Add-on azithromycin reduces sputum cytokines in non-eosinophilic asthma: an AMAZES substudy

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ABSTRACT

Add-on azithromycin (AZM) significantly reduces exacerbations in poorly controlled asthma irrespective of disease phenotype. In a predefined substudy of the original AMAZES protocol (500 mg, three times a week for 48 weeks), we report that AZM treatment reduces key sputum inflammatory proteins (interleukin (IL)-6, IL-1β and extracellular DNA), which is more evident in non-eosinophilic asthma (NEA). Moreover, AZM reduced *Haemophilus influenzae* load only in NEA. Our data support the anti-inflammatory effects of AZM in poorly controlled asthma. Prospective studies are required to identify patients that derive greatest benefit from AZM add-on therapy.

Adults with poorly controlled asthma experience exacerbations, despite maintenance treatment with inhaled corticosteroids and long-acting bronchodilators. This population is heterogeneous and includes those with eosinophilic asthma (EA), characterised by type 2 airway inflammation, and those with non-eosinophilic asthma (NEA), who exhibit predominantly neutrophilic inflammation[1] and whose airways are commonly colonised by high levels of Gammaproteobacteria, including *Haemophilus influenzae* and *Moraxella catarrhalis*.[2] For patients with EA, biologic therapies (anti-IgE, anti-interleukin (IL)-5 and anti-IL-5Ra) have proven transformative in preventing exacerbations by acting on eosinophilic inflammation. However, effective treatment options for NEA are limited.

We have previously shown that add-on oral azithromycin (AZM) reduces exacerbations in poorly controlled asthma, including NEA.[1] We now report an a priori substudy of the original trial protocol to assess the effect of AZM on soluble proinflammatory mediators IL-6, IL-1β and extracellular DNA (eDNA) in EA and NEA. Given the recent findings that AZM reduces *H. influenzae* abundance,[3] and high baseline *H. influenzae* predicts clinical benefit from AZM treatment,[4] we further relate *H. influenzae* to eosinophilic phenotype, and proinflammatory mediator levels.

Paired induced sputum samples from 212 participants before and after add-on AZM (n=109; 500 mg, three times a week for 48 weeks) or placebo (n=103) (table 1) were assessed. This subgroup showed a significant reduction in total exacerbations with AZM (p=0.001, table 1), consistent with the original study. Participants underwent a clinical assessment and gave written informed consent.[1] Sputum inflammatory cell counts were performed after dithiothreitol dispersion and characterised as eosinophilic or non-eosinophilic.[1] Supernatant concentrations of IL-6, IL-8, IL-1β and eDNA, and raw sputum *H. influenzae* abundance were measured as previously described[4–6] and detailed in the online supplemental. IL-8 and *H. influenzae* were measured in smaller subset of samples (IL-8: n=87 baseline sample, *H. influenzae* n=112 baseline and 61 endpoint samples) due to sample availability. The relationship between baseline measures were assessed by Spearman's rank correlation with p value adjusted for multiple comparisons using the Bonferroni test. Associations between end of treatment levels of inflammation and *H. influenzae* with treatment allocation were analysed using linear mixed models, with a fixed effect used to adjust for baseline levels and a random effect for study site, as defined a priori and performed in the initial Asthma and Macrolides: the Azithromycin Efficacy and Safety Study (AMAZES) trial.[1]

Markers of neutrophilic inflammation and *H. influenzae* were highly co-correlated at baseline (figure 1A, online supplemental figure 1). After stratification by inflammatory phenotype, *H. influenzae* was only significantly correlated with IL-1β in those with NEA (R_s=0.546, p=0.004, n=41, figure 1B, online supplemental figures 2 and 3). Eosinophil count correlated with neutrophil count, however not with any other markers of inflammation or *H. influenzae* (figure 1A–C).

