Supplemental Appendix:

Azithromycin for Prevention of Exacerbations in Severe Asthma:

the AZISAST study

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Methods

Study patients

Inclusion criteria encompassed the following criteria: 18-75 years of age; a diagnosis of persistent asthma for at least 1 year duration; a history consistent with Global Initiative for Asthma (GINA) step 4 or 5 clinical features; patients receiving high doses of inhaled corticosteroids (≥1000 µg fluticasone or equivalent) plus inhaled long-acting beta2-agonists for at least 6 months prior to screening; patients suffering multiple (at least two) independent, severe asthma exacerbations requiring treatment with systemic corticosteroids and/or LRTI requiring treatment with antibiotics, within the previous 12 months; never-smokers or ex-smokers with a smoking history of ≤ 10 pack-years; and a fractional excretion of exhaled nitric oxide (FeNO) level below the upper limit of normal according to gender, atopic status and smoking history.[1] Subjects with FeNO levels above the upper limit of normal were excluded, since high FeNO levels in symptomatic patients with asthma have been associated with eosinophilic airway inflammation due to persistent allergen exposure or poor adherence to inhaled corticosteroids.[2] All subjects were followed by respiratory physicians, checking and optimizing patient's inhalation technique prior to enrollment into the study. All eligible patients had to have a diagnosis of persistent asthma, according to the Global Initiative for Asthma (GINA) guidelines, implicating the presence of variable airflow obstruction as evidenced by (1) spirometry with acute reversibility testing (pre- and post-bronchodilator FEV_1), (2) bronchial provocation testing (positive histamine or methacholine challenge test) or (3) peak flow variability. The asthma patients had to fulfill at least one of these criteria of variable airflow obstruction in their medical history.

Exclusion criteria were a prolonged corrected QT interval, severe bronchiectasis, significant medical conditions or significant laboratory abnormalities that might interfere with the study conduct or patient's safety, pregnancy or breastfeeding, prohibited concomitant medication including anti-IgE treatment and treatment with macrolide antibiotics within the last three months.

Patients continued maintenance treatment with high doses inhaled corticosteroids (≥1000 µg fluticasone or equivalent) and long-acting beta2-agonists during the trial. Salbutamol 100 µg per puff was provided as rescue medication. In patients under chronic maintenance treatment with oral corticosteroids, the dose was kept stable until visit 3 (after 4 weeks of study drug treatment) and could then be tapered to the lowest possible dose, at the discretion of the investigator.

Study design and oversight

The AZIthromycin in Severe ASThma (AZISAST) study was a randomized, double-blind, placebo-controlled, parallel-group, multicenter study (see supplementary appendix Figure S1). After a 2 week run-in period, patients were randomly assigned, in a 1:1 ratio, to receive add-on therapy with either azithromycin or placebo using a central web-based randomisation tool, available at a secured study website to the central study staff. Patients who developed a severe asthma exacerbation or lower respiratory tract infection during the run-in period were to be randomized 6 weeks after recovery from the infection or exacerbation.

The hospital pharmacist at the site of the principal investigator formulated the study drugs: capsules with either 250 mg of azithromycin (prepared from capsules of Zitromax®) or placebo, indistinguishable without chemical analysis. After randomisation, the patients took one capsule per day during 5 days and then one capsule three times a week. Total treatment period was 26 weeks (until Visit 6), with a study-drug-free follow-up period of 4 weeks (Visit 7).

The AZISAST study was an academic clinical trial, without sponsorship from the pharmaceutical industry. The study has been funded by the Agency for Innovation by Science and Technology (IWT 70709), Flanders, Belgium. The principal investigator was the main author of the manuscript; all the authors reviewed the drafts and approved the final text for publication. All authors vouch for the accuracy of the reported data and the fidelity to the study protocol.

The study protocol was approved by the central ethics committee at the site of the principal investigator (Ghent University Hospital, Ghent, Belgium), and was reviewed by the local ethics committees at each participating site. All patients provided written informed consent.

Assessments

At screening, demographic information was recorded, as well as medical history, concomitant medication and an extensive asthma history, including information about atopy and comorbidities. An electrocardiography (ECG), a chest X-ray, a high resolution CT-scan of the chest and blood samples were taken to check exclusion criteria before randomisation.

At each visit, vital signs were measured and a physical examination was performed. Lab tests were repeated at visit 3, 4 and 6 (at week 4, 10 and 26 of the treatment period, respectively; Supplemental Figure S1). Adverse events were assessed at each visit.

Lung function

Assessments included pulmonary function tests, encompassing pre- and postbronchodilator spirometry (performed at each visit), and lung volumes and diffusing capacity of the lung (DLCO) (performed at the start and end of the treatment period). Spirometry was performed according to ATS / ERS task force on Standardisation of Lung Function Testing.[3] Before each spirometry, a FeNO measurement was performed with a Niox or Niox Mino analyser according to the ATS/ERS recommendations.[4]

Patient Diary

Patients kept a diary and recorded the following items during the 2 weeks preceding each study visit: Peak Expiratory Flow (PEF) in the morning and evening (highest of 3 values recorded), number of occasions of inhalation of rescue medication, day and night symptoms. Subjects also reported information about medical consumption related to asthma in their diary.

