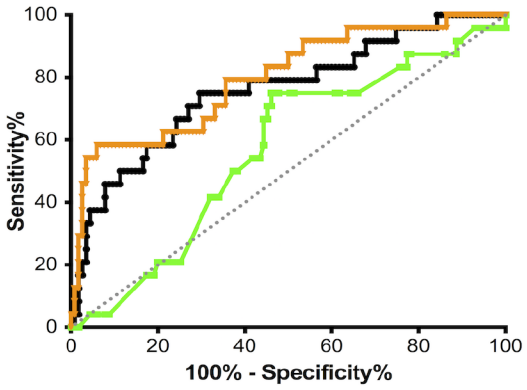
BIOMARKERS OF FUTURE FREQUENT (2+/YEAR) SEVERE EXACERBATIONS OF POORLY CONTROLLED ASTHMA IN THE AMAZES TRIAL



Sputum 6 Gene Signature AUC=76%***

Sputum 6 Gene Signature + 12 month OCS History AUC=80%***

Peripheral Blood Eosinophils AUC=57% ***NS***

**

**

AUC = Area Under Curve, OCS = Oral Corticosteroid
*** = p<0.001, ** = p<0.01, ***NS*** = not signficant

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

Author list:

Michael Fricker, BSc PhD[1], Peter G Gibson, MBBS FRACP[1,2,14], Heather Powell, MMedSci[1,2], Jodie L Simpson, BSc PhD[1], Ian A Yang, MBBS PhD FRACP[3,4], John W Upham, MBBS PhD[3,5], Paul N Reynolds, MBBS MD PhD FRACP[6,7,8], Sandra Hodge, PhD[6,7,8], Alan L James MBBS MD FRACP[9,10], Christine Jenkins, MBBS MD FRACP[11,12], Matthew J Peters, MD FRACP[12,13], Guy B Marks, MBBS PhD FRACP[14,15], Melissa Baraket MBBS PhD FRACP[16], Katherine J Baines, BSc PhD[1]

Affiliations:

[1]Priority Research Centre for Healthy Lungs, The University of Newcastle, Newcastle, NSW, Australia

[2]Department of Respiratory and Sleep Medicine, John Hunter Hospital, Newcastle, NSW, Australia

[3]Diamantina Institute, The University of Queensland, Brisbane, QLD, Australia

[4]Department of Thoracic Medicine, The Prince Charles Hospital, Brisbane, QLD, Australia

[5]Department of Respiratory Medicine, Princess Alexandra Hospital, Brisbane, QLD, Australia

[6]Department of Thoracic Medicine, Royal Adelaide Hospital, Adelaide, SA, Australia

[7]Lung Research Laboratory, Hanson Institute, Adelaide, SA, Australia

[8]School of Medicine, University of Adelaide, Adelaide, SA, Australia

[9]Department of Pulmonary Physiology and Sleep Medicine, Sir Charles Gairdner Hospital, Perth, WA, Australia

[10]School of Medicine and Pharmacology, The University of Western Australia, Perth, WA, Australia

[11]Respiratory Trials, The George Institute for Global Health, Sydney, NSW, Australia

[12]Department of Thoracic Medicine, Concord General Hospital, Sydney, NSW, Australia

[13]Faculty of Medicine and Health Sciences, Macquarie University, Sydney, NSW, Australia

[14]Woolcock Institute of Medical Research, Sydney, NSW, Australia

[15]South Western Sydney Clinical School, University of New South Wales, Sydney, NSW, Australia

[16]Respiratory Medicine Department and Ingham Institute Liverpool Hospital, University of New South Wales, Medicine Faculty, Sydney, NSW, Australia

Corresponding author contact details:

Michael Fricker

Address: Priority Research Centre for Healthy Lungs, Hunter Medical Research Institute, Lot 1 Kookaburra Circuit, New Lambton Heights, NSW, 2305, Australia.

Phone: +61 2 404 20207

Fax: +61 2 404 20046

Email: michael.fricker@newcastle.edu.au

Declaration of all funding sources: This study was funded by the National Health and Medical Research Council of Australia (NHMRC project identifiers 569246, 1058552 and 1078579) and the John Hunter Hospital Charitable Trust.

Conflict of interest statement: M.F. has received research and fellowship funding from the NHMRC, Thoracic Society of Australia and New Zealand and AstraZeneca, and declares no

conflict of interest in relation to this paper. P.G.G. has received research and fellowship funding from the NHMRC, research funding from AstraZeneca, GlaxoSmithKline, and Novartis, and speaker fees from AstraZeneca, GlaxoSmithKline, and Novartis, unrelated to the current manuscript. H.P. declares no conflict of interest. J.L.S. declares no conflict of interest in relation to this paper. I.A.Y. declares no conflict of interest in relation to this paper. J.U. has received speaker fees and consultancy fees from AstraZeneca, GlaxoSmithKline, Novartis, Boehringer Ingelheim and Menarini, none of these were related to the current manuscript. P.N.R. has received speaker fees from Boehringer Ingelheim and Roche, none of these were related to the current manuscript. S.H.'s institution has grants with NHMRC; and she has received royalties from the book *Lung Macrophages in Health and Disease*. A.L.J. has received speaker fees from AstraZeneca, GlaxoSmithKline and Menarini, none of these were related to the current manuscript. C.J. has received personal payments for advisory board membership, speaker engagement and educational resource development from AstraZeneca, GlaxoSmithKline, Boehringer Ingelheim, Novartis and Menarini. Her institution receives grants from GlaxoSmithKline and AstraZeneca. She has no conflict of interest in relation to this paper and received no payments in relation to the work undertaken. M.J.P. declares no conflict of interest in relation to this manuscript. G.M.'s institution has received research funding from AstraZeneca and GlaxoSmithKline and he has served an on an advisory board for AstraZeneca. He has no conflicts of interest in relation to this manuscript. M.B. declares no conflict of interest in relation to this paper. K.J.B. received research funding from the NHMRC CRE Severe Asthma for this work and a Lung Foundation Australia fellowship. K.J.B. and P.G.G. have a patent pending, "Biomarkers of asthma inflammatory phenotypes and response to therapy", regarding use of the 6GS as a phenotyping tool in asthma.

Abstract:

- **Background:** Improved diagnostic tools for predicting future exacerbation frequency in asthma are required. A sputum gene expression signature of 6 biomarkers (6GS - including *CLC, CPA3, DNASE1L3, ALPL, CXCR2, IL1B*) predicts inflammatory and treatment response phenotypes in stable asthma. We recently demonstrated that azithromycin (AZM) add-on treatment in uncontrolled moderate-to-severe asthma significantly reduced asthma exacerbations (AMAZES clinical trial).
- **Objectives:** To test whether the 6GS predicts future exacerbation and inflammatory phenotypes in a subpopulation of AMAZES. To test the impact of AZM therapy on 6GS expression and prognostic capacity.
- **Methods:** 142 patients (73 placebo-treated, 69 AZM-treated) had sputum stored for qPCR of 6GS markers at baseline and after 48 weeks of treatment. Logistic regression, ROC and AUC were performed on baseline measures, and in an exploratory analysis the predictive value of 6GS was compared with conventional biomarkers for exacerbation and inflammatory phenotypes.
- **Results:** The 6GS significantly predicted all future exacerbation phenotypes tested. Calculated AUCs for 6GS were significantly higher than AUCs for peripheral blood eosinophil counts, sputum neutrophil counts and combined sputum eosinophils and neutrophil counts. 6GS AUCs were also were numerically, but not significantly, higher than FeNO and sputum eosinophil counts. AZM treatment neither altered the 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 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.

Key Messages

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

Capsule Summary:

In this AMAZES RCT sub-analysis, the sputum 6GS predicts exacerbation and airway inflammatory phenotype of uncontrolled, moderate-to-severe asthma. Azithromycin appears to exert a therapeutic effect independently of 6GS-related airway inflammatory factors.

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

Abbreviations:

6GS	6 gene signature
ACQ	Asthma control questionnaire-6
ALPL	Alkaline Phosphatase, liver/bone/kidney
AUC	Area under curve
AZM	Azithromycin
CLC	Charcot-Leyden Crystal Galectin

128	**CPA3**	Carboxypeptidase 3
129	**CXCR2**	C-X-C motif chemokine receptor 2
130	**DNASE1L3**	Deoxyribonuclease 1 like 3
131	**EA**	Eosinophilic asthma
132	**FENO**	Fractional exhaled nitric oxide
133	**ICS**	Inhaled corticosteroid
134	**IL1B**	Interleukin-1β
135	**MGA**	Mixed granulocytic asthma
136	**NA**	Neutrophilic asthma
137	**NEA**	Non-eosinophilic asthma
138	**NNA**	Non-neutrophilic asthma
139	**NPGA**	Non-paucigranulocytic asthma
140	**OCS**	Oral corticosteroid
141	**PBE**	Peripheral blood eosinophil
142	**PGA**	Paucigranulocytic asthma
143	**RCT**	Randomized controlled trial
144	**ROC**	Receiver operating characteristic
145		
146		

Introduction

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 asthma 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 to conventional management[6-8].