Compared with placebo, add-on AZM resulted in a significant reduction in sputum *H. influenzae*, IL-6, IL-1β and eDNA (figure 1D, online supplemental table 1). A significant reduction in *H. influenzae*, IL-6, IL-1β and eDNA, remained in those with NEA (figure 1E, online supplemental table 1), while only IL-6 was significantly reduced following AZM in those with EA (estimate (95% CI)=−0.292 (−0.570 to −0.015); p=0.039, figure 1F, online supplemental table 1).

We further investigated whether changes in inflammatory markers related to changes in *H. influenzae* levels following AZM. In the 24 participants in whom *H. influenzae* and IL-1β were measured at baseline and following AZM, reduction in *H. influenzae was* strongly correlated with the reduction in IL-1β (R_s=0.949, p<0.001). This relationship remained strong for those with NEA (R_s=0.958, p<0.001, n=12), while weaker, although significant, for EA (R_s=0.790, p=0.046, n=12). Changes in IL-6, eDNA and neutrophils did not correlate with changes in *H. influenzae* following AZM.

Collectively, our findings show a relationship

Table 1 Baseline characteristics of participants

	Placebo N=103	Azithromycin N=109
Age (years)*	57.2 (16.5)	60.3 (12.4)
Gender (M/F)	44/59	50/59
Ex-smoker†	39 (38%)	39 (36%)
Pack years‡	7.5 (1.4–25.0)	9.3 (1.5–26.3)
Atopy	83 (81%)	79/107 (74%)
ACQ6 Score*	1.83 (0.78)	1.79 (0.84)
Eosinophilic†	53 (51%)	46 (42%)
Asthma history past year‡		
Emergency room visit or hospital admission	0 (0–0)	0 (0–0)
Unscheduled doctor visits	0 (0–2)	1 (0–2)
Oral corticosteroid courses	1 (0–2)	1 (0–2)
Medications†		
ICS daily dose as beclomethasone equivalent µg/day‡	1000 (200–4500)	1480 (200–4000)
ICS/LABA	102 (99%)	106 (97%)
Oral corticosteroid	2 (1.9%)	3 (2.8%)
Spirometry, pre β2-agonist*		
FEV_1% predicted	74.75 (15.84)	74.53 (19.68)
FVC% predicted	84.16 (14.13)	84.94 (14.24)
FEV_1/FVC%	68.77 (10.39)	67.31 (12.05)
Sputum cell counts‡		
Viability	72 (9–100)	67 (7–100)
Total cell count (10^6/mL)	4.41 (0.31–69.93)	4.05 (0.45–36.00)
Neutrophils%	34.38 (0.25–95.75)	35.25 (0.68–95.25)
Neutrophils×10^4/mL	116.8 (0.67–3486)	126.2 (3.04–3296)
Eosinophils%	2.13 (0.00–52.50)	1.50 (0.00–83.00)
Eosinophils×10^4/mL	8.34 (0.00–547.2)	6.23 (0.00–888.9)
Macrophages%	50.38 (2.25–93.75)	43.88 (2.00–93.00)
Lymphocytes%	0.50 (0.00–14.50)	0.50 (0.00–10.75)
Columnar epithelial%	3.25 (0.00–59.50)	2.13 (0.00–79.00)
Exacerbations/person-year during trial		
Total	1.58	0.95
Severe	0.70	0.51
Moderate	0.89	0.44

*Mean(SD).
†n/N(%); FEV_1.
‡Median(Q1,Q3).
ACQ6, Asthma Control Questionnaire 6; FEV_1, forced expiratory volume in one second; FVC, forced vital capacity; ICS, inhaled corticosteroid; LABA, long-acting beta agonist.

between inflammatory mediators associated with neutrophilic inflammation and *H. influenzae*, and that add-on AZM therapy reduces these inflammatory mediators. These relationships were more pronounced in NEA compared with EA. Our previous substudy, which examined RNA gene copy number, identified that AZM did not affect inflammatory gene expression.[7] The impact of AZM on inflammatory protein expression reported here may be due to a downstream mechanism, such as AZM-induced inhibition of protein translation.

It remains unclear whether the benefit provided by AZM occurs via different pathways in those with EA and NEA, or whether there is a common mechanism that is more pronounced in NEA. It is also yet to be determined whether relationships between reduction in *H. influenzae* and reduction in inflammatory marker levels represent causal interactions, and what the direction of any such relationship might be.

