At visit 10 (48 weeks of treatment), there was no significant difference in 6GS expression between the placebo- and AZM-treated groups (Fig 2, *A-F*). *CXCR2* mRNA expression was significantly increased at visit 10 versus baseline visits in both placebo- and AZM-treated patients (Fig 2, *E*). In a further exploratory subanalysis, we examined biomarker performance for the various exacerbator phenotypes, analyzing placebo- and AZM-treated groups separately. Of note, the 6GS retained statistically significant predictive capacity for all exacerbation phenotypes examined in both placebo- and AZM-treated groups, with the exception of prediction of frequent exacerbators (total) in AZM-treated patients (AUC = 62.7%, $P = .097$; Fig 2, *G-J*, and see Tables E5-E9 in this article's Online Repository at www.jacionline.org). Other biomarkers did not provide significant predictive capacity for any exacerbator phenotype in either the placebo- or AZM-treated groups, with the exception of sputum eosinophil counts, for predicting frequent severe exacerbators in the placebo group (AUC = 70.1%, $P = .004$; see Table E5).

6GS predicts airway inflammatory phenotype in a population with uncontrolled moderate-to-severe asthma

Airway expression of *CLC*, *CPA3*, and *DNASE1L3* was significantly increased in patients with EA (≥3% sputum eosinophils) versus noneosinophilic asthma (NEA; <3% sputum eosinophils),

TABLE II. AUC for each predictive marker by study population, exacerbation severity, and exacerbation frequency status

	6GS (n = 139)	Sputum eosinophils (n = 139)	Sputum neutrophils (n = 139)	Sputum eosinophils and neutrophils (n = 139)	PBEs (n = 139)	6GS (FENO [n = 67])*	FENO (n = 67)
Total exacerbations							
≥1 or 0	**AUC = 0.696, *P* < .0001**	**AUC = 0.630, *P* = .008**	**AUC = 0.633,† *P* = .005**	**AUC = 0.642, *P* = .003**	AUC = 0.596,† *P* = .058	**AUC = 0.691, *P* = .004**	AUC = 0.635, *P* = .056
≥2 or 0-1	**AUC = 0.664, *P* = .001**	**AUC = 0.606, *P* = .034**	**AUC = 0.608, *P* = .029**	**AUC = 0.621, *P* = .016**	AUC = 0.566,† *P* = .181	**AUC = 0.647, *P* = .029**	**AUC = 0.670, *P* = .010**
Severe exacerbations							
≥1 or 0	**AUC = 0.681, *P* < .0001**	AUC = 0.579, *P* = .126	AUC = 0.549,† *P* = .348	AUC = 0.588, *P* = .083	AUC = 0.503,‡ *P* = .957	**AUC = 0.736, *P* < .0001**	AUC = 0.618, *P* = .094
≥2 or 0-1	**AUC = 0.761, *P* < .0001**	**AUC = 0.703, *P* = .002**	AUC = 0.631,‡ *P* = .054	**AUC = 0.684,† *P* = .012**	AUC = 0.567,‡ *P* = .281	**AUC = 0.837, *P* < .0001**	**AUC = 0.756, *P* < .0001**

Values in boldface indicate statistical significance.
*Values were calculated in subpopulations where FENO measurements were made.
†$P < .05$ versus 6GS.
‡$P < .01$ versus 6GS.

whereas *IL1B* expression was lower in patients with EA (Fig 3, *A*). *CXCR2* and *ALPL* expression did not differ between patients with EA and those with NEA. Expression of *IL1B*, *CXCR2*, and *ALPL* was significantly increased in patients with NA versus those with nonneutrophilic asthma, whereas expression of *CLC*, *CPA3*, and *DNASE1L3* showed no significant differences between these groups (Fig 3, *B*).

We tested whether the 6GS values measured at baseline could predict airway inflammatory phenotype using multiple logistic regression and ROC curve analysis. In all analyses the sputum 6GS value discriminated airway inflammatory phenotypes to a statistically significant extent (patients with EA vs patients with NEA: AUC = 76.8%, $P < .0001$; patients with EA vs patients with NA: AUC = 92.9%, $P < .0001$; patients with EA vs patients with PGA: AUC = 76.4%, $P < .0001$; patients with NA vs patients with nonneutrophilic asthma: AUC = 89.5%, $P < .0001$; patients with NA vs patients with PGA: AUC = 88.0%, $P < .0001$; patients with PGA vs patients with nonpaucigranulocytic asthma: AUC = 74.0%, $P < .0001$; Table III).

We also examined 2 established biomarkers of type 2/eosinophilic inflammation in asthmatic patients, PBE counts and FENO values, and compared their performance with the 6GS in distinguishing sputum inflammatory phenotypes. Both PBE counts and FENO values discriminated patients with EA versus those with NEA, patients with EA versus those with NA, and patients with EA versus those with PGA to a statistically significant extent (see Table E10 in this article's Online Repository at www.jacionline.org). However, the 6GS significantly outperformed PBE (Fig 3, *C*) and FENO (Fig 3, *D*) measurement as a diagnostic test for predicting EA versus NA.

DISCUSSION

In this study we demonstrate that sputum 6GS values can predict future exacerbation phenotype in a cohort of patients with uncontrolled moderate-to-severe asthma. Furthermore, we find that AZM did not alter 6GS expression relative to placebo and that the 6GS retains its prognostic utility, even in patients who were treated with AZM add-on therapy, which reduced the overall rate of exacerbations compared with placebo treatment. The sputum 6GS had statistically better predictive capacity for future frequent severe exacerbations than PBE counts, sputum neutrophil counts, and combined sputum eosinophil and neutrophil counts. Numerically, but not statistically, superior AUC values were also observed for the 6GS compared with sputum eosinophil counts and FENO values in the prediction of future exacerbation phenotypes.