Sputum induction, processing and analysis is 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 (PBE) 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]. We previously reported a sputum gene expression signature comprised of 6 transcripts (*CLC, CPA3, DNASE1L3, ALPL, CXCR2, IL1B*) which distinguished airway inflammatory phenotypes of asthma with high specificity and sensitivity[13]. *CLC*, *CPA3* and *DNASE1L3* expression are increased in eosinophilic asthma. *ALPL*, *CXCR2* and *IL1B* are

increased in neutrophilic asthma and mark innate inflammatory signaling pathways relating to TNFα, CXCL1 and IL-1β respectively. This 6 gene signature (6GS) also predicts responsiveness to inhaled[13] and oral corticosteroids (OCS)[14], which suppress *CLC*, *CPA3* and *DNASE1L3* expression. The development of sputum gene signatures may increase the feasibility of use of sputum-based measures in the clinic, as the sample processing (RNA extraction, cDNA synthesis, qPCR) can be automated, and the markers have high specificity.

We recently published findings from a clinical trial (AMAZES) which demonstrated that treatment of moderate-to-severe, uncontrolled asthma with the macrolide 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 whereby AZM reduces asthma exacerbations remains unclear, and could be related to its anti-inflammatory, anti-bacterial or anti-viral properties.

In the present study, we evaluate the ability of the sputum 6GS to predict asthma exacerbation frequency and to differentiate 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 to sputum cell count, PBE and FENO. We hypothesized that the 6GS would provide superior prediction of exacerbation and inflammatory phenotype compared to other biomarkers.

Methods

The AMAZES study[15] was a double-blind placebo-controlled trial where 420 adults with persistent symptomatic asthma despite current use of ICS and long-acting bronchodilator were randomized to receive AZM 500mg 3 times per week or identical placebo for 48 weeks (Online Repository). Induced sputum was collected prior to 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 was performed using our previously described methods (see Online Repository). Inflammatory phenotypes were defined as follows: eosinophilic asthma (EA, sputum eosinophils $\geq 3\%$[7]); neutrophilic asthma (NA, sputum neutrophils $\geq 61\%$[1]); mixed granulocytic asthma (MGA, sputum neutrophils $\geq$ 61% and eosinophils $\geq 3\%$); paucigranulocytic asthma (PGA, sputum neutrophils $< 61\%$ and eosinophils $< 3\%$). In the AMAZES trial exacerbation occurrence and type (severe or moderate) were determined by structured interview. Decisions regarding treatment of trial participants during exacerbation were determined by the treating physicians, and were not part of the trial. Severe exacerbations were defined as a worsening of asthma symptoms requiring ≥3 days of systemic corticosteroid treatment ≥10mg/day, or an asthma-specific hospitalization or emergency department visit requiring systemic corticosteroids. Moderate exacerbations were defined as any temporary increase in ICS or antibiotics in conjunction with a deterioration in asthma symptoms or both (change in ACQ6 of at least 0.5 or increased

diary symptom score), or 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, IL1B* was quantified as previously described[9] (see Online Repository). Statistical analysis of diagnostic ability was performed on the change in cycle threshold (ΔCt) between the target gene and housekeeping 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 non-exacerbator, was modelled by logistic regression (STATA 13, StataCorp, College Station, Texas, USA) using single (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 the severity of exacerbations. These included one or more vs none (any exacerbations) and two or more vs one or none (frequent exacerbations), where exacerbations included all exacerbations (total moderate and severe) or were limited to severe exacerbations[15]. To examine the potential effect of AZM treatment on the relationship between 6GS and future exacerbation, each model was adjusted for AZM treatment and conducted with and without interaction terms for treatment and the individual gene expression. The models with and without the interaction terms were then compared using a log likelihood ratio test and, if non-significant, $p>0.05$, the 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 which was generated by 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 the logistic model adjusted for AZM treatment.

Receiver Operating Characteristic (ROC) curves were generated of the 6GS and other biomarker predicted values by exacerbator (outcome) status for each exacerbation model. 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 %, PBE and FENO. 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 $p<0.05$.

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

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

Results

Subject Characteristics

Most patients were classified as GINA step 4 (85.9%) and 48.6% as having severe asthma (ERS/ATS guidelines)[16] and all had persistent symptomatic (ACQ6 ≥ 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, gender, asthma control, asthma severity, spirometry 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).

The 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 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 = 0.036) and future frequent severe exacerbations (model P = 0.022).

The 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, sputum eosinophils and/or neutrophils, PBE and FENO quantified at baseline to

evaluate their relative prognostic value for various exacerbation phenotypes based on exacerbations recorded during the trial period. As there was no interaction between AZM treatment and the association of 6GS with future exacerbations, we combined placebo and AZM-treated patients in our initial analysis.

Sputum eosinophils, sputum neutrophils, PBE and FENO did not provide statistically significant discriminatory capacity for those patients that experienced at least one severe exacerbation (exacerbators) during the trial from those that experienced none (non-exacerbators) (figure 1A and table II). In contrast the sputum 6GS provided modest but significant prediction of severe exacerbators vs non-exacerbators (AUC = 68.1%, $P < 0.0001$) (table II and supplemental table E1).

Sputum eosinophils, eosinophils and neutrophils combined and the 6GS provided significant discriminatory capacity of patients who experienced frequent (≥ 2) vs infrequent (< 2) severe exacerbations (6GS AUC = 76.1%, $P < 0.0001$; sputum eosinophil AUC = 70.3%, $P = 0.002$; sputum eosinophils and neutrophils AUC = 68.4%, $P = 0.012$) (figure 1B and table II). In the subset of patients where FENO was measured, both 6GS and FENO provided significant prognostic capacity (6GS AUC = 83.7%, $P < 0.0001$; FENO AUC = 75.6%, $P < 0.0001$). Of all biomarkers examined, the sputum 6GS gave the highest AUC values and significantly outperformed sputum neutrophils, eosinophils and neutrophils combined and PBE in predicting the frequent severe exacerbation phenotype (table II and supplemental table E2).

In the AMAZES study moderate exacerbations were also quantified[15]. We performed logistic regression analysis comparing exacerbator vs non-exacerbator and frequent vs infrequent exacerbator phenotypes for total (sum of moderate and severe) exacerbations. The 6GS and

sputum eosinophils and/or neutrophils significantly predicted exacerbators vs non-exacerbators (total) (6GS AUC = 69.6%, P < 0.0001; eosinophils AUC = 63%, P = 0.008; neutrophils AUC = 63.3%, P = 0.005; eosinophils and neutrophils AUC = 64.2%, P = 0.003) (figure 1C, table II, supplemental table E3). In this analysis 6GS statistically outperformed sputum neutrophils and PBE. The 6GS and sputum eosinophils and/or neutrophils significantly discriminated frequent (≥ 2) total exacerbators from infrequent exacerbators (6GS AUC = 66.4%, P = 0.001; eosinophils AUC = 60.6%, P = 0.034; neutrophils AUC = 60.8%, P = 0.029; eosinophils and neutrophils AUC = 62.1%, P = 0.016) (figure 1D, table II, supplemental table E4), and the 6GS significantly outperformed PBE.

History of OCS use for the 12-month period prior to the study baseline visit was recorded. Prior OCS history alone could significantly predict the future severe exacerbation frequency with a similar AUC to the 6GS (Prior OCS use AUC = 76.5%, p < 0.0001). When 6GS was evaluated and the data 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 < 0.0001) (figure 1E).

AZM treatment does not alter 6GS expression nor prediction of future exacerbation status

We evaluated the effect of 48 weeks AZM treatment on 6GS transcript expression. At visit 10 (48 weeks of treatment), there was no significant difference in 6GS expression between placebo and AZM treatment groups (figure 2, A-F). *CXCR2* mRNA was significantly increased at visit 10 vs baseline visits in both placebo and AZM-treated patients (figure 2, E). In a further exploratory sub-analysis, we examined biomarker performance for the various exacerbator phenotypes, analyzing placebo- and AZM-treated groups separately. Of note,

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 the AZM-treated patients (AUC = 62.7%, P = 0.097) (figure 2, G-J, supplemental tables E5-9). Other biomarkers did not provide significant predictive capacity for any exacerbator phenotype in either placebo or AZM-treated groups, with the exception of sputum eosinophils for predicting frequent severe exacerbators in the placebo group (AUC = 70.1%, P = 0.004) (supplemental table E5).

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

Airway expression of *CLC*, *CPA3* and *DNASE1L3* were significantly elevated in eosinophilic (EA; ≥ 3% sputum eosinophils) vs non-eosinophilic (NEA; < 3% sputum eosinophils) asthma, whilst *IL1B* was lower in EA (figure 3A). *CXCR2* and *ALPL* expression did not differ between EA and NEA. *IL1B*, *CXCR2* and *ALPL* were significantly elevated in neutrophilic (NA) vs non-neutrophilic (NNA) asthma, whilst expression of *CLC*, *CPA3* and *DNASE1L3* showed no significant differences between these groups (figure 3B).

We tested whether the 6GS measured at baseline could predict airway inflammatory phenotype, using multiple logistic regression and ROC curve analysis. In all analyses, the sputum 6GS discriminated airway inflammatory phenotypes to a statistically significant extent (EA vs NEA: AUC = 76.8%, P < 0.0001; EA vs NA: AUC = 92.9%, P < 0.0001; EA vs PGA: AUC = 76.4%, P < 0.0001; NA vs NNA: AUC = 89.5%, P < 0.0001; NA vs PGA: AUC = 88.0%, P < 0.0001; PGA vs NPGA: AUC = 74.0%, P < 0.0001) (table III). We also examined two established biomarkers of type 2/eosinophilic inflammation in asthma, PBE and FENO, and compared their performance with the 6GS in distinguishing sputum

inflammatory phenotypes. Both PBE and FENO discriminated EA vs NEA, EA vs NA and EA vs PGA to a statistically significant extent (supplemental table E10). However, the 6GS significantly outperformed PBE (figure 3C) and FENO (figure 3D) as a diagnostic test for predicting EA vs NA.