In adults with NEA, the reduction in IL-1β, eDNA, IL-6 and *H. influenzae* following AZM is a potential mechanism of clinical benefit. As reviewed in detail elsewhere,[8] the reduction in inflammatory markers by macrolides such as AZM can reduce an overt neutrophil response that is associated with corticosteroid-resistant asthma exacerbations, as well as improve monocyte/macrophage activity leading to clearance of proinflammatory material, including *H. influenzae*.

Our study focused on identifying the effects of AZM in those with NEA, given the current lack of effective therapies for this patient group. However, AZM was found to reduce IL-6 in both EA and NEA. IL-6 may be involved in a conserved mechanism

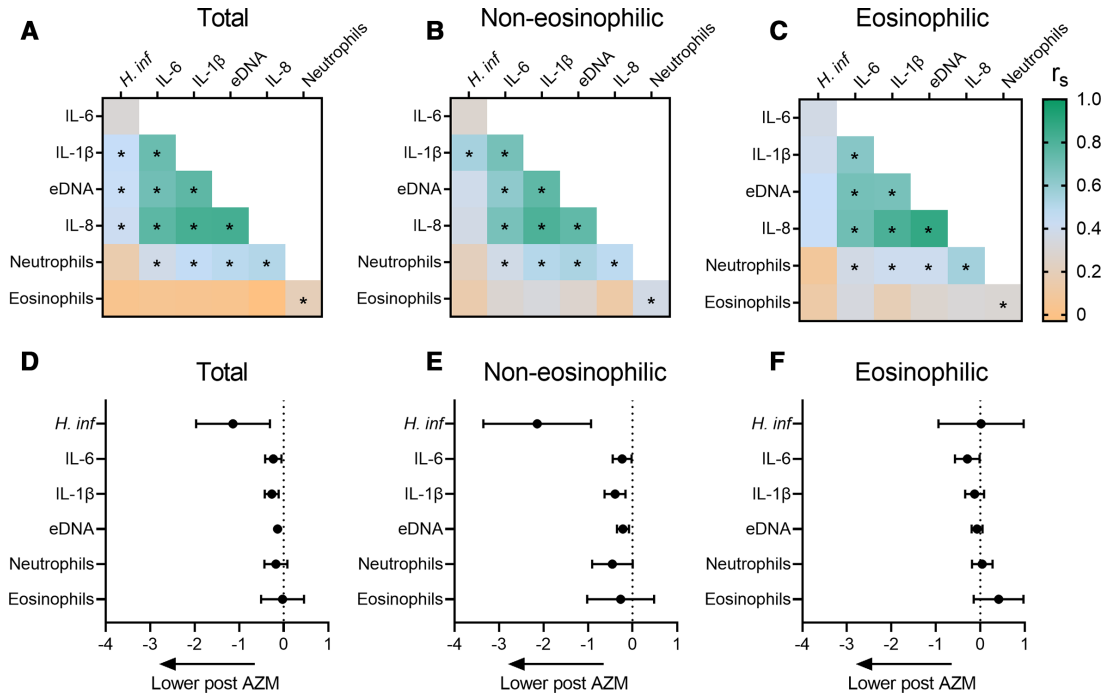

Figure 1 Relationship among azithromycin (AZM), markers of airway inflammation and airway *Haemophilus influenzae* levels in adults with persistent uncontrolled asthma, stratified by eosinophilic phenotype. (A–C) Heatmap showing Spearman's correlations of baseline measures for (A) all participants, (B) non-eosinophilic asthma and (C) eosinophilic asthma. Each cell represents a correlation between the measure on the left of the heat map and the top measure, coloured by the strength of the correlation coefficient (r_s). The asterisk (*) indicates p<0.05 (Bonferroni corrected). (D,E) Linear mixed model regression analyses assessing the effect of AZM on endpoint measures adjusting for baseline levels and study site for (D) all participants, (E) non-eosinophilic asthma and (F) eosinophilic asthma. Negative values indicate lower post treatment levels in the AZM group. eDNA, extracellular DNA; *H. inf*, *Haemophilus influenzae*; IL, interleukin.