Sputum 6GS values predict future exacerbations more effectively than conventional biomarkers

The development of biomarkers that can identify asthmatic patients most likely to experience frequent exacerbations would be useful to target treatment for this at-risk population. At present, the best indicator of future exacerbation probability is past exacerbation frequency[17,18]; however this does not assist in selecting treatment options. Patients with increased eosinophilic or type 2 inflammatory biomarkers, including sputum eosinophil counts, PBE counts, and FENO values, experience more frequent severe exacerbations.[19-22]

In the present study, using ROC analysis to evaluate biomarker potential, we demonstrate that the sputum 6GS can discriminate future exacerbators from nonexacerbators and frequent from nonfrequent exacerbators when either severe exacerbations or total exacerbations were modeled. In all but 1 ROC analysis performed, sputum 6GS generated higher AUC values than conventional biomarkers. Performance of conventional biomarkers was inconsistent, and in exploratory comparative analyses the 6GS frequently statistically outperformed sputum neutrophil and PBE measurement. The sputum 6GS matched past courses of OCSs as a predictor of frequent severe exacerbations over the following 48 weeks. The ability of the 6GS to identify patients who would go on to experience frequent severe exacerbations was further enhanced when we adjusted for prior OCS courses, resulting in an AUC of 80%, which corresponds to a good performance as a prognostic tool. To our knowledge, this is the best such score reported for the identification of patients who would go on to experience frequent severe exacerbations over the following year. Of note, the 6GS was initially developed as an inflammatory phenotyping tool,[13] and thus although these results demonstrate the promise of sputum gene signatures to identify patients most at risk of exacerbation, improved biomarker

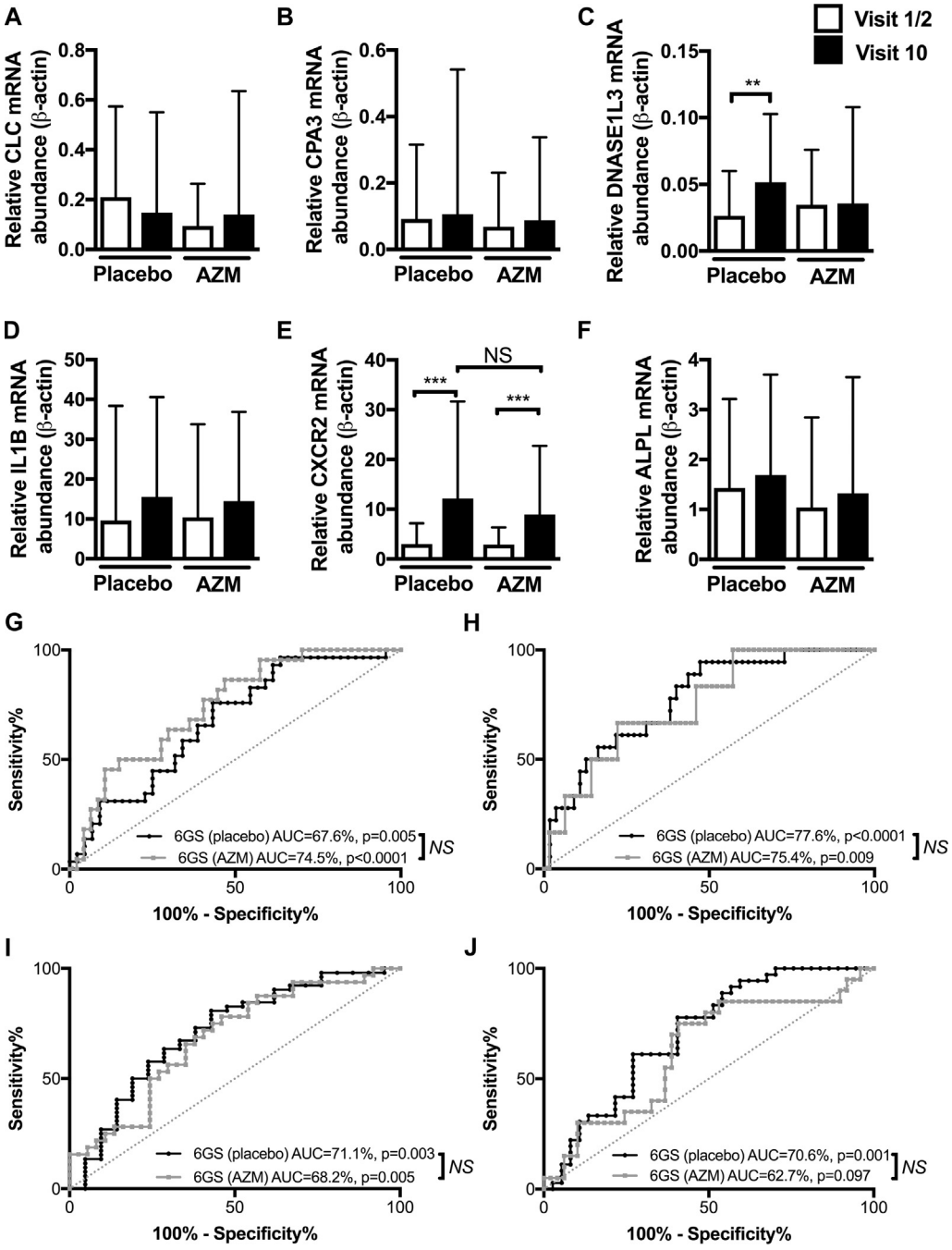


FIG 2. AZM treatment does not alter sputum 6GS expression or prognostic capacity compared with placebo. qPCR was performed on cDNA generated from raw sputum samples collected during screening visits (visit 1/2) and a visit at the end of the treatment period (week 48, visit 10) for the AMAZES trial. **A-F,** *CLC* (Fig 2, *A*), *CPA3* (Fig 2, *B*), *DNASE1L3* (Fig 2, *C*), *IL1B* (Fig 2, *D*), *CXCR2* (Fig 2, *E*), and *ALPL* (Fig 2, *F*) data are reported as relative abundance normalized to expression of the housekeeping gene β-actin. $^{**}P < .01$ and $^{***}P < .001$, Mann-Whitney test). **G-J,** ROC curve comparison for sputum 6GS in the placebo arm *(black line)* compared with sputum 6GS in the AZM arm *(grey line)*. Comparisons shown are for nonexacerbators versus exacerbators (severe exacerbations only; Fig 2, *G*), infrequent exacerbators versus frequent exacerbators (severe exacerbations only; Fig 2, *H*), nonexacerbators versus exacerbators (sum of moderate and severe exacerbations; Fig 2, *I*), and infrequent exacerbators versus frequent exacerbators (sum of moderate and severe exacerbations; Fig 2, *J*).

performance could potentially be achieved in the future through further gene signature optimization.