Discussion

In this study, we demonstrate that the sputum 6GS 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 whom were treated with AZM add-on therapy, which reduced overall rate of exacerbations compared to the placebo treatment. The sputum 6GS had statistically better predictive capacity for future frequent severe exacerbations than PBE, sputum neutrophils and combined sputum eosinophil and neutrophil count. Numerically, but not statistically, superior AUC values were also observed for 6GS compared to sputum eosinophils and FENO in the prediction of future exacerbation phenotypes.

Sputum 6GS predicts future exacerbations more effectively than conventional biomarkers

Development of biomarkers that can identify asthma 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 elevated eosinophilic or type 2 inflammatory biomarkers including sputum eosinophils, PBE and FENO 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 non-exacerbators and frequent from non-frequent exacerbators, when either severe exacerbations or total exacerbations were modeled. In all but one ROC analyses performed, the sputum 6GS generated higher AUC values than conventional biomarkers. Performance of conventional biomarkers was inconsistent, and in exploratory comparative analysis the 6GS frequently statistically outperformed sputum

neutrophils and PBE. The sputum 6GS matched past courses of OCS 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, giving an AUC value 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], thus whilst these results demonstrate the promise of sputum gene signatures to identify patients most at risk of exacerbation, improved biomarker performance may be achieved in the future through further gene signature optimization.

Why might the sputum 6GS 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 count in this study was a poor prognostic marker for future exacerbation status, high sputum neutrophil count has been linked to more severe forms of asthma in cluster analysis, associated with higher healthcare burden and hospitalization, particularly when accompanied by elevated sputum eosinophils[23]. Combinatorial use of biomarkers reporting on distinct disease endotypes or markers could improve prognostic potential. In agreement with this hypothesis, combinatorial use of type 2-related biomarkers FENO, PBE and serum periostin improves prediction of exacerbation risk when compared to 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 to each variable in isolation.

The 6GS may 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 elevated in frequent exacerbators in both COPD and asthma[25, 26]. It is also possible that the 6GS improves on sputum eosinophil and neutrophil count 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 humans[11, 27, 28]. Our and others' sputum transcriptomic analyses identified a number of mast cell-related genes that were upregulated in eosinophilic asthma[9, 10, 11]. A recent study reported flow cytometry-based quantification of sputum mast cells and demonstrated positive correlation with sputum eosinophil count[29]. Of the 6GS genes, *CPA3* and *CLC* were the most effective at predicting the frequent severe exacerbator phenotype (supplemental table E2). *CLC* may 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 may 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 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 anti-bacterial, anti-viral 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 the sputum 6GS genes. However, the lack of effect of AZM on sputum 6GS 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 the sputum 6GS could significantly predict future exacerbation phenotypes in most analyses conducted in AZM-treated patients, despite the fact that exacerbation rate was significantly reduced by AZM treatment. The implications of our findings are that the mechanism of action whereby AZM treatment reduces 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 moderate-to-severe asthma

The findings of the present study further consolidate and broaden the potential use of 6GS as an inflammatory phenotyping tool in asthma. Here we demonstrate for the first time 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 the sputum 6GS in stable, mild-to-moderate asthma[13] and as a predictor of positive response to ICS and OCS[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 RCT[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, due to the requirement of sufficient sputum sample to allow RNA isolation and qPCR analysis for our present study, only those patients that produced sufficient sputum were included, and this could be a source of biological bias. In this sub-population of the AMAZES RCT, FENO data was not available for all patients, and thus our analysis of FENO 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.

In conclusion, the sputum 6GS can predict future exacerbation phenotype in 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 may still retain use in identifying a subset of patients who may experience frequent severe exacerbations despite AZM therapy.

Acknowledgements:

This study was funded by the National Health and Medical Research Council of Australia (NHMRC project identifiers 569246, 1058552 and 1078579), the NHMRC Centre of Research Excellence in Severe Asthma, and the John Hunter Hospital Charitable Trust. The authors would like to 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.

Tables

Table I. Subject Characteristics

	All	Placebo Group	Azithromycin Group
N	142	73	69
Age[Ω]	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[Ω]	9.15 (1.30, 24.0)	9.2 (1.4, 25.0)	9.15 (1.3, 22.0)
ACQ score[Ω]	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% [Ψ]	73.85 (18.84)	73.23 (18.93)	74.52 (18.85)
Pre-b2 FVC% [Ψ]	84.07 (14.62)	82.70 (14.57)	85.55 (14.64)
Pre-b2 FEV_1/FVC% [Ψ]	67.73 (11.19)	68.15 (10.92)	67.28 (11.54)
ICS dose (BDP mcg/day) [Ω]	1000 (800, 2000)	1000 (800, 2000)	1280 (800, 2000)
FENO ppb[Ω]	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 (x 10^9/L) [Ω]	0.29 (0.2, 0.4)	0.3 (0.2, 0.4)	0.2 (0.12, 0.4)
Sputum cell counts			
Sputum cell viability[Ω]	72.1 (55.4, 84.0)	78.0 (61.0, 86.1)	68.2 (53.3, 82.7)
Total cell count (x 10^6/ml) [Ω]	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 % [Ω]	32.5 (14.0, 54.0)	33.5 (18.0, 55.0)	31.75 (12.50, 52.75)
Eosinophils % [Ω]	1.75 (0.50, 9.50)	2.0 (0.50, 6.25)	1.63 (0.25, 11.50)
Macrophages % [Ω]	50.50 (31.60, 69.0)	51.0 (31.75, 68.75)	45.63 (31.60, 69.0)
Lymphocytes % [Ω]	0.75 (0.25, 1.75)	0.75 (0.25, 1.50)	0.75 (0.25, 2.0)
Columnar epithelial % [Ω]	2.50 (1.0, 5.75)	2.50 (0.75, 4.75)	2.38 (1.25, 6.50)
C2R stained eosinophils % [Ω]	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 [∞]

[Ω]Median (q1,q3); [¥](n(%); [Ψ]Mean (SD); [∞]Negative binomial regression p<0.03.

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

		6GS	Sputum eosinophils	Sputum neutrophils	Sputum Eosinophils & Neutrophils	PBE	6GS (FENO) $^{\Omega}$	FENO
		N=139	N=139	N=139	N=139	N=139	N=67	N=67
Total exacerbations	≥ 1 or 0	**AUC=0.696 P<0.0001**	**AUC=0.630 P=0.008**	**AUC=0.633* P=0.005**	**AUC=0.642 P=0.003**	AUC=0.596* P=0.058	**AUC=0.691 P=0.004**	AUC=0.635 P=0.056
	≥ 2 or 0-1	**AUC=0.664 P=0.001**	**AUC=0.606 P=0.034**	**AUC=0.608 P=0.029**	A**UC=0.621 P=0.016**	AUC=0.566* P=0.181	**AUC=0.647 P=0.029**	**AUC=0.670 P=0.010**
Severe exacerbations	≥ 1 or 0	**AUC=0.681 P<0.0001**	AUC=0.579 P=0.126	AUC=0.549* P=0.348	AUC=0.588 P=0.083	AUC=0.503$^{¥}$ P=0.957	**AUC=0.736 P<0.0001**	AUC=0.618 P=0.094
	≥ 2 or 0-1	**AUC=0.761 P<0.0001**	**AUC=0.703 P=0.002**	AUC=0.631$^{¥}$ P=0.054	**AUC=0.684* P=0.012**	AUC=0.567$^{¥}$ P=0.281	**AUC=0.837 P<0.0001**	**AUC=0.756 P<0.0001**

$^{\Omega}$values calculated in subpopulation where FENO measurement was made *p<0.05 vs 6GS; $^{¥}$p<0.01 vs 6GS;

$^{\Pi}$p<0.05 vs 6GS (FENO subpopulation); §p<0.01 vs 6GS (FENO subpopulation)

499 **Table III. Analysis of diagnostic value of the sputum 6GS for asthma airway**

500 **inflammatory phenotype**

501

	Marker *	Logistic Regression			
		Constant	Coefficient	Model P value	AUC (95%CI)
EA vs NEA N=139	ALPL CLC CPA3 CXCR2 DNASE1L3 IL1B	3.016208	0.1233148 -0.0929966 -0.3150406 0.0323884 -0.0486184 0.1429728	**<0.0001**	0.7684 (0.6898, 0.8469) **P<0.0001**
EA vs NA N=79	ALPL CLC CPA3 CXCR2 DNASE1L3 IL1B	3.645667	1.529648 0.0586264 -0.0971249 -0.298909 -0.9454183 0.5770762	**<0.0001**	0.9294 (0.8637, 0.9951) **P<0.0001**
EA vs PGA N=118	ALPL CLC CPA3 CXCR2 DNASE1L3 IL1B	4.249046	0.0063321 -0.1013124 -0.3867286 -0.0325417 0.0516468 0.0999352	**0.0002**	0.7636 (0.6775, 0.8498) **P<0.0001**
NA vs NNA N=139	ALPL CLC CPA3 CXCR2 DNASE1L3 IL1B	-2.034031	-0.8416046 -0.2829233 0.0170649 -0.1026321 0.766542 -0.3135194	**<0.0001**	0.8948 (0.8294, 0.9603) **P<0.0001**
NA vs PGA N=81	ALPL CLC CPA3 CXCR2 DNASE1L3 IL1B	1.820331	-0.5331606 -0.4059778 -0.0610565 -0.2789399 0.565322 -0.2033842	**<0.0001**	0.8804 (0.8055, 0.9553) **P<0.0001**
PGA vs Granulocytic N=139	ALPL CLC CPA3 CXCR2 DNASE1L3 IL1B	-4.316154	0.0509944 0.1325146 0.2796407 0.1651778 -0.1245273 -0.0157521	**0.0004**	0.7396 (0.6561, 0.5232) **P<0.0001**