by which AZM acts across asthma. For example, in EA, an AZM-dependent reduction in IL-6 could reduce its role in promoting T helper (Th)2 differentiation over Th1,[9] while in NEA, an AZM-dependent reduction in IL-6 could impair Th17 differentiation, limiting neutrophilic activity.[9] Alternatively, IL-6 could contribute to asthma inflammation via trans-signalling of the soluble IL-6 receptor, which is crucial in expression of genes involved in regulation of airway remodelling and innate immune activation.[10]

In conclusion, our findings indicate that long-term administration of AZM attenuates key sputum inflammatory markers (IL-6, IL-1β and eDNA) as well as levels of

H. influenzae, especially in patients with NEA. Sputum IL-6 levels were significantly reduced in both EA and NEA. These anti-inflammatory effects of AZM may contribute to the reduction in asthma exacerbations observed in the main study.[1] Achieving a better understanding of the mechanistic basis of AZM benefit should now be prioritised as a means to enable identification of those patients most likely to benefit.

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Competing interests JWU reports personal fees from AstraZeneca, personal fees from GSK, personal fees from Novartis, personal fees from Boehringer Ingelheim, personal fees from Sanofi, outside the submitted work. PGG reports personal fees from AstraZeneca, GlaxoSmithKline, Novartis, grants from AstraZeneca, GlaxoSmithKline, outside the submitted work.

Patient consent for publication Not required.

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Add-on Azithromycin reduces sputum cytokines in non-eosinophilic asthma: an AMAZES sub-study

ONLINE SUPPLEMENT

SUPPLEMENTARY METHODS

Sub-study design

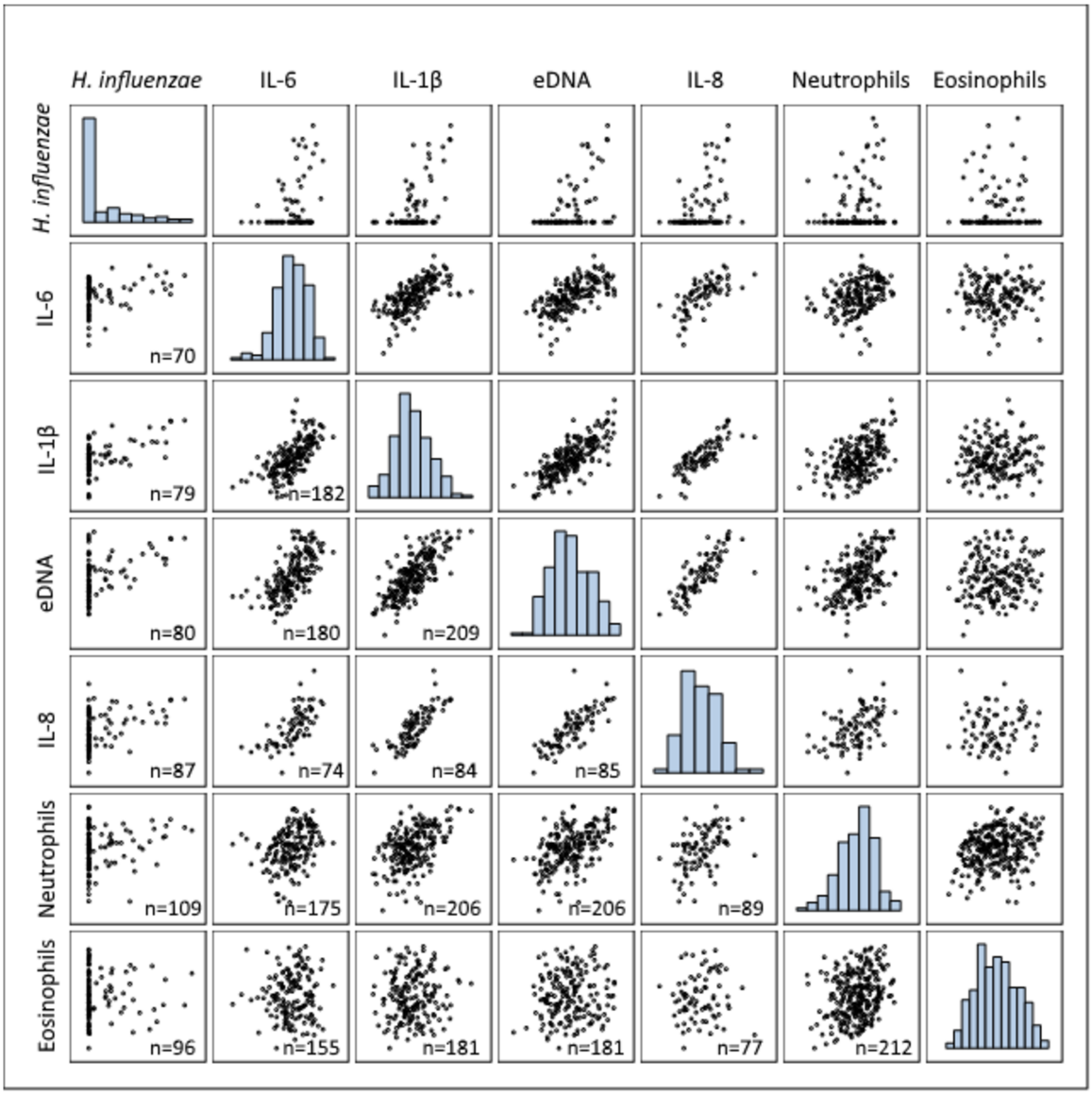
This was a pre-specified sub-study from the initial AMAZES trial protocol (Section 11.2, Appendix I) https://www.severeasthma.org.au/wp-content/uploads/2017/04/AMAZES-Protocol-V15-25.02.14-final.pdf