Why might sputum 6GS values outperform conventional inflammatory biomarkers as a prognostic tool in this instance? One possibility is that the 6GS reports on multiple inflammatory variables that impact on asthma exacerbation frequency, as opposed to a single variable in isolation. For example, although sputum neutrophil counts in this study were a poor prognostic

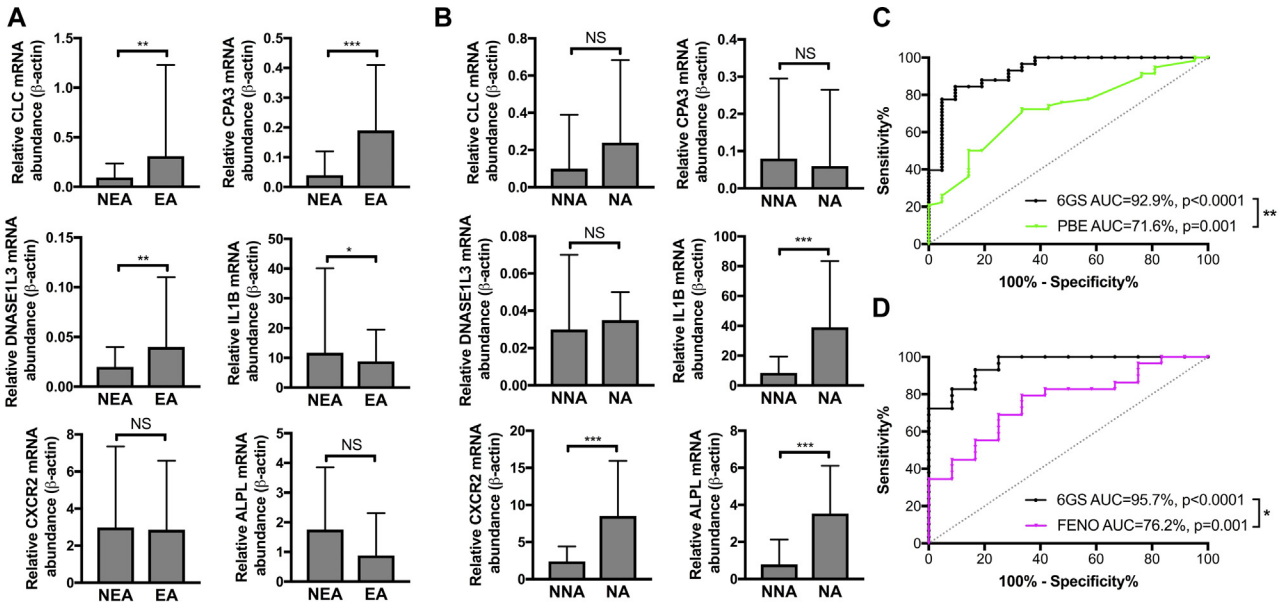


FIG 3. Sputum 6GS expression in eosinophilic and neutrophilic subtypes of asthma and prediction of airway inflammatory phenotype. qPCR was performed on cDNA generated from raw sputum samples collected during screening visits for the AMAZES trial. *CLC*, *CPA3*, *DNASE1L3*, *CXCR2*, *IL1B*, and *ALPL* data are reported as relative abundance normalized to expression of the housekeeping gene β-actin. **A,** Patients are separated into NEA (sputum eosinophils < 3%) and EA (≥3%) groups. **B,** patients are separated into non-neutrophilic asthma (*NNA*; sputum neutrophils < 61%) and NA (≥61%) groups. Data are expressed as median values with interquartile ranges. **P* < .05, ***P* < .01, and ****P* < .001, Mann-Whitney test). **C** and **D,** ROC curve comparison for sputum 6GS values *(black line)* compared with PBE counts (*green line*; Fig 3, *C*) and compared with FENO values (*pink line*; Fig 3, *D*; patient subgroup where FENO data was available). 6GS values were better at distinguishing the EA from NA phenotypes than PBE counts or FENO values. **P* < .05 and ***P* < .01.

marker for future exacerbation status, high sputum neutrophil counts have been linked to more severe forms of asthma in cluster analysis and associated with greater health care burden and hospitalization, particularly when accompanied by increased sputum eosinophil counts.[23] Combinatorial use of biomarkers reporting on distinct disease endotypes or markers could improve prognostic potential. In agreement with this hypothesis, combinatorial use of the type 2–related biomarkers FENO, PBEs, and serum periostin improves prediction of exacerbation risk when compared with each variable in isolation.[24] The individual gene markers within the 6GS combine information about the eosinophilic and neutrophilic inflammatory status of the airways. However, in our study combinatorial use of sputum eosinophil and neutrophil proportions provided little or no improvement compared with each variable in isolation.

The 6GS might provide information relating to airway inflammatory status beyond merely relating to the cellular composition of the sputum sample. We have shown previously that airway *IL1B* expression is increased in frequent exacerbators in both patients with chronic obstructive pulmonary disease and those with asthma.[25,26] It is also possible that the 6GS improves on use of sputum eosinophil and neutrophil counts in prognostic tests because it reflects cellular inflammation or processes not reported in conventional sputum analysis. *CPA3* encodes a carboxypeptidase expressed exclusively in mast cells in human subjects.[11,27,28] Our and others' sputum transcriptomic analyses identified a number of mast cell–related genes that were upregulated in patients with EA.[9-11] A recent study reported flow cytometry–based quantification of sputum mast cells and demonstrated positive correlation with sputum eosinophil counts.[29] Of the 6GS genes, *CPA3* and *CLC* were the most effective at predicting the frequent severe exacerbator phenotype (see Table E2). *CLC* can be expressed in both eosinophils and basophils, which are correlated in sputum samples.[29,30] Thus the potential of the sputum 6GS to provide information relating to mast cell– and basophil-related inflammation in addition to eosinophils and neutrophils might explain its superior performance as a predictor of exacerbation phenotype.