502 *Markers are normalized to beta-actin mRNA expression (ΔCT)

Figures

Figure 1. ROC analysis of diagnostic performance of 6GS, sputum eosinophils and/or neutrophils and PBE for predicting asthma exacerbation phenotypes. (double column, color)

Figure 2. AZM treatment does not alter sputum 6GS expression or prognostic capacity compared to placebo. (double column)

Figure 3. Sputum 6 gene signature expression in eosinophilic and neutrophilic subtypes of asthma and prediction of airway inflammatory phenotype. (double column, color)

References

1. Simpson JL, Scott R, Boyle MJ, Gibson PG. Inflammatory subtypes in asthma: assessment and identification using induced sputum. Respirology 2006; 11:54-61.
2. McGrath KW, Icitovic N, Boushey HA, Lazarus SC, Sutherland ER, Chinchilli VM, et al. A large subgroup of mild-to-moderate asthma is persistently noneosinophilic. Am J Respir Crit Care Med 2012; 185:612-9.
3. Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. Nat Med 2012; 18:716-25.
4. Fricker M, Heaney LG, Upham JW. Can biomarkers help us hit targets in difficult-to-treat asthma? Respirology 2017; 22:430-42.
5. Russell RJ, Brightling C. Pathogenesis of asthma: implications for precision medicine. Clin Sci (Lond) 2017; 131:1723-35.
6. Green RH, Brightling CE, McKenna S, Hargadon B, Parker D, Bradding P, et al. Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial. Lancet 2002; 360:1715-21.
7. Pavord ID, Brightling CE, Woltmann G, Wardlaw AJ. Non-eosinophilic corticosteroid unresponsive asthma. Lancet 1999; 353:2213-4.
8. Jayaram L, Pizzichini MM, Cook RJ, Boulet LP, Lemiere C, Pizzichini E, et al. Determining asthma treatment by monitoring sputum cell counts: effect on exacerbations. Eur Respir J 2006; 27:483-94.
9. Baines KJ, Simpson JL, Wood LG, Scott RJ, Gibson PG. Transcriptional phenotypes of asthma defined by gene expression profiling of induced sputum samples. J Allergy Clin Immunol 2011; 127:153-60, 60 e1-9.

10. Kuo CS, Pavlidis S, Loza M, Baribaud F, Rowe A, Pandis I, et al. T-helper cell type 2 (Th2) and non-Th2 molecular phenotypes of asthma using sputum transcriptomics in U-BIOPRED. Eur Respir J 2017; 49.

11. Peters MC, Mekonnen ZK, Yuan S, Bhakta NR, Woodruff PG, Fahy JV. Measures of gene expression in sputum cells can identify TH2-high and TH2-low subtypes of asthma. J Allergy Clin Immunol 2014; 133:388-94.

12. Yan X, Chu JH, Gomez J, Koenigs M, Holm C, He X, et al. Noninvasive analysis of the sputum transcriptome discriminates clinical phenotypes of asthma. Am J Respir Crit Care Med 2015; 191:1116-25.

13. Baines KJ, Simpson JL, Wood LG, Scott RJ, Fibbens NL, Powell H, et al. Sputum gene expression signature of 6 biomarkers discriminates asthma inflammatory phenotypes. J Allergy Clin Immunol 2014; 133:997-1007.

14. Berthon BS, Gibson PG, Wood LG, MacDonald-Wicks LK, Baines KJ. A sputum gene expression signature predicts oral corticosteroid response in asthma. Eur Respir J 2017; 49.

15. Gibson PG, Yang IA, Upham JW, Reynolds PN, Hodge S, James AL, et al. Effect of azithromycin on asthma exacerbations and quality of life in adults with persistent uncontrolled asthma (AMAZES): a randomised, double-blind, placebo-controlled trial. Lancet 2017; 390:659-68.

16. Chung KF, Wenzel SE, Brozek JL, Bush A, Castro M, Sterk PJ, et al. International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. Eur Respir J 2014; 43:343-73.

17. Miller MK, Lee JH, Miller DP, Wenzel SE, Group TS. Recent asthma exacerbations: a key predictor of future exacerbations. Respir Med 2007; 101:481-9.

18. Al-ani S, Spigt M, Hofset P, Melbye H. Predictors of exacerbations of asthma and COPD during one year in primary care. Fam Pract 2013; 30:621-8.

19. Dweik RA, Sorkness RL, Wenzel S, Hammel J, Curran-Everett D, Comhair SA, et al. Use of exhaled nitric oxide measurement to identify a reactive, at-risk phenotype among patients with asthma. Am J Respir Crit Care Med 2010; 181:1033-41.

20. Lemiere C, Ernst P, Olivenstein R, Yamauchi Y, Govindaraju K, Ludwig MS, et al. Airway inflammation assessed by invasive and noninvasive means in severe asthma: eosinophilic and noneosinophilic phenotypes. J Allergy Clin Immunol 2006; 118:1033-9.

21. Denlinger LC, Phillips BR, Ramratnam S, Ross K, Bhakta NR, Cardet JC, et al. Inflammatory and Comorbid Features of Patients with Severe Asthma and Frequent Exacerbations. Am J Respir Crit Care Med 2017; 195:302-13.

22. Wenzel S, Swanson S, Teper A, Hamilton J, Izuhara J, Ohta S, et al. Dupilumab reduces severe exacerbations in periostin-high and periostin-low asthma patients. Eur Respir J 2016; 48:OA1798.

23. Moore WC, Hastie AT, Li X, Li H, Busse WW, Jarjour NN, et al. Sputum neutrophil counts are associated with more severe asthma phenotypes using cluster analysis. J Allergy Clin Immunol 2014; 133:1557-63 e5.

24. Heaney LG, Djukanovic R, Woodcock A, Walker S, Matthews JG, Pavord ID, et al. Research in progress: Medical Research Council United Kingdom Refractory Asthma Stratification Programme (RASP-UK). Thorax 2016; 71:187-9.

25. Baines KJ, Fu JJ, McDonald VM, Gibson PG. Airway gene expression of IL-1 pathway mediators predicts exacerbation risk in obstructive airway disease. Int J Chron Obstruct Pulmon Dis 2017; 12:541-50.

588 26. Fu JJ, McDonald VM, Baines KJ, Gibson PG. Airway IL-1beta and Systemic
589 Inflammation as Predictors of Future Exacerbation Risk in Asthma and COPD. Chest
590 2015; 148:618-29.
591 27. Dwyer DF, Barrett NA, Austen KF, Immunological Genome Project C. Expression
592 profiling of constitutive mast cells reveals a unique identity within the immune
593 system. Nat Immunol 2016; 17:878-87.
594 28. Wang G, Baines KJ, Fu JJ, Wood LG, Simpson JL, McDonald VM, et al. Sputum mast
595 cell subtypes relate to eosinophilia and corticosteroid response in asthma. Eur Respir
596 J 2016; 47:1123-33.
597 29. Suzuki Y, Wakahara K, Nishio T, Ito S, Hasegawa Y. Airway basophils are increased
598 and activated in eosinophilic asthma. Allergy 2017.
599 30. Brooks CR, van Dalen CJ, Hermans IF, Gibson PG, Simpson JL, Douwes J. Sputum
600 basophils are increased in eosinophilic asthma compared with non-eosinophilic
601 asthma phenotypes. Allergy 2017.
602 31. Wong EH, Porter JD, Edwards MR, Johnston SL. The role of macrolides in asthma:
603 current evidence and future directions. Lancet Respir Med 2014; 2:657-70.
604 32. Fricker M, Gibson PG. Macrophage dysfunction in the pathogenesis and treatment of
605 asthma. Eur Respir J 2017; 50.
606
607