Inflammatory marker assessment

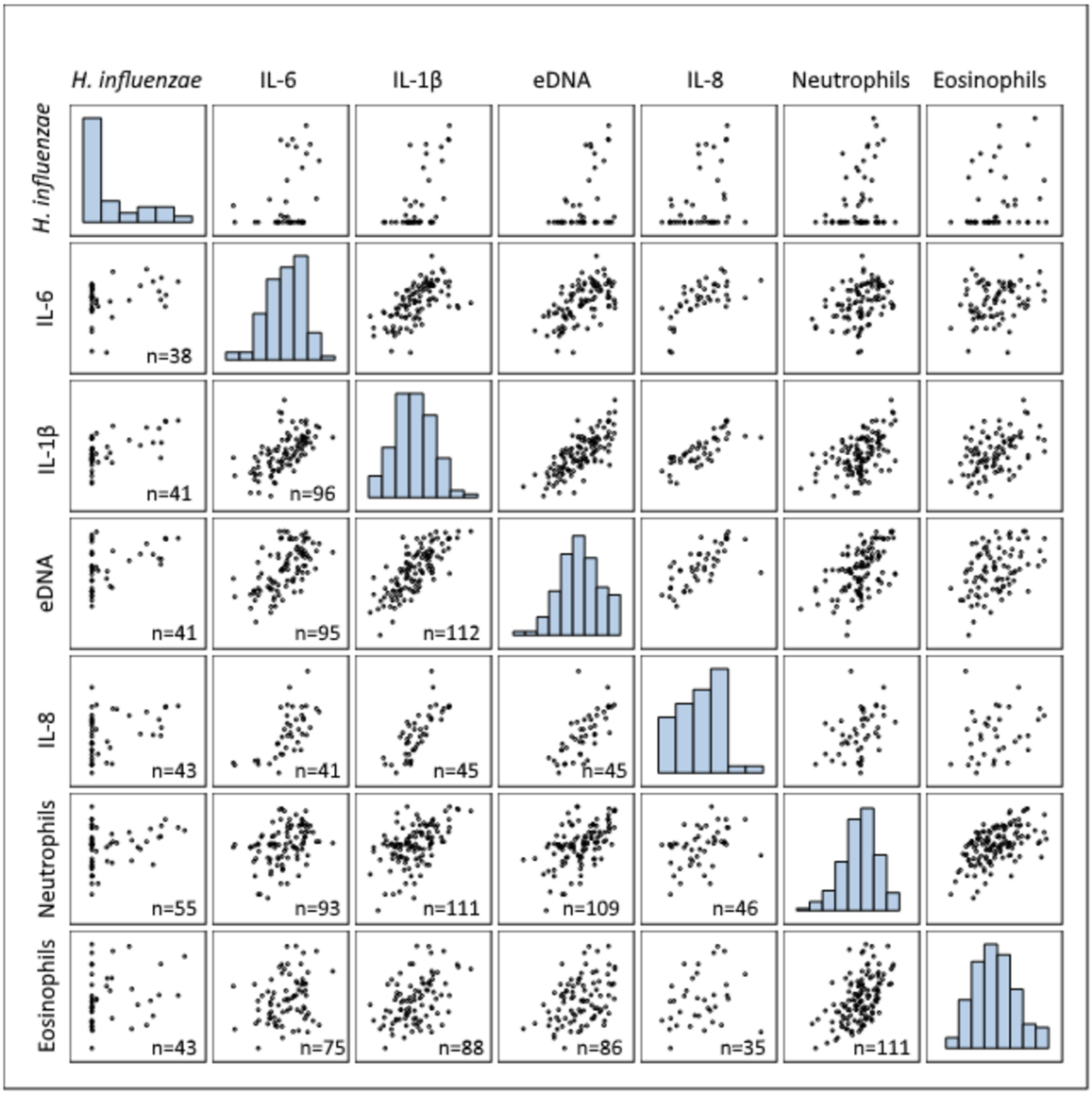
Sputum IL-6, IL-8 and IL-1β levels were determined by ELISA according to the manufacturer's instructions (R&D Systems; Minneapolis, MS, USA). The validity of IL-6, IL-8 and IL-1β in sputum supernatant has been reported previously.[1-3] The limits of detection of each assay were as following; sputum IL-6: 168.84 pg/ml; IL-8: 31.3 pg/ml; IL-1β: 35.19 pg/ml. IL-6 and IL-1β were assessed in all available sputum supernatant pairs. The abundance of eDNA in the cell-free sputum supernatant was quantitated using the Quant-iT PicoGreen dsDNA Assay Kit (P7589, Invitrogen, Carlsbad, CA) as per manufacturer's instructions.[4] This assay selectively detects double-stranded DNA, which was quantitated in 10 µL of sputum supernatant against a DNA standard curve of 0–200 ng/µL.