AZM add-on treatment does not modify sputum 6GS expression or prognostic capacity despite significantly reducing exacerbation rate

In the primary analysis of the AMAZES trial, we demonstrated that AZM add-on therapy in patients with uncontrolled moderate-to-severe asthma reduced asthma exacerbations by approximately 40% and improved asthma-related quality-of-life scores.[15] In our initial analysis we were unable to identify asthma-related variables (clinical, inflammatory, or microbiological) that predicted AZM response.[15] AZM treatment did not alter most systemic and airway inflammatory variables measured, with the exception being a significant reduction in the absolute number (but not proportion) of sputum eosinophils. Macrolides, including AZM, exert antibacterial, antiviral, and anti-inflammatory effects, all of which could explain the reduction in asthma exacerbations observed.[31,32] Our present analysis concludes that AZM add-on treatment did not significantly affect expression of sputum 6GS genes. However, the lack of effect of AZM on sputum 6GS

TABLE III. Analysis of diagnostic value of the sputum 6GS values for asthma airway inflammatory phenotype

		Logistic regression			
	Marker*	Constant	Coefficient	Model *P* value	AUC (95% CI)
EA vs NEA (n = 139)	*ALPL*	3.016208	0.1233148	**<.0001**	0.7684 (0.6898-0.8469),
	CLC		−0.0929966		***P* < .0001**
	CPA3		−0.3150406		
	CXCR2		0.0323884		
	DNASE1L3		−0.0486184		
	IL1B		0.1429728		
EA vs NA (n = 79)	*ALPL*	3.645667	1.529648	**<.0001**	0.9294 (0.8637-0.9951)
	CLC		0.0586264		***P* < .0001**
	CPA3		−0.0971249		
	CXCR2		−0.298909		
	DNASE1L3		−0.9454183		
	IL1B		0.5770762		
EA vs PGA (n = 118)	*ALPL*	4.249046	0.0063321	**.0002**	0.7636 (0.6775-0.8498)
	CLC		−0.1013124		***P* < .0001**
	CPA3		−0.3867286		
	CXCR2		−0.0325417		
	DNASE1L3		0.0516468		
	IL1B		0.0999352		
NA vs NNA (n = 139)	*ALPL*	−2.034031	−0.8416046	**<.0001**	0.8948 (0.8294-0.9603)
	CLC		−0.2829233		***P* < .0001**
	CPA3		0.0170649		
	CXCR2		−0.1026321		
	DNASE1L3		0.766542		
	IL1B		−0.3135194		
NA vs PGA (n = 81)	*ALPL*	1.820331	−0.5331606	**<.0001**	0.8804 (0.8055-0.9553)
	CLC		−0.4059778		***P* < .0001**
	CPA3		−0.0610565		
	CXCR2		−0.2789399		
	DNASE1L3		0.565322		
	IL1B		−0.2033842		
PGA vs granulocytic (n = 139)	*ALPL*	−4.316154	0.0509944	**.0004**	0.7396 (0.6561-0.5232)
	CLC		0.1325146		***P* < .0001**
	CPA3		0.2796407		
	CXCR2		0.1651778		
	DNASE1L3		−0.1245273		
	IL1B		−0.0157521		

Values in boldface indicate statistical significance.
NNA, Nonneutrophilic asthma.
*Markers are normalized to β-actin mRNA expression (ΔCT).

expression is in agreement with the prior analysis that AZM did not affect sputum or systemic inflammatory biomarkers. Consistent with this, we also found that sputum 6GS values could significantly predict future exacerbation phenotypes in most analyses conducted in AZM-treated patients, despite the fact that exacerbation rates were significantly reduced by AZM treatment. The implications of our findings are that the mechanism of action through which AZM treatment reduces the exacerbation rate is discrete from the inflammatory pathways reflected by the sputum 6GS, including sputum eosinophils, neutrophils, and mast cells.

Sputum 6GS is a useful tool for discriminating asthma inflammatory phenotypes in patients with moderate-to-severe asthma

The findings of the present study further consolidate and broaden the potential use of the 6GS as an inflammatory phenotyping tool in asthmatic patients. Here, for the first time, we demonstrate that the 6GS is effective as a diagnostic predictor of inflammatory phenotype in a cohort of patients with uncontrolled moderate-to-severe asthma. These results add to our prior work assessing the utility of sputum 6GS values in patients with stable mild-to-moderate asthma[13] and as a predictor of positive response to ICSs and OCSs.[13,14] Thus we establish that the sputum 6GS provides excellent airway inflammatory phenotyping capacity across all asthma severities.

This study does have limitations. This was a secondary analysis of our previously published AMAZES randomized controlled trial.[15] Our comparative analysis of biomarkers was exploratory, and further validation of the 6GS as a prognostic tool for future exacerbation phenotypes would require prospective recruitment of patients in a study designed to address this specific question. Notably, because of the requirement of sufficient sputum sample to allow RNA isolation and qPCR analysis for our present study, only those patients who produced sufficient sputum were included, and this could be a source of biological bias. In this subpopulation of the AMAZES randomized controlled trial, FENO data were not available for all patients, and thus our analysis of

FENO values as a prognostic tool and comparisons of its performance with the sputum 6GS are likely underpowered and thus not definitive. We cannot exclude that integration of gene signatures with cell counts could provide superior performance by better reflecting the activation status of key immune pathways, and this should be explored in future studies.

Conclusion

The sputum 6GS can predict future exacerbation phenotypes in patients with moderate-to-severe asthma, demonstrating the prognostic potential of gene signatures. We also conclude that AZM exerts a therapeutic mechanism independent of the inflammatory factors reported by the sputum 6GS and that the 6GS still has use in identifying a subset of patients who might experience frequent severe exacerbations despite AZM therapy.