608 **Figure legends**

609

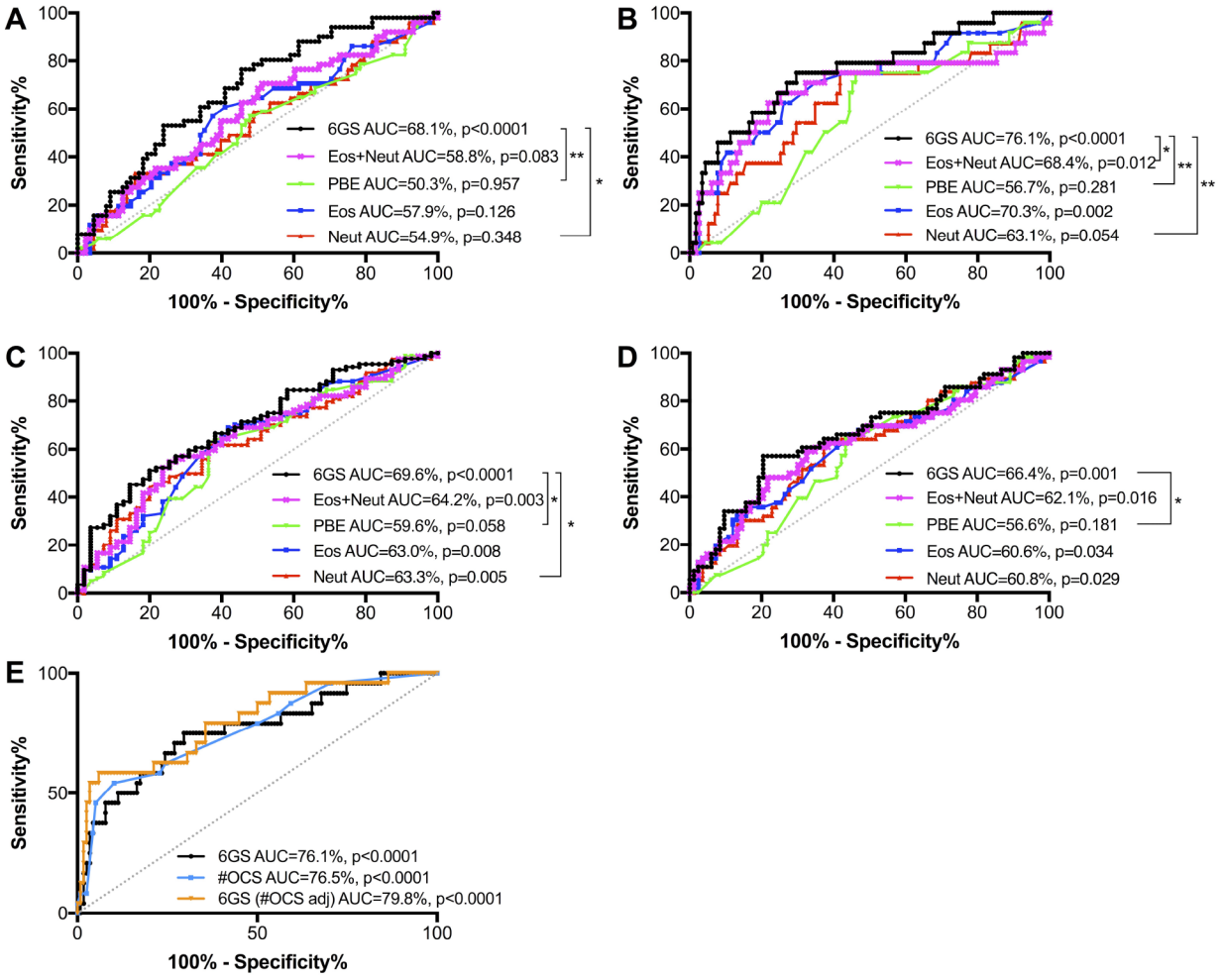
610 **Figure 1. ROC analysis of diagnostic performance of 6GS, sputum eosinophils and/or**
611 **neutrophils and PBE for predicting asthma exacerbation phenotypes.** ROC curve
612 comparison performed in both placebo- and AZM-treated patients (combined) enrolled in the
613 AMAZES trial. Biomarkers examined: the sputum 6GS (black line), combined sputum
614 eosinophils and neutrophils (pink line), sputum eosinophils (blue line), sputum neutrophils
615 (red line) and PBE (green line). Comparisons shown are non-exacerbator vs exacerbator
616 (severe exacerbations only) (**A**), infrequent exacerbator vs frequent exacerbator (severe
617 exacerbations only) (**B**), non-exacerbator vs exacerbator (sum moderate and severe
618 exacerbations) (**C**) and infrequent exacerbator vs frequent exacerbator (sum moderate and
619 severe exacerbations) (**D**). ROC analysis was also performed to compare prognostic capacity
620 of sputum 6GS, OCS courses (prior 12 months) and 6GS adjusted for prior OCS courses to
621 identify frequent vs non-frequent severe exacerbators (**E**). (* = $P < 0.05$, ** = $P < 0.01$).