Questionnaires

The Asthma Control Questionnaire (ACQ) and the Asthma Quality of Life Questionnaire (AQLQ) had to be completed by the patient at visit 2, 4 and 6 (at randomisation, at week 10 and 26 of the treatment period; see Supplemental Figure S1). The EuroQol 5D questionnaire (EQ-5D), a standardised instrument as a measure of health outcome, was completed at visit 2 and visit 6.

Serology

Serology for *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae* were determined at enrollment and at the end of the treatment phase. Separate blood samples taken at screening (visit 1) and at the end of the treatment period (visit 6) were sent to the central laboratory at Ghent University Hospital to determine IgG and IgM antibodies against *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae* by SeroCP™ Recombinant IgG, SeroCP™ Recombinant IgM, SeroMP™ Recombinant IgG and SeroMP™ Recombinant IgM respectively, all from Savyon Diagnostics (St. Ashdod, Israel). All serological tests were

performed according to the manufacturer's instructions on the BEPIII Behring ELISA processor (Siemens, Munich, Germany).

Bacteriological substudy

In two centers (Ghent University Hospital and OLV Hospital Aalst), we performed a bacteriological sub-study to determine macrolide resistance in streptococci with oro-pharyngeal swabs [5], taken at four different visits: visit 2, 3, 6 and 7 (before randomisation, after 4 and 26 weeks of study drug treatment and at the final visit, after a washout period of 4 weeks, respectively; see Supplemental Figure S1).

Statistical analysis

Primary efficacy outcome analysis

Mean primary endpoint rates and mean exacerbation rates per treatment group were investigated using Poisson or negative binomial regression as appropriate.[6] When subjects withdrew from the study, their number of primary endpoints (exacerbations and LRTI requiring treatment with antibiotics) was adjusted as follows: recorded number of observations + (days remaining/total study period in days) x mean primary endpoint frequency in the study group. As a supporting analysis, and to alternatively account for varying lengths of treatment for patients who dropped out from the trial prematurely, primary endpoint rates and exacerbation rates were calculated for each patient separately and compared between treatment groups using the Wilcoxon rank-sum test.

Sample size calculation

To have a power of 0.80 (the maximum likelihood of making a type II [false-negative] error being $\beta = 20\%$) and to have a level of statistical significance of 0.05 (two-sided testing with $\alpha = 0.05$), and taking into account an estimated standardized effect size of 0.22 on the primary outcome (the rate of primary endpoints), a sample size of 54 evaluable patients per treatment arm was required. For both groups together, 108 evaluable patients were thus required. Estimating the drop out rate at $\pm$ 10%, approximately 120 patients with severe asthma needed to be randomized in the AZISAST study.

Results

Predefined subgroup analysis of response to macrolide treatment according to severe asthma phenotype

Since severe asthma is biologically heterogenous, and since macrolides have anti-inflammatory effects in noneosinophilic (neutrophilic) chronic airway diseases, we performed a predefined subgroup analysis comparing the efficacy of azithromycin versus placebo depending on the presence or absence of blood eosinophilia at baseline. In subjects with severe asthma and blood eosinophilia ≤ 200/µL (i.e. noneosinophilic severe asthma), azithromycin significantly reduced the rate of primary endpoints compared to placebo (Figure 2C). Based on a Poisson regression model, the estimated primary endpoint rate for non-eosinophilic severe asthma was 0.44 (95% CI 0.25 to 0.78) in the azithromycin group and 1.03 (95% CI 0.72 to 1.48) in the placebo group (P=0.013). In contrast, the primary endpoint rate for eosinophilic severe asthma was 0.96 (95% CI 0.66 to 1.41) in the azithromycin group compared to 0.50 (95% CI 0.28 to 0.88) in the placebo group (P=0.058). Importantly, in the Poisson regression model, there is a statistical significant interaction between the (non)eosinophilic phenotype of severe asthma and the treatment arm (P=0.002).

In subjects with noneosinophilic asthma, azithromycin also significantly decreased the number of patients with at least one primary endpoint (9 out of 27 [33%] of azithromycin-treated subjects, compared with 18 out of 29 [62%] of placebo-treated subjects; relative risk: 0.54, 95% CI, 0.29 to 0.98, P=0.037). In contrast, there was a trend towards a higher percentage of subjects experiencing at least one primary endpoint in patients with severe asthma and blood eosinophilia > 200/µL (relative risk: 1.67, 95% CI, 0.98 to 2.83, P=0.058).