We acknowledge technical assistance from Heather Macdonald, Bridgette Ridewood, Kellie Fakes, Michelle Gleeson, Erin Harvey, Catherine Delahunty, Gabrielle LeBrocq, the AMAZES study participants, clinical research offers, and laboratory technicians.

Key messages

- **Sputum gene signatures can offer a superior means to predict future exacerbations of asthma compared with conventional biomarkers.**
- **Our data suggest a therapeutic mechanism of AZM that is independent of inflammatory factors associated with the 6GS (airway eosinophilia, neutrophilia, mast cells).**

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METHODS

Trial design

The AMAZES trial was a multicenter, randomized, double-blind, placebo-controlled parallel-group trial designed to evaluate the efficacy and safety of 500 mg of oral AZM 3 times weekly for 48 weeks as add-on therapy in adults with persistent symptomatic asthma, despite maintenance controller therapy with ICS/long-acting bronchodilator. Four hundred twenty patients were allocated to AZM or identical-looking placebo in a 1:1 ratio centrally by using concealed random allocation from a computer-generated random-numbers table with permuted blocks of 4 or 6 and stratification for center and past smoking.

Trial oversight

A national steering committee of investigators designed the trial and was responsible for its conduct, analysis, interpretation, and reporting. Stenlake Compounding Pharmacy (Bondi Junction, Australia) prepared the study drug and matching placebo. The trial was funded by the Australian Government's National Health and Medical Research Council, and there was no commercial input into any aspect of the trial. The trial was registered (ANZCTR no. 12609000197235) and approved by institutional ethics committees. All patients provided written informed consent.

Patients

Patients were eligible if they had asthma defined as a compatible history and objective evidence of variable airflow obstruction from bronchodilator response (n = 307 [74.5%]), airway hyperresponsiveness (n = 129 [56%]),[E1,E2] or increased peak flow variability (n = 73 [44.7%]); were currently symptomatic with at least partial loss of asthma control (ACQ-6 score > 0.74)[E3] despite treatment with maintenance ICS/long-acting bronchodilator; were clinically stable with no recent exacerbation, infection, or change in maintenance medication for at least 4 weeks before study entry; and were nonsmokers (exhaled carbon monoxide, <10 ppm). Ex-smokers with a greater than 10 pack-year smoking history underwent gas transfer testing and were excluded if their carbon monoxide gas transfer coefficient was less than 65% of predicted value.

Procedures

After a screening visit, patients entered a 2-week run-in period. Those with optimized asthma treatment and adherence to greater than 80% of doses and who remained stable with a change in ACQ-6 score of less than 0.5 were randomized. Patients were treated for 48 weeks and attended the clinic for assessment at weeks 6, 12, 24, 36, 48, and 52. Study visits assessed symptoms, medication use, asthma exacerbations, adherence, adverse events, and spirometry. Telephone assessments were conducted at weeks 18, 30, and 42. Induced sputum[E4] was performed before randomization and at the end of the treatment visit (week 48). Adherence was assessed by tablet count returns at each visit. For safety monitoring, we assessed liver function tests and an electrocardiogram at screening, after 6 weeks of treatment, and at the end of treatment. QTc prolongation of greater than 480 ms resulted in withdrawal from the trial. Microbiological assessments involved sputum culture for recognized pulmonary pathogens (5 sites) and throat swab and nose swabs (2 sites) at randomization and the end of treatment.

Outcomes

Our primary outcome was the rate of severe asthma exacerbations over 48 weeks.[E5,E6] Severe exacerbations were worsening of asthma symptoms requiring 3 or more days of systemic corticosteroid treatment of 10 mg/d or greater or an asthma-specific hospitalization or emergency department visit requiring systemic corticosteroids. Exacerbations were captured at all visits by using structured interviewing. Secondary efficacy variables were ACQ-6 score, asthma-related quality of life,[E7] lung function, and induced sputum cell counts.

Sputum induction and analysis

Airflow limitation was assessed by using spirometry (Medgraphics, CPFS/D^{TM} usb Spirometer, BreezeSuite version 7.1; MGC Diagnostics, Saint Paul, Minn). Sputum induction with hypertonic saline (4.5%) was performed in participants whose FEV_1 was 1 L or greater by using our previously described methods.[E8] In those with FEV_1 of less than 1 L, 0.9% saline was used. For gene expression, Buffer RLT (Qiagen, Hilden, Germany) was immediately added to 100 μL of selected sputum and stored at −80°C until RNA extraction. For inflammatory cell counts, selected sputum was dispersed by using dithiothreitol, and total cell counts and viability were performed. Cytospins were prepared and stained (May-Grunwald–Giemsa), and a differential cell count was obtained from 400 nonsquamous cells.

Gene expression analysis

Sputum gene expression of *CLC*, *CPA3*, *DNASE1L3*, *ALPL*, *CXCR2*, and *IL1B* was performed, as previously described.[E9] Briefly, sputum RNA was extracted by using the Qiagen RNeasy Mini Kit, quantified, reverse transcribed to cDNA, and used to detect gene expression by using standard TaqMan real-time qPCR methods (Applied Biosystems, Foster City, Calif). Statistical analysis of diagnostic ability was performed on ΔCt between the target gene and the housekeeping gene β-actin. For relative gene expression levels, data were log-transformed ($2^{-\Delta Ct}$).

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TABLE E1. Analysis of prognostic value of sputum 6GS values for discriminating patients who experience none or some severe asthma exacerbations in the following 48 weeks

		Logistic regression			
	Marker*	**Constant**	**Coefficient**	**Model *P* value**	**AUC**
N = 142 (51/142 [35.9%] severe exacerbators)					
Individual					
	ALPL	0.6821072	−0.1729284	.0888	**0.6132** **$P = .026$**
	CLC	1.157094	−0.1759621	**.0179**	**0.6391** **$P = .003$**
	CPA3	1.238786	−0.1685756	**.0379**	**0.6152** **$P = .016$**
	CXCR2	−0.3657592	−0.0095939	.6181	0.5490 $P = .354$
	DNASE1L3	1.33276	−0.1507057	.1685	0.5893 $P = .074$
	IL1B	−0.1105435	−0.1005466	.3594	0.5652 $P = .204$
Combination					
6GS	*ALPL*	1.107549	−0.2649145	.0522	0.6889
	CLC		−0.1413991		**$P < .0001$**
	CPA3		−0.1169522		
	CXCR2		0.2247225		
	DNASE1L3		0.1312494		
	IL1B		−0.049104		

Values in boldface indicate statistical significance.
*Markers are normalized to β-actin mRNA expression (ΔCT).

