

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

**ONLINE SUPPLEMENT**

## SUPPLEMENTARY METHODS

### Sub-study design

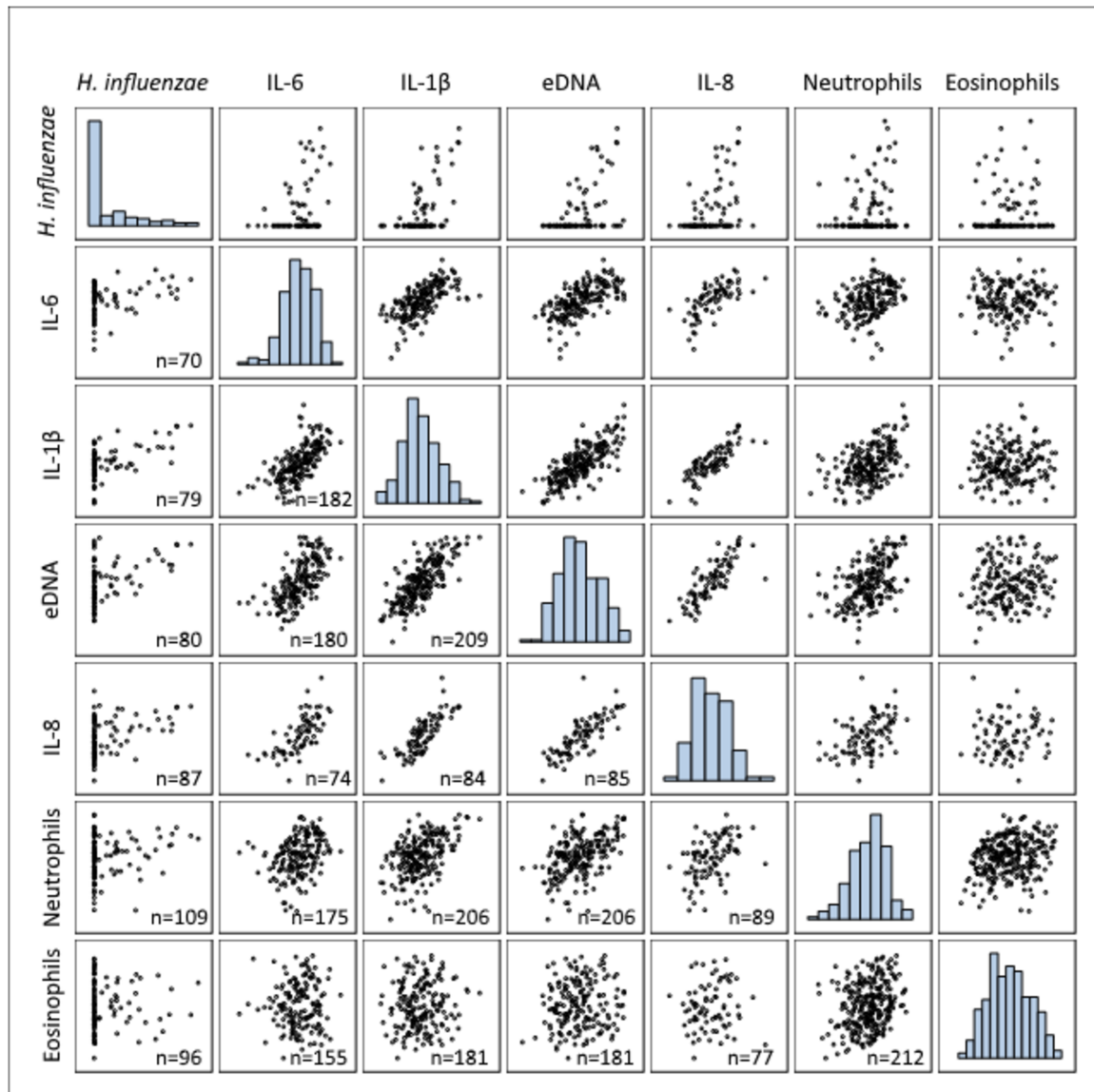
This was a pre-specified sub-study from the initial AMAZES trial protocol (Section 11.2, Appendix I) <https://www.severeasthma.org.au/wp-content/uploads/2017/04/AMAZES-Protocol-V15-25.02.14-final.pdf>