Haemophilus influenzae detection in raw sputum

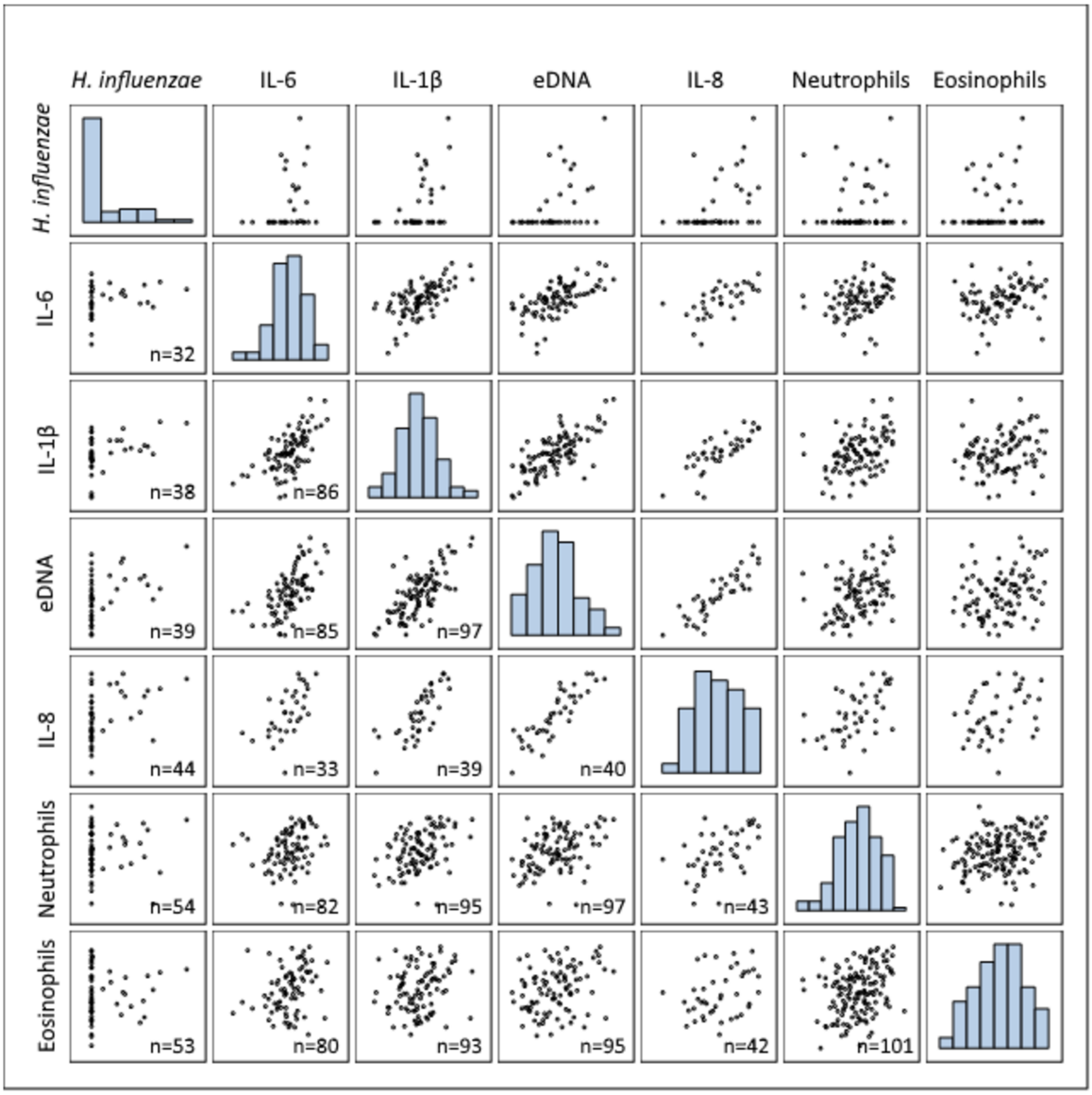
The total DNA was extracted from frozen raw sputum aliquots using a combination of physical, enzymatic, and heat-based cell lysis method that was followed by phenol-chloroform extraction and DNA recovery using EZ-10 Spin columns (Bio Basic, Inc.) as previously described.[5] *H. influenzae* abundance was measured using a previously published, validated, species-specific qPCR assay with a standard curve of *H. influenzae* strain NTCC8468 of known quantities.[6,7] Briefly, 1 µL of DNA extract, 0.25 µM of primers (F: ATTAAATGTTGCATCAACGC, R: GACTTTTGCCCACGCAC), 0.2 µM of target probe (FAM-ACGRTTTTACCATAGTTGCACTTTCTC-BHQ1), 17.5 µL of 2X KAPA Probe Fast qPCR Master Mix (KAPA Biosystems Inc., Wilmington, USA) and the appropriate volume of water was added to a 35 µL total reaction volume. Quantitative real-time PCR were performed on three technical replicates, at 10 µL reaction volume per replicate, on a QuantStudio 6 and 7 Flex Real-Time PCR system (Applied Biosystems, Carlsbad, USA). Cycle conditions were 50 cycles of 95 °C for 10 s and 63 °C for 30 s. Serial dilutions of a known concentration of *H. influenzae* strain NTCC8468 was run with every PCR reaction and a copy number per mL of sputum was calculated by comparing sample Ct to the standard curve.