TABLE E2. Analysis of diagnostic value of the sputum 6GS for discriminating patients who experience infrequent (<2) or frequent (≥2) severe asthma exacerbations in the following 48 weeks

		Logistic regression			
	Marker*	**Constant**	**Coefficient**	**Model *P* value**	**AUC**
N = 142 (24/142 [16.9%] frequent severe)					
Individual					
	ALPL	−0.4141298	−0.1112192	**.0210**	**0.6780** ***P* = .002**
	CLC	1.133079	−0.2618302	**.0004**	**0.7444** ***P* < .0001**
	CPA3	1.799899	−0.3078733	**.0002**	**0.7429** ***P* < .0001**
	CXCR2	−0.8829863	−0.0442772	**.0323**	**0.6480** ***P* = .016**
	DNASE1L3	2.309992	−0.2999175	**.0021**	**0.7270** ***P* < .0001**
	IL1B	−0.9561744	−0.0529377	**.0320**	**0.6448** ***P* = .013**
Combination					
6GS	*ALPL*	1.653505	−0.0366669	**.0091**	**0.7613** ***P* < .0001**
	CLC		−0.1419366		
	CPA3		−0.2420405		
	CXCR2		0.0328808		
	DNASE1L3		0.0800461		
	IL1B		−0.0380849		

Values in boldface indicate statistical significance.
*Markers are normalized to β-actin mRNA expression (ΔCT).

TABLE E3. Analysis of the prognostic value of the sputum 6GS for discriminating patients who experience none or some total (moderate and severe) asthma exacerbations in the following 48 weeks

		Logistic regression			
	Marker*	**Constant**	**Coefficient**	**Model *P* value**	**AUC**
N = 142 (84/142 [59.2%] total exacerbators)					
Individual					
	ALPL	2.021271	−0.1683247	**.0014**	**0.6741** ***P* < .0001**
	CLC	1.116	−0.0229152	**.0096**	**0.6332** ***P* = .006**
	CPA3	0.9125818	−0.00059	**.0102**	**0.6172** ***P* = .016**
	CXCR2	0.9289623	−0.0041664	**.0102**	**0.6361** ***P* = .004**
	DNASE1L3	0.5852789	0.0276092	**.0098**	**0.6404** ***P* = .003**
	IL1B	0.9864461	−0.0257423	**.0099**	**0.6381** ***P* = .003**
Combination					
6GS	*ALPL*	0.9350901	−0.4127687	**.0115**	**0.7114** ***P* < .0001**
	CLC		−00.0361907		
	CPA3		−.0101578		
	CXCR2		0.2094708		
	DNASE1L3		0.1425817		
	IL1B		0.1086857		

Values in boldface indicate statistical significance.
*Markers are normalized to β-actin mRNA expression (ΔCT).

TABLE E4. Analysis of the diagnostic value of the sputum 6GS for discriminating patients who experience infrequent or frequent total (moderate and severe) asthma exacerbations in the following 48 weeks

		Logistic regression			
	Marker*	Constant	Coefficient	Model *P* value	AUC
N = 142 (56/142 [39.4%] frequent total exacerbators)					
Individual					
	ALPL	0.7881897	−0.1265727	**.0148**	**0.6433** ***P* = .004**
	CLC	1.152437	−0.1299279	**.0060**	**0.6555** ***P* = .002**
	CPA3	1.413362	−0.1452072	**.0054**	**0.6530** ***P* = .002**
	CXCR2	0.3325545	−0.0675409	**.0332**	**0.6219** ***P* = .015**
	DNASE1L3	1.606988	−0.1402179	**.0145**	**0.6453** ***P* = .003**
	IL1B	0.2776118	−0.0991223	.0264	**0.6292** ***P* = .007**
Combination					
6GS	*ALPL*	1.462895	−0.0890073	.0876	**0.6649** ***P* = .001**
	CLC		−0.068176		
	CPA3		−0.1237933		
	CXCR2		0.039212		
	DNASE1L3		0.074877		
	IL1B		−0.0496472		

Values in boldface indicate statistical significance.
*Markers are normalized to β-actin mRNA expression (ΔCT).

TABLE E5. AUC for each predictive marker by study population, exacerbation severity, and exacerbation frequency status