### Inflammatory marker assessment

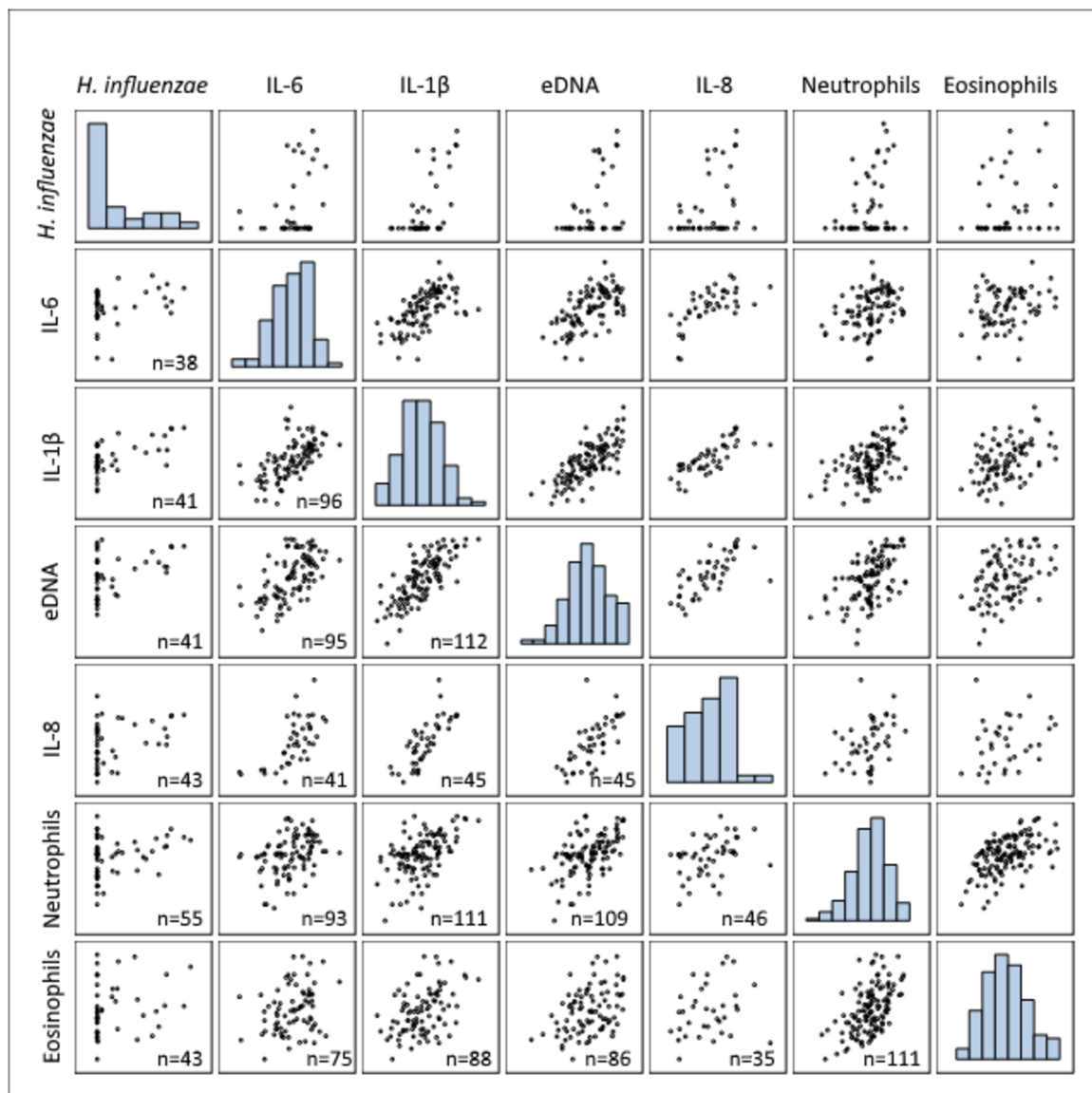
Sputum IL-6, IL-8 and IL-1 $\beta$  levels were determined by ELISA according to the manufacturer's instructions (R&D Systems; Minneapolis, MS, USA). The validity of IL-6, IL-8 and IL-1 $\beta$  in sputum supernatant has been reported previously.<sup>1-3</sup> The limits of detection of each assay were as following; sputum IL-6: 168.84 pg/ml; IL-8: 31.3 pg/ml; IL-1 $\beta$ : 35.19 pg/ml. IL-6 and IL-1 $\beta$  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.<sup>4</sup> This assay selectively detects double-stranded DNA, which was quantitated in 10  $\mu$ L of sputum supernatant against a DNA standard curve of 0–200 ng/ $\mu$ L.