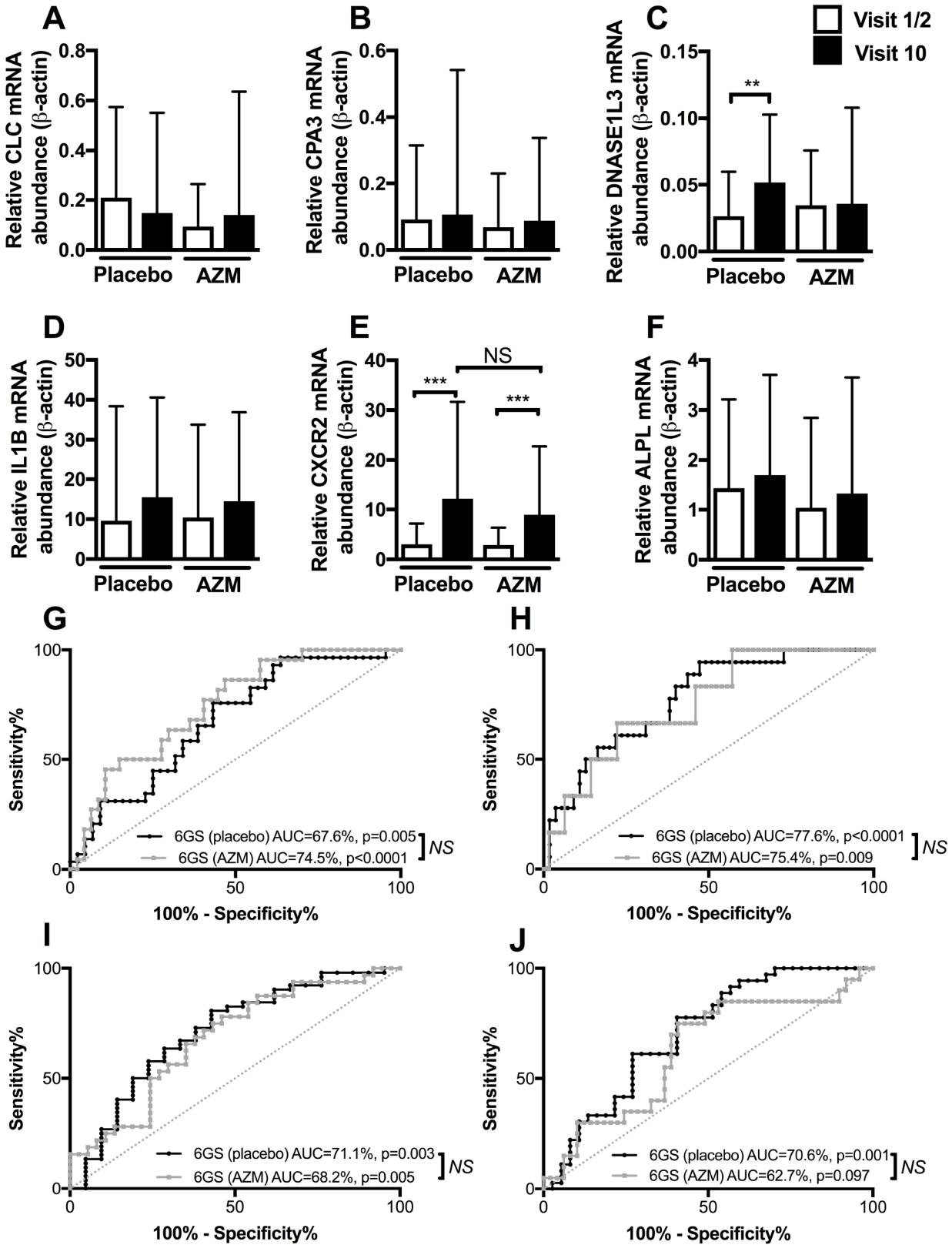
622

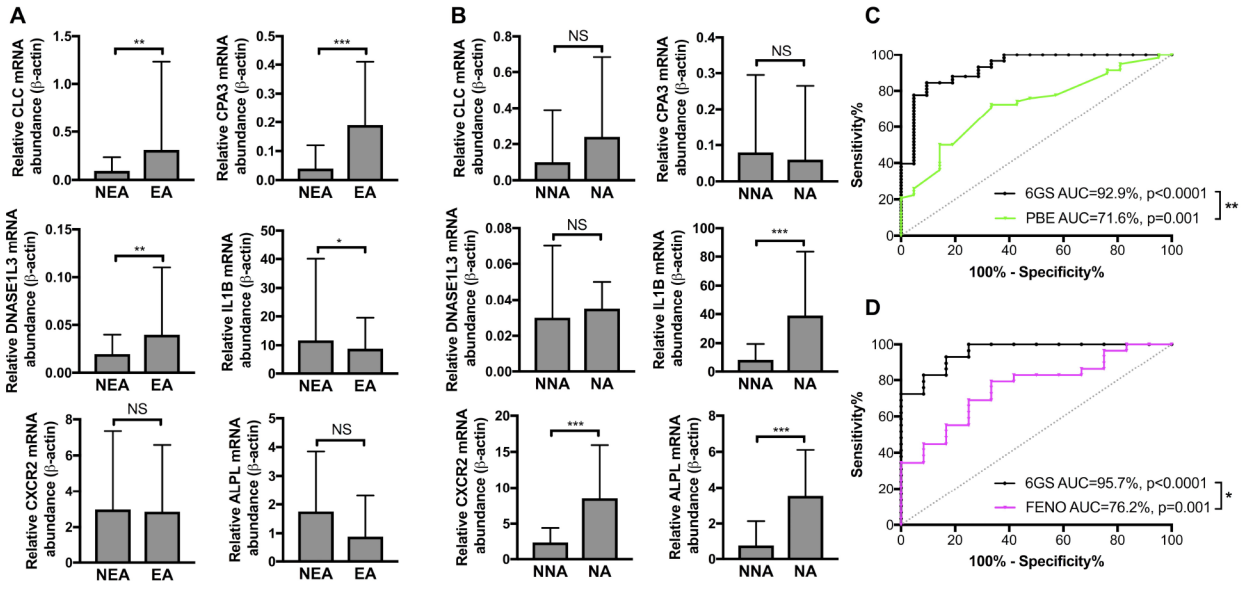
623 **Figure 2. AZM treatment does not alter sputum 6GS expression or prognostic capacity**
624 **compared to placebo.** qPCR was performed on cDNA generated from raw sputum samples
625 collected during screening visits (visit 1/2) and a visit at end of the treatment period (week
626 48, visit 10) for the AMAZES trial. CLC (**A**), CPA3 (**B**), DNASE1L3 (**C**), IL1B (**D**), CXCR2
627 (**E**) and ALPL (**F**) data are reported as relative abundance normalized to expression of the
628 housekeeping gene B-ACTIN (** = $P < 0.01$, *** = $P < 0.001$, Mann-Whitney). ROC curve
629 comparison for the sputum 6GS in placebo arm (black line) compared with sputum 6GS in
630 AZM arm (grey line). Comparisons shown are non-exacerbator vs exacerbator (severe
631 exacerbations only) (**G**), infrequent exacerbator vs frequent exacerbator (severe
632 exacerbations only) (**H**), non-exacerbator vs exacerbator (sum moderate and severe

exacerbations) (**I**) and infrequent exacerbator vs frequent exacerbator (sum moderate and severe exacerbations) (**J**).

Figure 3. Sputum 6 gene signature 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 B-ACTIN. **A)** patients are separated into non-eosinophilic asthma (sputum eosinophils < 3%) and eosinophilic asthma (≥ 3%) groups. **B)** patients are separated into non-neutrophilic asthma (sputum neutrophils < 61%) and neutrophilic asthma (≥ 61%) groups. Data are expressed as median value with interquartile range. (* = P < 0.05, ** = P < 0.01, *** = P < 0.001, Mann-Whitney). ROC curve comparison for the sputum 6GS (black line) compared with PBE (green line, **panel C**) and compared with FENO (pink line, **panel D**) (patient subgroup where FENO data was available). 6GS was better at distinguishing the EA from NA phenotypes than PBE or FENO (* = P < 0.05, ** = P < 0.01).


A
Sensitivity%
100% - Specificity%
6GS AUC=68.1%, p<0.0001
Eos+Neut AUC=58.8%, p=0.083
PBE AUC=50.3%, p=0.957
Eos AUC=57.9%, p=0.126
Neut AUC=54.9%, p=0.348
**
*
B
Sensitivity%
100% - Specificity%
6GS AUC=76.1%, p<0.0001
Eos+Neut AUC=68.4%, p=0.012
PBE AUC=56.7%, p=0.281
Eos AUC=70.3%, p=0.002
Neut AUC=63.1%, p=0.054
*
**
**
C
Sensitivity%
100% - Specificity%
6GS AUC=69.6%, p<0.0001
Eos+Neut AUC=64.2%, p=0.003
PBE AUC=59.6%, p=0.058
Eos AUC=63.0%, p=0.008
Neut AUC=63.3%, p=0.005
*
*
D
Sensitivity%
100% - Specificity%
6GS AUC=66.4%, p=0.001
Eos+Neut AUC=62.1%, p=0.016
PBE AUC=56.6%, p=0.181
Eos AUC=60.6%, p=0.034
Neut AUC=60.8%, p=0.029
*
E
Sensitivity%
100% - Specificity%
6GS AUC=76.1%, p<0.0001
#OCS AUC=76.5%, p<0.0001
6GS (#OCS adj) AUC=79.8%, p<0.0001


A
Relative CLC mRNA abundance (β-actin)
0.8
0.6
0.4
0.2
0.0
Placebo
AZM
B
Relative CPA3 mRNA abundance (β-actin)
0.6
0.4
0.2
0.0
Placebo
AZM
C
Visit 1/2
Visit 10
Relative DNASE1L3 mRNA abundance (β-actin)
0.15
0.10
0.05
0.00
**
Placebo
AZM
D
Relative IL1B mRNA abundance (β-actin)
50
40
30
20
10
0
Placebo
AZM
E
Relative CXCR2 mRNA abundance (β-actin)
40
30
20
10
0
NS
***
***
Placebo
AZM
F
Relative ALPL mRNA abundance (β-actin)
4
3
2
1
0
Placebo
AZM
G
Sensitivity%
100
50
0
0
50
100
100% - Specificity%
6GS (placebo) AUC=67.6%, p=0.005
6GS (AZM) AUC=74.5%, p<0.0001
NS
H
Sensitivity%
100
50
0
0
50
100
100% - Specificity%
6GS (placebo) AUC=77.6%, p<0.0001
6GS (AZM) AUC=75.4%, p=0.009
NS
I
Sensitivity%
100
50
0
0
50
100
100% - Specificity%
6GS (placebo) AUC=71.1%, p=0.003
6GS (AZM) AUC=68.2%, p=0.005
NS
J
Sensitivity%
100
50
0
0
50
100
100% - Specificity%
6GS (placebo) AUC=70.6%, p=0.001
6GS (AZM) AUC=62.7%, p=0.097
NS



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

Author list:

Michael Fricker, BSc PhD[1], Peter G Gibson, MBBS FRACP[1,2,14], Heather Powell, MMedSci[1,2], Jodie L Simpson, BSc PhD[1], Ian A Yang, MBBS PhD FRACP[3,4], John W Upham, MBBS PhD[3,5], Paul N Reynolds, MBBS MD PhD FRACP[6,7,8], Sandra Hodge, PhD[6,7,8], Alan L James MBBS MD FRACP[9,10], Christine Jenkins, MBBS MD FRACP[11,12], Matthew J Peters, MD FRACP[12,13], Guy B Marks, MBBS PhD FRACP[14,15], Melissa Baraket MBBS PhD FRACP[16], Katherine J Baines, BSc PhD[1]

ONLINE REPOSITORY

SUPPLEMENTAL METHODS

Trial Design

The AMAZES trial was a multicentre, randomized, double-blind, placebo controlled parallel group trial that was designed to evaluate the efficacy and safety of oral azithromycin 500mg, three times weekly for 48 weeks, as add-on therapy in adults with persistent symptomatic asthma despite maintenance controller therapy with ICS/LABD. 420 patients were allocated to azithromycin or identical-looking placebo in a 1:1 ratio centrally using concealed random allocation from a computer-generated random numbers table with permuted blocks of 4 or 6 and stratification for centre 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, NSW, 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%) [1, 2], or increased peak flow variability (n=73, 44.7%); were currently symptomatic with at least partial loss of asthma control (asthma control score (ACQ6)>0.74) [3] despite treatment with maintenance ICS/LABD; were clinically stable with no recent exacerbation, infection or change in maintenance medication for at least 4 weeks prior to study entry; and were non-smokers (exhaled carbon monoxide < 10ppm). Exsmokers with a >10pack year smoking history underwent gas transfer testing and were excluded if their carbon monoxide gas transfer coefficient was <65% predicted.

Procedures

After a screening visit patients entered a 2 week run-in period. Those with optimised asthma treatment, adherence to >80% of doses and who remained stable with change in ACQ6 of <0.5 were randomized. Patients were treated for 48 weeks and attended the clinic for assessment at weeks 6,12,24,36,48,52. 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[4] was performed before randomization and at the end of 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 >480mSec resulted in withdrawal from the trial. Microbiological assessments involved sputum culture for recognised pulmonary pathogens (5 sites), and throat swab and nose swabs (2 sites) at randomisation and end of treatment.

Outcomes

Our primary outcome was the rate of severe asthma exacerbations over 48 weeks[5, 6]. Severe exacerbations were worsening of asthma symptoms requiring ≥3 days of systemic corticosteroid treatment ≥10mg/day, or an asthma-specific hospitalization or emergency department visit requiring systemic corticosteroids. Exacerbations were captured at all visits using structured interviewing. Secondary efficacy variables were ACQ6, asthma-related quality of life (AQLQ[7], lung function, and induced sputum cell counts.

Sputum induction and analysis

Airflow limitation was assessed using spirometry (Medgraphics, CPFS/D^{TM} usb Spirometer, BreezeSuite v7.1, Saint Paul, USA). Sputum induction with hypertonic saline (4.5%) was performed in participants whose FEV_1 was ≥1L using our previously described methods[8]. In those with FEV_1 <1L, 0.9% saline was used. For gene expression, Buffer RLT (Qiagen, Hilden, Germany) was immediately added to 100 • L of selected sputum and stored at -80°C until RNA extraction. For inflammatory cell counts, selected sputum was dispersed using dithiothreitol, and total cell count and viability were performed. Cytospins were prepared, stained (May-Grunwald–Giemsa) and a differential cell count obtained from 400 non-squamous cells.

