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

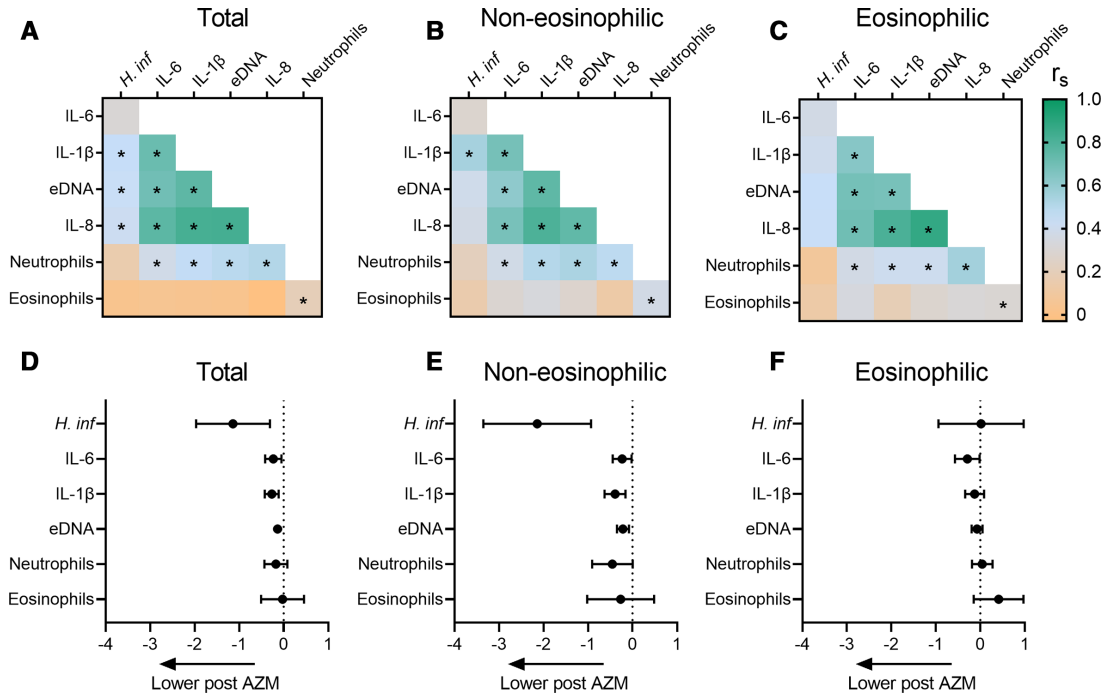

Figure 1 Relationship among azithromycin (AZM), markers of airway inflammation and airway *Haemophilus influenzae* levels in adults with persistent uncontrolled asthma, stratified by eosinophilic phenotype. (A–C) Heatmap showing Spearman's correlations of baseline measures for (A) all participants, (B) 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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