### *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.<sup>5</sup> *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.<sup>6,7</sup> Briefly, 1  $\mu$ L of DNA extract, 0.25  $\mu$ M of primers (F: ATTAAATGTTGCATCAACGC, R: GACTTTTGCCCACGCAC), 0.2  $\mu$ M of target probe (FAM-ACGRTTTTACCATAGTTGCACTTTCTC-BHQ1), 17.5  $\mu$ L of 2X KAPA Probe Fast qPCR Master Mix (KAPA Biosystems Inc., Wilmington, USA) and the appropriate volume of water was added to a 35  $\mu$ L total reaction volume. Quantitative real-time PCR were performed on three technical replicates, at 10  $\mu$ L 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  $^{\circ}$ C for 10 s and 63  $^{\circ}$ 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.

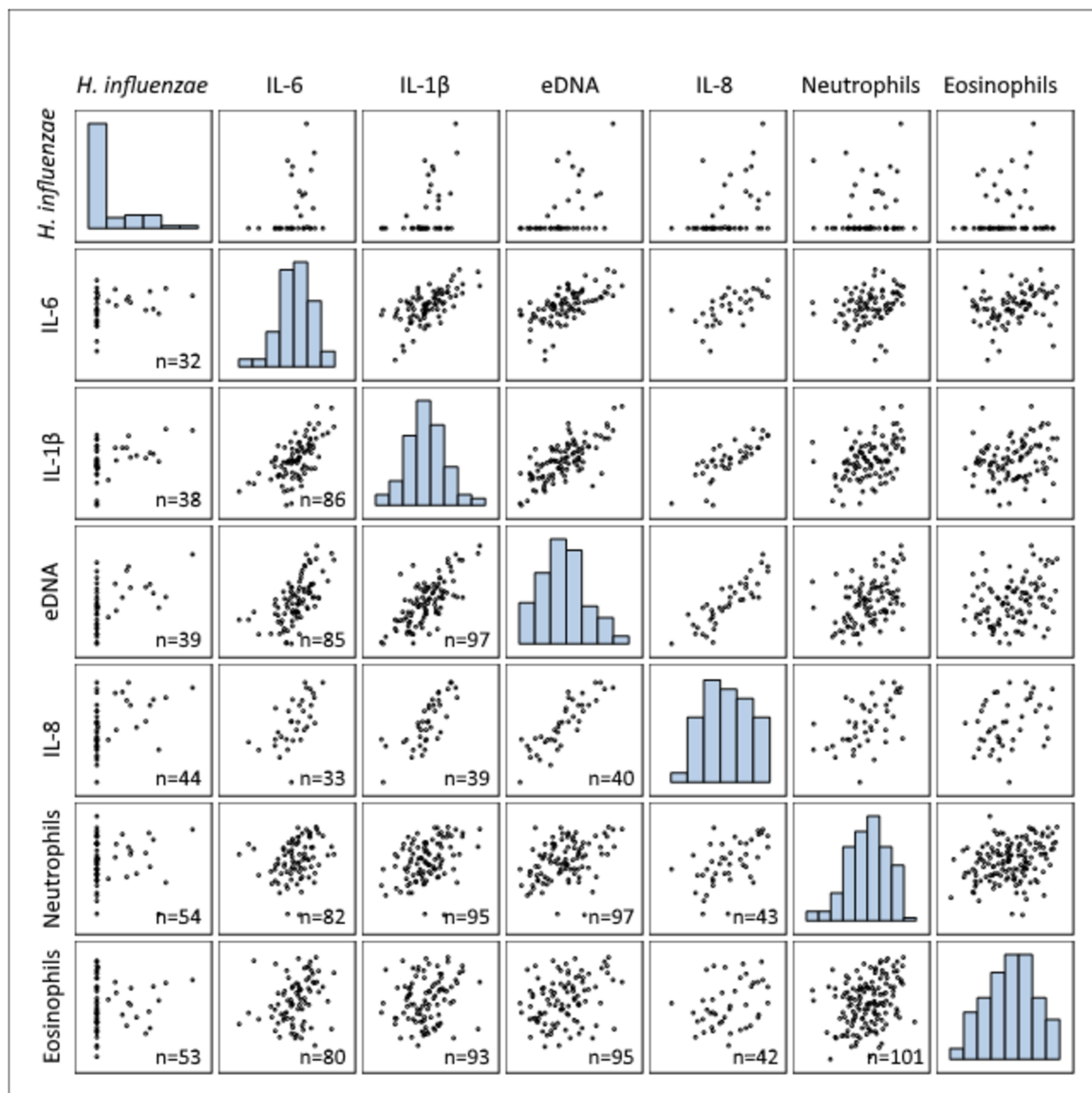


**Supplementary Figure 1:** Scatter plot matrix of inflammatory mediators and *Haemophilus influenzae* in all participants. X-axis from left to right and y-axis from top to bottom display  $\log_{10}$  values of *H. influenzae* (copies/mL), IL-6 (pg/mL), IL-1 $\beta$  (pg/mL), extracellular DNA (ng/mL), IL-8 (pg/mL), neutrophils (count  $\times 10^4$ /mL), and eosinophils (count  $\times 10^4$ /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-1 $\beta$  (pg/mL), extracellular DNA (ng/mL), IL-8 (pg/mL), neutrophils (count  $\times 10^4$ /mL), and eosinophils (count  $\times 10^4$ /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-1 $\beta$  (pg/mL), extracellular DNA (ng/mL), IL-8 (pg/mL), neutrophils (count  $\times 10^4$ /mL), and