Gene expression analysis

Sputum gene expression of *CLC*, *CPA3*, *DNASE1L3*, *ALPL*, *CXCR2, IL1B* was performed as previously described[9] (see Online Repository). Briefly, sputum RNA was extracted using the

Qiagen RNeasy Mini Kit, quantified, reverse-transcribed to cDNA and used to detect gene expression using standard Taqman real-time qPCR methods (Applied Biosystems, Foster City, USA). Statistical analysis of diagnostic ability was performed on the change in cycle threshold (• Ct) between the target gene and housekeeping • -actin. For relative gene expression levels, data were log transformed ($2^{-\bullet Ct}$).

SUPPLEMENTAL RESULTS

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

		Marker *	Logistic Regression			
			Constant	Coefficient	Model P value	AUC
N=142 (51/142, 35.9% severe exacerbators)	*Individual*					
		ALPL	0.6821072	-0.1729284	0.0888	**0.6132 P=0.026**
		CLC	1.157094	-0.1759621	**0.0179**	**0.6391 P=0.003**
		CPA3	1.238786	-0.1685756	**0.0379**	**0.6152 P=0.016**
		CXCR2	-0.3657592	-0.0095939	0.6181	0.5490 P=0.354
		DNASE1L3	1.33276	-0.1507057	0.1685	0.5893 P= 0.074
		IL1B	-0.1105435	-0.1005466	0.3594	0.5652 P=0.204
	Combination					
	6GS	ALPL CLC CPA3 CXCR2 DNASE1L3 IL1B	1.107549	-0.2649145 -0.1413991 -0.1169522 0.2247225 0.1312494 -0.049104	0.0522	**0.6889 P<0.0001**

*Markers are normalized to beta-actin mRNA expression (• CT)

81 **Supplemental Table E2. Analysis of diagnostic value of the sputum 6GS for discriminating**
82 **patients who experience infrequent (< 2) or frequent (• 2) severe asthma exacerbations in the**
83 **following 48 weeks**

84

		Marker *	Logistic Regression			
			Constant	Coefficient	Model P value	AUC
N=142 (24/142, 16.9% frequent severe)	*Individual*					
		ALPL	-0.4141298	-0.1112192	**0.0210**	**0.6780 P=0.002**
		CLC	1.133079	-0.2618302	**0.0004**	**0.7444 P<0.0001**
		CPA3	1.799899	-0.3078733	**0.0002**	**0.7429 P<0.0001**
		CXCR2	-0.8829863	-0.0442772	**0.0323**	**0.6480 P=0.016**
		DNASE1L3	2.309992	-0.2999175	**0.0021**	**0.7270 P<0.0001**
		IL1B	-0.9561744	-0.0529377	**0.0320**	**0.6448 P=0.013**
	Combination					
	6GS	ALPL CLC CPA3 CXCR2 DNASE1L3 IL1B	1.653505	-0.0366669 -0.1419366 -0.2420405 0.0328808 0.0800461 -0.0380849	**0.0091**	**0.7613 P<0.0001**

85 *Markers are normalized to beta-actin mRNA expression (• CT)

Supplemental Table E3. Analysis of 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

		Marker *	Logistic Regression			
			Constant	Coefficient	Model P value	AUC
N=142 (84/142, 59.2% total exacerbators)	*Individual*					
		ALPL	2.021271	-0.1683247	**0.0014**	**0.6741 P<0.0001**
		CLC	1.116	-0.0229152	**0.0096**	**0.6332 P=0.006**
		CPA3	0.9125818	-0.00059	**0.0102**	**0.6172 P=0.016**
		CXCR2	0.9289623	-0.0041664	**0.0102**	**0.6361 P=0.004**
		DNASE1L3	0.5852789	0.0276092	**0.0098**	**0.6404 P=0.003**
		IL1B	0.9864461	-0.0257423	**0.0099**	**0.6381 P=0.003**
	Combination					
	6GS	ALPL CLC CPA3 CXCR2 DNASE1L3 IL1B	0.9350901	-0.4127687 -00.0361907 -.0101578 0.2094708 0.1425817 0.1086857	**0.0115**	**0.7114 P<0.0001**

*Markers are normalized to beta-actin mRNA expression (• CT)

Supplemental Table E4. 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

		Marker *	Logistic Regression			
			Constant	Coefficient	Model P value	AUC
N=142 (56/142, 39.4% frequent total exacerbators)	*Individual*					
		ALPL	.7881897	-0.1265727	**0.0148**	**0.6433 P=0.004**
		CLC	1.152437	-0.1299279	**0.0060**	**0.6555 P=0.002**
		CPA3	1.413362	-0.1452072	**0.0054**	**0.6530 P=0.002**
		CXCR2	0.3325545	-0.0675409	**0.0332**	**0.6219 P=0.015**
		DNASE1L3	1.606988	-0.1402179	**0.0145**	**0.6453 P=0.003**
		IL1B	0.2776118	-0.0991223	0.0264	**0.6292 P=0.007**
	Combination					
	6GS	ALPL CLC CPA3 CXCR2 DNASE1L3 IL1B	1.462895	-0.0890073 -0.068176 -0.1237933 0.039212 0.074877 -0.0496472	0.0876	**0.6649 P=0.001**

*Markers are normalized to beta-actin mRNA expression (• CT)

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

			6GS	Sputum eosinophils	Sputum neutrophils	PBE	6GS (FENO)•	FENO
Placebo	Total exacerbations	• 1 or 0	**AUC=0.711 P=0.003** N=73	AUC=0.505 P=0.947 N=73	AUC=0.582 P=0.263 N=73	AUC=0.602 P=0.165 N=73	**AUC=0.738 P=0.026** N=34	AUC=480 P=0.846 N=34
		• 2 or 0-1	**AUC=0.706 P=0.001** N=73	AUC=0.575 P=0.269 N=73	AUC=0.554 P=0.428 N=73	AUC=0.606 P=0.110 N=73	AUC=0.561 P=0.576 N=34	AUC=0.550 P=0.627 N=34
	Severe exacerbations	• 1 or 0	**AUC=0.676 P=0.005** N=73	AUC=0.547 P=0.499 N=73	AUC=0.593 P=0.181 N=73	AUC=0.558 P=0.397 N=73	**AUC=0.712 P=0.020** N=34	AUC=0.535 P=0.741 N=34
		• 2 or 0-1	**AUC=0.776 P<0.0001** N=73	**AUC=0.701 P=0.004** N=73	AUC=0.563* P=0.436 N=73	AUC=0.628 P=0.091 N=73	**AUC=0.810 P<0.0001** N=34	AUC=0.634 P=0.213 N=34
AZM	Total exacerbations	• 1 or 0	**AUC=0.643 P=0.038** N=66	AUC=0.544 p=0.540 N=66	AUC=0.479 p=0.776 N=66	AUC=0.524 p=0.740 N=66	AUC=0. 640 p=0.160 N=33	AUC=0.529 p=0.783 N=33
		• 2 or 0-1	AUC=0.610 P=0.150 N=66	AUC=0.431 P=0.386 N=66	AUC=0.470 P=0.698 N=66	AUC=0.511 P=0.891 N=66	AUC=0.609 P=0.348 N=33	AUC=0.565 P=0.536 N=33
	Severe exacerbations	• 1 or 0	**AUC=0.741 P<0.0001** N=66	AUC=0.534* P=0.669 N=66	AUC=0.469 ¥ P=0.675 N=66	AUC=0.592 P=0.229	**AUC=0.804 P<0.0001** N=33	AUC=0.583 § P=0.444 N=33
		• 2 or 0-1	**AUC=0.750 P=0.013** N=66	AUC=0.467* P=0.807 N=66	AUC=0.293 ¥ P=0.055 N=66	AUC=0.678 P=0.081 N=66	AUC=0.767 P=0.125 N=33	AUC=0.600 P=0.394 N=33

• values calculated in subpopulation where FENO measurement was made, *p<0.05 vs 6GS; ¥p<0.01 vs 6GS; §p<0.01 vs 6GS (FENO subpopulation)

Supplemental 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

		Marker *	Logistic Regression			
			Constant	Coefficient	Model P value	AUC (95%CI)
Placebo group. N=73 (29/73, 39.7% severe exacerbators)	*Individual*					
		ALPL	1.174711	-0.2524459	**0.0335**	**0.6552, p=0.017**
		CLC	1.311412	-0.1935234	**0.0293**	**0.6481, p=0.021**
		CPA3	1.121902	-0.1565537	0.1088	0.5862, p=0.204
		CXCR2	0.0996914	-0.0977394	0.3993	0.5846, p=0.217
		DNASE1L3	2.178457	-0.2240271	0.1038	0.6042, p=0.122
		IL1B	0.1822344	-0.1986743	0.1491	0.5799, p=0.259
	Combination					
	6GS	ALPL CLC CPA3 CXCR2 DNASE1L3 IL1B	2.553787	-0.2441983 -0.1503987 -0.0236053 0.0817315 -0.0199114 -0.0210936	0.2311	**0.6763 P=0.005**
AZM group. N=69 (22/69, 31.9% severe exacerbators)	*Individual*					
		ALPL	-0.2789161	-0.0735923	0.5923	0.5184, p=0.813
		CLC	0.7462753	-0.1546867	0.1193	0.5841 p=0.244
		CPA3	1.087588	-0.182063	0.0805	0.6190, p=0.088
		CXCR2	-1.351341	0.1113008	0.4124	0.5754, p=0.346
		DNASE1L3	0.2451273	-0.0881535	0.4917	0.5368, p=0.622
		IL1B	-0.7417235	-0.0056191	0.9669	0.5029, p=0.971
	Combination					
	6GS	ALPL CLC CPA3 CXCR2 DNASE1L3 IL1B	-0.2663717	-0.5786811 -0.0886016 -0.3606021 0.6540632 0.4044179 -0.0915628	0.0966	**0.7447 P<0.0001**