	6GS	Sputum eosinophils	Sputum neutrophils	PBEs	6GS (FENO)*	FENO
Placebo						
Total exacerbations						
≥1 or 0	**AUC = 0.711, *P* = .003, n = 73**	AUC = 0.505, *P* = .947, n = 73	AUC = 0.582, *P* = .263, n = 73	AUC = 0.602, *P* = .165, n = 73	**AUC = 0.738, *P* = .026, n = 34**	AUC = 480, *P* = .846, n = 34
≥2 or 0-1	**AUC = 0.706, *P* = .001, n = 73**	AUC = 0.575, *P* = .269, n = 73	AUC = 0.554, *P* = .428, n = 73	AUC = 0.606, *P* = .110, n = 73	AUC = 0.561, *P* = .576, n = 34	AUC = 0.550, *P* = .627, n = 34
Severe exacerbations						
≥1 or 0	**AUC = 0.676, *P* = .005, n = 73**	AUC = 0.547, *P* = .499, n = 73	AUC = 0.593, *P* = .181, n = 73	AUC = 0.558, *P* = .397, n = 73	**AUC = 0.712, *P* = .020, n = 34**	AUC = 0.535, *P* = .741, n = 34
≥2 or 0-1	**AUC = 0.776, *P* < .0001, n = 73**	**AUC = 0.701, *P* = .004, n = 73**	AUC = 0.563,† *P* = .436, n = 73	AUC = 0.628, *P* = .091, n = 73	**AUC = 0.810, *P* < .0001, n = 34**	AUC = 0.634, *P* = .213, n = 34
AZM						
Total exacerbations						
≥1 or 0	**AUC = 0.643, *P* = .038, n = 66**	AUC = 0.544, *P* = .540, n = 66	AUC = 0.479, *P* = .776, n = 66	AUC = 0.524, *P* = .740, n = 66	AUC = 0.640, *P* = .160, n = 33	AUC = 0.529, *P* = .783, n = 33
≥2 or 0-1	AUC = 0.610, *P* = .150, n = 66	AUC = 0.431, *P* = .386, n = 66	AUC = 0.470, *P* = .698, n = 66	AUC = 0.511, *P* = .891, n = 66	AUC = 0.609, *P* = .348, n = 33	AUC = 0.565, *P* = .536, n = 33
Severe exacerbations						
≥1 or 0	**AUC = 0.741, *P* < .0001, n = 66**	AUC = 0.534,† *P* = .669, n = 66	AUC = 0.469,‡ *P* = .675, n = 66	AUC = 0.592, *P* = .229	**AUC = 0.804, *P* < .0001, n = 33**	AUC = 0.583,§ *P* = .444, n = 33
≥2 or 0-1	**AUC = 0.750, *P* = .013, n = 66**	AUC = 0.467,† *P* = .807, n = 66	AUC = 0.293,‡ *P* = .055, n = 66	AUC = 0.678, *P* = .081, n = 66	AUC = 0.767, *P* = .125, n = 33	AUC = 0.600, *P* = .394, n = 33

Values in boldface indicate statistical significance.

*Values were calculated in subpopulations in which FENO measurements were made.

†$P < .05$ versus 6GS.

‡$P < .01$ versus 6GS.

§$P < .01$ versus 6GS (FENO subpopulation).

TABLE E6. Analysis of prognostic value of the sputum 6GS for discriminating patients who experience none or some severe asthma exacerbations in following 48 weeks

		Logistic regression			
	Marker*	**Constant**	**Coefficient**	**Model *P* value**	**AUC (95% CI)**
Placebo group; n = 73 (29/73 [39.7%] severe exacerbators)					
Individual					
	ALPL	1.174711	−0.2524459	**.0335**	**0.6552** ***P* = .017**
	CLC	1.311412	−0.1935234	**.0293**	**0.6481** ***P* = .021**
	CPA3	1.121902	−0.1565537	.1088	0.5862 *P* = .204
	CXCR2	0.0996914	−0.0977394	.3993	0.5846 *P* = .217
	DNASE1L3	2.178457	−0.2240271	.1038	0.6042 *P* = .122
	IL1B	0.1822344	−0.1986743	.1491	0.5799 *P* = .259
Combination					
6GS	*ALPL*	2.553787	−0.2441983	.2311	**0.6763** ***P* = .005**
	CLC		−0.1503987		
	CPA3		−0.0236053		
	CXCR2		0.0817315		
	DNASE1L3		−0.0199114		
	IL1B		−0.0210936		
AZM group; n = 69 (22/69 [31.9%] severe exacerbators)					
Individual					
	ALPL	−0.2789161	−0.0735923	.5923	0.5184 *P* = .813
	CLC	0.7462753	−0.1546867	.1193	0.5841 *P* = .244
	CPA3	1.087588	−0.182063	.0805	0.6190 *P* = .088
	CXCR2	−1.351341	0.1113008	.4124	0.5754 *P* = .346
	DNASE1L3	0.2451273	−0.0881535	.4917	0.5368 *P* = .622
	IL1B	−0.7417235	−0.0056191	.9669	0.5029 *P* = .971
Combination					
6GS	*ALPL*	−0.2663717	−0.5786811	.0966	**0.7447** ***P* < .0001**
	CLC		−0.0886016		
	CPA3		−0.3606021		
	CXCR2		0.6540632		
	DNASE1L3		0.4044179		
	IL1B		−0.0915628		

Values in boldface indicate statistical significance.
*Markers are normalized to β-actin mRNA expression (ΔCT).

TABLE E7. Analysis of diagnostic value of the sputum 6GS for discriminating patients who experience infrequent (<2) or frequent (≥2) severe asthma exacerbations in the following 48 weeks

		Logistic regression			
	Marker*	**Constant**	**Coefficient**	**Model *P* value**	**AUC (95% CI)**
Placebo group; n = 73 (18/73 [24.7%] frequent severe)					
Individual					
	ALPL	−0.665708	−0.0708267	.5671	0.5747 *P* = .342
	CLC	1.79629	−0.3441432	**.0017**	**0.7434** ***P* < .0001**
	CPA3	2.70536	−0.4086174	**.0009**	**0.7354** ***P* < .0001**
	CXCR2	−0.879162	−0.0450079	.7307	0.5242 *P* = .753
	DNASE1L3	2.292987	−0.2984015	.0615	**0.6525** ***P* = .039**
	IL1B	−1.010963	−0.0347323	.8202	0.4828 *P* = .844
Combination					
6GS	*ALPL* *CLC* *CPA3* *CXCR2* *DNASE1L3* *IL1B*	1.910656	0.000224 −0.2322196 −0.4001086 −0.0767063 0.2721843 −0.0170972	**.0305**	**0.7758** ***P* < .0001**
AZM group; n = 69 (6/69 [8.7%] frequent severe)					
Individual					
	ALPL	−0.8723939	−0.2375642	.2961	0.5714 *P* = .571
	CLC	−1.152898	−0.1256578	.4212	0.5132 *P* = .907
	CPA3	−0.78702	−0.1579249	.3320	0.6032 *P* = .329
	CXCR2	−2.132548	−0.0422384	.8482	0.4815 *P* = .907
	DNASE1L3	0.9566618	−0.3022422	.1425	0.6534 *P* = .111
	IL1B	−2.078326	−0.0922804	.6817	0.5661 *P* = .531
Combination					
6GS	*ALPL* *CLC* *CPA3* *CXCR2* *DNASE1L3* *IL1B*	2.057035	−0.6730842 0.0791463 0.2137911 0.6367583 −0.5728786 −0.0484114	.5772	**0.7540** ***P* = .009**

Values in boldface indicate statistical significance.
*Markers are normalized to β-actin mRNA expression (ΔCT).