eosinophils (count  $\times 10^4$ /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)	<b>0.007</b>	AZM: 11 Placebo: 14	-2.142 (-3.352 to -0.932)	<b>0.001</b>	AZM: 12 Placebo: 16	0.017 (-0.940 to 0.975)	0.971
$\log_{10}(\text{IL-6})$	AZM: 91 Placebo: 86	-0.234 (-0.416 to -0.052)	<b>0.012</b>	AZM: 47 Placebo: 41	-0.235 (-0.446 to -0.023)	<b>0.029</b>	AZM: 39 Placebo: 43	-0.292 (-0.57 to -0.015)	<b>0.039</b>
$\log_{10}(\text{IL-1}\beta)$	AZM: 108 Placebo: 100	-0.268 (-0.423 to -0.114)	<b>0.001</b>	AZM: 57 Placebo: 47	-0.393 (-0.627 to -0.158)	<b>0.001</b>	AZM: 44 Placebo: 50	-0.130 (-0.341 to 0.081)	0.227
$\log_{10}(\text{eDNA})$	AZM: 103 Placebo: 98	-0.139 (-0.229 to -0.049)	<b>0.002</b>	AZM: 54 Placebo: 45	-0.215 (-0.351 to -0.080)	<b>0.002</b>	AZM: 42 Placebo: 50	-0.075 (-0.198 to 0.048)	0.234
$\log_{10}(\text{Neutrophils})$	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
$\log_{10}(\text{Eosinophils})$	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  $\geq 3\%$ .

## References

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2. Fu JJ, McDonald VM, Gibson PG, Simpson JL. Systemic Inflammation in Older Adults With Asthma-COPD Overlap Syndrome. *Allergy Asthma Immunol Res*. 2014;6(4):316-324.
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4. Wright TK, Gibson PG, Simpson JL, McDonald VM, Wood LG, Baines KJ. Neutrophil extracellular traps are associated with inflammation in chronic airway disease. *Respirology*. 2016;21(3):467-475.
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