*Markers are normalized to beta-actin mRNA expression (• CT)

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

		Marker *	Logistic Regression			
			Constant	Coefficient	Model P value	AUC (95%CI)
Placebo group. N=73 (x18/73, 24.7% frequent severe)	*Individual*					
		ALPL	-0.665708	-0.0708267	0.5671	0.5747, p=0.342
		CLC	1.79629	-0.3441432	**0.0017**	**0.7434, p<0.0001**
		CPA3	2.70536	-0.4086174	**0.0009**	**0.7354, p<0.0001**
		CXCR2	-0.879162	-0.0450079	0.7307	0.5242, p=0.753
		DNASE1L3	2.292987	-0.2984015	0.0615	**0.6525, p=0.039**
		IL1B	-1.010963	-0.0347323	0.8202	0.4828, p=0.844
	Combination					
	6GS	ALPL CLC CPA3 CXCR2 DNASE1L3 IL1B	1.910656	0.000224 -0.2322196 -0.4001086 -0.0767063 0.2721843 -0.0170972	**0.0305**	**0.7758 P<0.0001**
AZM group. N=69 (6/69, 8.7% frequent severe)	*Individual*					
		ALPL	-0.8723939	-0.2375642	0.2961	0.5714 P=0.571
		CLC	-1.152898	-0.1256578	0.4212	0.5132 P=0.907
		CPA3	-0.78702	-0.1579249	0.3320	0.6032 P=0.329
		CXCR2	-2.132548	-0.0422384	0.8482	0.4815 P=0.907
		DNASE1L3	0.9566618	-0.3022422	0.1425	0.6534 P=0.111
		IL1B	-2.078326	-0.0922804	0.6817	0.5661 P=0.531
	Combination					
	6GS	ALPL CLC CPA3 CXCR2 DNASE1L3 IL1B	2.057035	-0.6730842 0.0791463 0.2137911 0.6367583 -0.5728786 -0.0484114	0.5772	**0.7540 P=0.009**

*Markers are normalized to beta-actin mRNA expression (• CT)

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

		Marker *	Logistic Regression			
			Constant	Coefficient	Model P value	AUC (95%CI)
Placebo group. N=73 (52/73, 71.2% total exacerbators)	*Individual*					
		ALPL	2.744964	-0.2750237	**0.0184**	**0.6896, p=0.015**
		CLC	1.044878	-0.0151497	0.8676	0.5238 P=0.776
		CPA3	0.9904325	-0.0084182	0.9344	0.4707 P=0.703
		CXCR2	1.54365	-0.1171154	0.3326	0.6200 P=0.125
		DNASE1L3	0.9053008	0.0001218	0.9993	0.5156 p=0.847
		IL1B	1.433011	-0.1650851	0.2606	0.5925 P=0.222
	Combination					
	6GS	ALPL CLC CPA3 CXCR2 DNASE1L3 IL1B	1.82736	-0.3766049 0.0077007 -0.0338994 -0.0014933 0.123925 0.1294379	0.3862	**0.7106 P=0.003**
AZM group. N=69 (32/69, 46.4% total exacerbators)	*Individual*					
		ALPL	0.1277557	-0.041565	0.7463	0.5025 p=0.972
		CLC	0.1613086	-0.0309722	0.7376	0.4975 p=0.971
		CPA3	-0.2102336	0.0062969	0.9476	0.5194 p=0.785
		CXCR2	-0.7677189	0.1182769	0.3501	0.5794 P=0.259
		DNASE1L3	-0.6843071	0.0470349	0.6959	0.5346 P=0.627
		IL1B	-0.3903965	0.0789144	0.5345	0.5456 P=0.521
	Combination					
	6GS	ALPL CLC CPA3 CXCR2 DNASE1L3 IL1B	-0.5183495	-0.6414888 -0.0522411 0.0004421 0.5629461 0.1618661 0.0880836	0.3576	**0.6816 P=0.005**

108 *Markers are normalized to beta-actin mRNA expression (• CT)

Supplemental 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 following 48 weeks

		Marker *	Logistic Regression			
			Constant	Coefficient	Model P value	AUC (95%CI)
Placebo. N=73 (36/73, 49.3% frequent total	*Individual*					
		ALPL	1.37584	-0.2187011	0.0495	**0.6569 P=0.018**
		CLC	2.287965	-0.255561	**0.0042**	**0.6959 P=0.002**
		CPA3	2.55715	-0.2607424	**0.0089**	**0.6569 P=0.015**
		CXCR2	0.9757104	-0.1889486	0.1021	0.6081 P=0.112
		DNASE1L3	3.415742	-0.2956417	0.0316	**0.6404 P=0.033**
		IL1B	0.4134511	-0.1433317	0.2820	0.5548 P=0.425
	Combination					
	6GS	ALPL CLC CPA3 CXCR2 DNASE1L3 IL1B	3.8649	-0.1726814 -0.1481676 -0.1704555 -0.1120212 0.0449441 0.0985971	0.0640	**0.7057 P=0.001**
AZM group. N=69 (20/69, 29.0% frequent total	*Individual*					
		ALPL	-0.9968258	0.0153026	0.9137	0.5378 p=0.635
		CLC	-1.157131	0.026261	0.7973	0.5633 P=0.395
		CPA3	-0.6788128	-0.0210921	0.8409	0.4796 P=0.784
		CXCR2	-1.463457	0.1064686	0.4452	0.5704 P=0.379
		DNASE1L3	-0.8950519	0.0000905	0.9995	0.4765 P=0.762
		IL1B	-0.7406066	-0.0508403	0.7153	0.5378 0.625
	Combination					
	6GS	ALPL CLC CPA3 CXCR2 DNASE1L3 IL1B	-0.9970808	-0.2584836 0.0914051 -0.0990941 0.4106918 0.0193966 -0.1623113	0.8283	0.6265 P=0.097

*Markers are normalized to beta-actin mRNA expression (• CT)

Supplemental Table E10. Comparison of diagnostic value of sputum 6GS vs PBE & FENO for asthma airway inflammatory phenotyping

Phenotype	6-Gene Signature	PBE	P value 6GS vs PBE
EA vs NEA N=139	AUC=0.7684 P<0.0001	AUC=0.7591 P<0.0001	0.858
EA vs NA N=79	AUC= 0.9294 P<0.0001	AUC= 0.7159 P=0.001	**0.002**
EA vs PGA N=118	AUC=0.7636 P<0.0001	AUC= 0.7726 P<0.0001	0.873
Phenotype	**6-Gene Signature**	**FENO**	**P value 6GS vs FENO**
EA vs NEA N=67	AUC=0.8152 P<0.0001	AUC=0.7268 P<0.0001	0.242
EA vs NA N=41	AUC= 0.9569 P<0.0001	AUC= 0.7615 P=0.001	**0.015**
EA vs PGA N=55	AUC=0.8383 P<0.0001	AUC= 0.7147 P=0.003	0.136

Supplemental References

1. Joos G, O'Connor B, Anderson S, Chung F, Cockcroft D, Dahlén B, et al. Indirect airway challenges Eur Respir J 2003; 21:1050-68.
2. Anderson SD. Indirect challenge tests: Airway hyperresponsiveness in asthma: its measurement and clinical significance. Chest 2010; 138:25S-30S.
3. Juniper E, O'Byrne P, Guyatt G, Ferrie P, King D. Development and validation of a questionnaire to measure asthma control. Eur Respir J 1999; 14:902-7.
4. Simpson J, Scott R, Boyle M, Gibson P. Inflammatory subtypes in asthma: assessment and identification using induced sputum. Respirology 2006; 11:54-61.
5. Brusselle GG, Vanderstichele C, Jordens P, Deman R, Slabbynck H, Ringoet V, et al. Azithromycin for prevention of exacerbations in severe asthma (AZISAST): a multicentre randomised double-blind placebo-controlled trial. Thorax 2013; 68:322-9.
6. Reddel H, Taylor D, Bateman E, Boulet L, Boushey H, WW B, et al. An official American Thoracic Society/European Respiratory Society statement: asthma control and exacerbations: standardizing endpoints for clinical asthma trials and clinical practice. Am J Respir Crit Care Med 2009; 180:59-99.
7. Juniper E, Buist AS, Cox FM, Ferrie PJ, King DR. Validation of a standardized version of the Asthma Quality of Life Questionnaire. Chest 1999; 115:1265-70.
8. Gibson PG, Wlodarczyk JW, Hensley MJ, Gleeson M, Henry RL, Cripps AW, et al. Epidemiological association of airway inflammation with asthma symptoms and airway hyperresponsiveness in childhood. Am J Respir Crit Care Med 1998; 158:36-41.
9. Baines KJ, Simpson JL, Wood LG, Scott RJ, Gibson PG. Transcriptional phenotypes of asthma defined by gene expression profiling of induced sputum samples. J Allergy Clin Immunol 2011; 127:153-60, 60 e1-9.