TABLE E8. Analysis of the prognostic value of the sputum 6GS for discriminating patients who experience none or some total (moderate and severe) asthma exacerbations in the following 48 weeks

		Logistic regression			
	Marker*	**Constant**	**Coefficient**	**Model *P* value**	**AUC (95 %CI)**
Placebo group; n = 73 (52/73 [71.2%] total exacerbators)					
Individual					
	ALPL	2.744964	−0.2750237	**.0184**	**0.6896 *P* = .015**
	CLC	1.044878	−0.0151497	.8676	0.5238 *P* = .776
	CPA3	0.9904325	−0.0084182	.9344	0.4707 *P* = .703
	CXCR2	1.54365	−0.1171154	.3326	0.6200 *P* = .125
	DNASE1L3	0.9053008	0.0001218	.9993	0.5156 *P* = .847
	IL1B	1.433011	−0.1650851	.2606	0.5925 *P* = .222
Combination					
6GS	*ALPL*	1.82736	−0.3766049	.3862	**0.7106 *P* = .003**
	CLC		0.0077007		
	CPA3		−0.0338994		
	CXCR2		−0.0014933		
	DNASE1L3		0.123925		
	IL1B		0.1294379		
AZM group; n = 69 (32/69 [46.4%] total exacerbators)					
Individual					
	ALPL	0.1277557	−0.041565	.7463	0.5025 *P* = .972
	CLC	0.1613086	−0.0309722	.7376	0.4975 *P* = .971
	CPA3	−0.2102336	0.0062969	.9476	0.5194 *P* = .785
	CXCR2	−0.7677189	0.1182769	.3501	0.5794 *P* = .259
	DNASE1L3	−0.6843071	0.0470349	.6959	0.5346 *P* = .627
	IL1B	−0.3903965	0.0789144	.5345	0.5456 *P* = .521
Combination					
6GS	*ALPL*	−0.5183495	−0.6414888	.3576	**0.6816 *P* = .005**
	CLC		−0.0522411		
	CPA3		0.0004421		
	CXCR2		0.5629461		
	DNASE1L3		0.1618661		
	IL1B		0.0880836		

Values in boldface indicate statistical significance.
*Markers are normalized to β-actin mRNA expression (ΔCT).

TABLE E9. Analysis of diagnostic value of the sputum 6GS for discriminating patients who experience infrequent or frequent total (moderate and severe) asthma exacerbations in the following 48 weeks

		Logistic regression			
	Marker*	**Constant**	**Coefficient**	**Model *P* value**	**AUC (95% CI)**
Placebo; n = 73 (36/73 [49.3%] frequent total exacerbators)					
Individual					
	ALPL	1.37584	−0.2187011	.0495	**0.6569** ***P* = .018**
	CLC	2.287965	−0.255561	**.0042**	**0.6959** ***P* = .002**
	CPA3	2.55715	−0.2607424	**.0089**	**0.6569** ***P* = .015**
	CXCR2	0.9757104	−0.1889486	.1021	0.6081 *P* = .112
	DNASE1L3	3.415742	−0.2956417	.0316	**0.6404** ***P* = .033**
	IL1B	0.4134511	−0.1433317	.2820	0.5548 *P* = .425
Combination					
6GS	*ALPL*	3.8649	−0.1726814	.0640	**0.7057** ***P* = .001**
	CLC		−0.1481676		
	CPA3		−0.1704555		
	CXCR2		−0.1120212		
	DNASE1L3		0.0449441		
	IL1B		0.0985971		
AZM group; n = 69 (20/69 [29.0%] frequent total exacerbators)					
Individual					
	ALPL	−0.9968258	0.0153026	.9137	0.5378 *P* = .635
	CLC	−1.157131	0.026261	.7973	0.5633 *P* = .395
	CPA3	−0.6788128	−0.0210921	.8409	0.4796 *P* = .784
	CXCR2	−1.463457	0.1064686	.4452	0.5704 *P* = .379
	DNASE1L3	−0.8950519	0.0000905	.9995	0.4765 *P* = .762
	IL1B	−0.7406066	−0.0508403	.7153	0.5378 *P* = .625
Combination					
6GS	*ALPL*	−0.9970808	−0.2584836	.8283	0.6265 *P* = .097
	CLC		0.0914051		
	CPA3		−0.0990941		
	CXCR2		0.4106918		
	DNASE1L3		0.0193966		
	IL1B		−0.1623113		

Values in boldface indicate statistical significance.
*Markers are normalized to β-actin mRNA expression (ΔCT).

TABLE E10. Comparison of the diagnostic value of the sputum 6GS versus PBE and FENO measurement for asthma airway inflammatory phenotyping

Phenotype	6GS	PBE count	*P* value, 6GS vs PBEs
EA vs NEA (n = 139)	AUC = 0.7684, $P < .0001$	AUC = 0.7591, $P < .0001$	.858
EA vs NA (n = 79)	AUC = 0.9294, $P < .0001$	AUC = 0.7159, $P = .001$	**.002**
EA vs PGA (n = 118)	AUC = 0.7636, $P < .0001$	AUC = 0.7726, $P < .0001$	.873

Phenotype	6GS	FENO value	*P* value, 6GS vs FENO value
EA vs NEA (n = 67)	AUC = 0.8152, $P < .0001$	AUC = 0.7268, $P < .0001$	.242
EA vs NA (n = 41)	AUC = 0.9569, $P < .0001$	AUC = 0.7615, $P = .001$	**.015**
EA vs PGA (n = 55)	AUC = 0.8383, $P < .0001$	AUC = 0.7147, $P = .003$	.136

Values in boldface indicate statistical significance.