Predefined subgroup analysis of response to macrolide treatment according to *Chlamydophila pneumoniae* serology

Since *Chlamydophila pneumoniae* has been associated with severe asthma and accelerated progression of disease, and since macrolides have antibiotic effects towards atypical bacteria, we performed a predefined subgroup analysis comparing the efficacy of azithromycin versus placebo depending on the presence or absence of *C. pneumoniae* immunoglobulin G (IgG) in serum at baseline. A positive *C. pneumoniae* serology, demonstrated in 64% of the ITT population, did not affect the therapeutic response to azithromycin or placebo, as compared with subjects with a negative *C. pneumoniae* serology at baseline (see Supplemental Figure S2). During the 26-weeks treatment phase of the study, one acute *C. pneumoniae* infection and two acute *M. pneumoniae* infections were

demonstrated by IgG seroconversion (as recommended by the IDSA/ATS consensus guidelines).[7]

Tapering of oral corticosteroids during the 26-week treatment phase

At randomization, twelve subjects (nine in the azithromycin group and three in the placebo group) were corticodependent, receiving chronic maintenance treatment with oral corticosteroids at a median dose of 10 mg prednisolone per day. In these patients, the dose was kept stable until visit 3 (after 4 weeks of study drug treatment) and could then be tapered to the lowest possible dose, at the discretion of the investigator. At the end of the treatment phase, the median dose of prednisolone had been reduced to 5 mg per day in the azithromycin arm, whereas the median dose remained 10 mg per day in the placebo arm. In two subjects of the azithromycin group, oral corticosteroid treatment could be stopped.

Safety

Supplemental Appendix Table S1: Adverse events

	Placebo (n=54)	Azithromycin (n=55)	
Adverse events *			
- Subjects (%)	39 (72%)	37 (67%)	0.678
- asthma exacerbation	41	40	
- LRT infection	34	24	
- diarrhoea	8	3	
- nausea	2	1	
- abdominal pain	8	2	
- vertigo	1	2	
- headache	3	4	
- allergic reactions	1	2	
- elevated liver function tests	0	2	
- thrombocytopenia	0	1	
- leucopenia	0	1	
- other	7	6	
- Total adverse events	105	88	
Serious adverse events			
- Subjects (%)	6(11%)	6 (11%)	1.000
- # SAE :			
0	48 (89%)	49 (89%)	
1	6 (11%)	5 (9%)	
2	0 (0%)	1 (2%)	
Drug related adverse events			
- Subjects (%)	3 (6%)	4 (7%)	1.000
- # Drug related adverse event :			
0	51 (94%)	51 (93%)	
1	2 (4%)	2 (4%)	
2	1 (2%)	2 (4%)	
Discontinuation	5 (9%)	2 (4%)	0.271
Discontinuation due to adverse event	2 (4%)	1 (2%)	0.618

Abbreviations: LRT: lower respiratory tract; SAE: serious adverse event.
* all adverse events till end of study.

Discussion

Similarities between severe asthma and bronchiolitis obliterans syndrome concerning response to treatment with macrolides (azithromycin)

The differential effect of azithromycin in severe asthma according to the asthma phenotype (noneosinophilic versus eosinophilic asthma) is reminiscent of the effect of azithromycin in chronic rejection or bronchiolitis obliterans syndrome (BOS) after lung transplantation.[8] In observational studies, we have revealed a dichotomy in the pathogenesis and clinical phenotype of BOS, encompassing a neutrophilic reversible allograft dysfunction, responding to azithromycin, and a fibroproliferative BOS, not responding to azithromycin.[8] Azithromycin significantly reduced airway interleukin-8 and neutrophilia in patients with BOS.[9] Importantly, in a double-blind randomized placebo-controlled trial, we have shown that chronic treatment with low-dose azithromycin reduced the prevalence of BOS after lung transplantation.[10]

Mechanisms of action of macrolides in non-eosinophilic severe asthma

The beneficial effects of azithromycin in noneosinohilic severe asthma might be due to antibiotic properties or anti-inflammatory and immunomodulatory effects. Several observations favour the antimicrobial activities of macrolides as mechanism of action: recurrent respiratory infections are associated with frequent exacerbations in adults with difficult-to-treat asthma [11]; severe asthma has been shown to be an independent risk factor for invasive pneumococcal disease [12]; and the microbiome of the lower airways and lungs is altered in patients with asthma compared to healthy controls.[13] Chronic respiratory infection with atypical bacteria such as *Mycoplasma pneumoniae* or *Chlamydophila pneumoniae* might play a role in the pathogenesis of severe asthma.[14] A trial of roxithromycin in subjects with asthma and serological evidence of infection with *C. pneumoniae* did not lead to sustained improvements of asthma control [15], which is in line with our observations that positive IgG antibodies to *C. pneumoniae* did not predict therapeutic efficacy of azithromycin in severe asthma. Although application of polymerase chain reaction (PCR) on bronchoalveolar lavage fluid or endobronchial biopsies is considered the gold standard to differentiate between subjects with true chronic *C. pneumoniae* infection and those previously exposed but not

9

currently infected [16], only 12 out of 92 patients in the Asthma Clinical Research Network trial with clarithromycin had PCR evidence of infection.[17]

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