Supplementary Figure 1: Scatter plot matrix of inflammatory mediators and *Haemophilus influenzae* in all participants. X-axis from left to right and y-axis from top to bottom display $\log_{10}$ values of *H. influenzae* (copies/mL), IL-6 (pg/mL), IL-1β (pg/mL), extracellular DNA (ng/mL), IL-8 (pg/mL), neutrophils (count × 10^4/mL), and eosinophils (count × 10^4/mL)



Supplementary Figure 2: Scatter plot matrix of inflammatory mediators and *Haemophilus influenzae* in non-eosinophilic asthma. X-axis from left to right and y-axis from top to bottom display $\log_{10}$ values of *H. influenzae* (copies/mL), IL-6 (pg/mL), IL-1β (pg/mL), extracellular DNA (ng/mL), IL-8 (pg/mL), neutrophils (count × 10^4/mL), and eosinophils (count × 10^4/mL)



Supplementary Figure 3: Scatter plot matrix of inflammatory mediators and *Haemophilus influenzae* in eosinophilic asthma. X-axis from left to right and y-axis from top to bottom display $\log_{10}$ values of *H. influenzae* (copies/mL), IL-6 (pg/mL), IL-1β (pg/mL), extracellular DNA (ng/mL), IL-8 (pg/mL), neutrophils (count × 10^4/mL), and eosinophils (count × 10^4/mL)

Supplementary Table 1. Azithromycin (AZM) effects on inflammatory mediators and *Haemophilus influenzae* in all, eosinophilic and non-eosinophilic asthma. Estimated difference (95% CI) between AZM and placebo at week 48 is shown.

	All			**Non-eosinophilic**			**Eosinophilic**		
	Observations	Estimate (95% CI)	p-value	Observations	Estimate (95% CI)	p-value	Observations	Estimate (95% CI)	p-value
$log_{10}(H.influenzae)$	AZM: 23 Placebo: 30	-1.140 (-1.970 to -0.309)	**0.007**	AZM: 11 Placebo: 14	-2.142 (-3.352 to -0.932)	**0.001**	AZM: 12 Placebo: 16	0.017 (-0.940 to 0.975)	0.971
log_{10}(IL-6)	AZM: 91 Placebo: 86	-0.234 (-0.416 to -0.052)	**0.012**	AZM: 47 Placebo: 41	-0.235 (-0.446 to -0.023)	**0.029**	AZM: 39 Placebo: 43	-0.292 (-0.57 to -0.015)	**0.039**
log_{10}(IL-1β)	AZM: 108 Placebo: 100	-0.268 (-0.423 to -0.114)	**0.001**	AZM: 57 Placebo: 47	-0.393 (-0.627 to -0.158)	**0.001**	AZM: 44 Placebo: 50	-0.130 (-0.341 to 0.081)	0.227
log_{10}(eDNA)	AZM: 103 Placebo: 98	-0.139 (-0.229 to -0.049)	**0.002**	AZM: 54 Placebo: 45	-0.215 (-0.351 to -0.080)	**0.002**	AZM: 42 Placebo: 50	-0.075 (-0.198 to 0.048)	0.234
log_{10}(Neutrophils)	AZM: 92 Placebo: 98	-0.175 (-0.431 to 0.082)	0.183	AZM: 52 Placebo: 47	-0.453 (-0.909 to 0.004)	0.052	AZM: 40 Placebo: 51	0.042 (-0.188 to 0.273)	0.719
log_{10}(Eosinophils)	AZM: 92 Placebo: 98	-0.024 (-0.507 to 0.459)	0.922	AZM: 52 Placebo: 47	-0.267 (-1.020 to 0.486)	0.487	AZM: 40 Placebo: 51	0.410 (-0.149 to 0.969)	0.151

* All models adjust for the baseline level. Negative values of the difference indicate lower post treatment levels in the AZM group. Eosinophilic asthma was defined as sputum eosinophils ≥3%.

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