Effect of Azithromycin on Systemic Markers of Inflammation in Patients With Cystic Fibrosis Uninfected With *Pseudomonas aeruginosa*

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Background: While the mechanism of action by which azithromycin exerts positive effects in patients with cystic fibrosis remains unclear, evidence suggests that azithromycin may act as an immunomodulatory agent. We examined changes in systemic inflammatory markers in a double-blind, randomized, controlled trial of oral azithromycin in patients 6-18 years of age with cystic fibrosis who were uninfected with *Pseudomonas aeruginosa*.

Methods: WBC counts and differential, serum myeloperoxidase (MPO), high-sensitivity C reactive protein (hsCRP), intracellular adhesion molecule 1, IL-6, calprotectin, serum amyloid A (SAA), and granulocyte colony-stimulating factor (G-CSF) were measured at baseline and after 28 and 168 days of treatment in patients receiving either oral azithromycin or placebo.

Results: Inflammatory markers were similar in both groups at baseline. HsCRP, MPO, SAA, calprotectin, and the absolute neutrophil count (ANC) significantly decreased from baseline to day 28 in the azithromycin group compared with the placebo group ($P < .05$). This treatment effect was sustained at day 168 for ANC, calprotectin, and SAA ($P < .05$). Changes in hsCRP, calprotectin, and SAA at day 28 were negatively correlated with changes in FEV₁ (L) and FEV₁ (% predicted), as well as both absolute and relative changes in weight ($P < .05$). Except for weight (%), the associations remained significant for calprotectin; FEV₁(L) and weight (%) remained significantly correlated with the 168-day change in hsCRP. The 168-day change in ANC was significantly correlated with changes in lung function, but not in weight; the change in G-CSF was significantly correlated with the change in weight (%) only.

Conclusions: In patients not infected with *P aeruginosa*, oral azithromycin significantly reduced neutrophil counts and serum inflammatory markers within 28 days of initiating treatment.

Trial registry: ClinicalTrials.gov; No.: NCT00431964; URL: www.clinicaltrials.gov

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Abbreviations: ANC = absolute neutrophil count; CF = cystic fibrosis; G-CSF = granulocyte colony-stimulating factor; hsCRP = high-sensitivity C reactive protein; ICAM = intercellular adhesion molecule; LOD = limit of detection; MPO = myeloperoxidase; SAA = serum amyloid

Cystic fibrosis (CF) is characterized by chronic airway infection and an excessive inflammatory response.¹ Current treatment strategies include antibiotic therapy for intercurrent exacerbations and maintenance therapy with inhaled antibiotics for patients chronically infected with *Pseudomonas aeruginosa*.² Azithromycin, a macrolide antibiotic, reduces pulmonary exacerbations and improves lung function in patients with CF, but its mechanism of action is still unclear.³⁻⁶ Modulation of oxidant production by

neutrophils and of proinflammatory cytokine synthesis and release by leukocytes are the two main effects observed in vitro.⁴⁻⁶ Macrolides accumulate within neutrophils and macrophages, and intracellular levels are markedly higher than extracellular levels.⁷ Macrolides reduce proinflammatory cytokine production in vitro by neutrophils, monocytes, and bronchial epithelial cells presumptively via the ERK1/2 pathway.⁸⁻¹² Macrolides may also reduce neutrophil accumulation by inhibiting intercellular adhesion molecule

(ICAM)-1 on epithelial cell surfaces.¹³ In addition, macrolides could exert an antibiotic effect by reducing bacterial burden in the airways, which could indirectly reduce inflammation.

Multiple markers and techniques have been used to quantify airway inflammation in patients with CF. BAL is considered the gold standard, but its use in longitudinal studies is limited by the invasiveness of the procedure.¹⁴ Sputum is an alternative way of assessing material directly derived from the area of inflammatory activity, but both spontaneously expectorated- and induced-sputum parameters are highly variable, making inflammation difficult to track in patients over time.¹⁵ There has been a renewed interest in assessing systemic markers of inflammation.^{16,17} An important question is whether systemic biomarkers are sensitive enough to detect a meaningful change in lung disease, given that the inflammatory response to infection in CF is largely confined to the lung. Here, we studied a pediatric CF population with relatively mild disease and uninfected with *P aeruginosa*, based on baseline pulmonary function, who participated in an intervention trial of oral azithromycin to assess the changes in circulating markers of inflammation during the 6-month study period.

MATERIALS AND METHODS

Details of the AZ0004 study have been described elsewhere.¹⁸ The trial was conducted at 31 Cystic Fibrosis Foundation-accredited care centers in the United States and at nine Canadian Cystic Fibrosis Foundation-accredited care centers coordinated by the CF Therapeutics Development Network Coordinating Center located in Seattle, Washington. Respective institutional review boards and ethics committees at each center approved the study and each participant and/or their parent voluntarily consented/assented

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to participate in the trial (Hospital for Sick Children Research Ethics Board file number: 1000009573).

Eligibility criteria included a documented diagnosis of CF, age 6-18 years, weight ≥ 18 kg, FEV₁ $\geq 50\%$ predicted,¹⁹ and respiratory cultures negative for *P aeruginosa* for ≥ 1 year. Exclusion criteria included the use of antibiotics, "high-dose" systemic steroids (defined as ≥ 1 mg/kg if participant's weight < 20 kg or ≥ 20 mg/d if participant's weight ≥ 20 kg) within 14 days of screening; initiation of dornase alfa, ibuprofen, aerosolized antibiotics, or hypertonic saline within 30 days of screening; a positive respiratory culture for *Burkholderia cepacia* complex or nontuberculous mycobacteria; or any of the following laboratory abnormalities: γ -glutamyltransferase phosphate, aspartate serum transferase, or alanine transferase levels ≥ 2 times the upper limit of normal; creatinine level > 1.5 times the upper limit of normal for age; or absolute neutrophil count (ANC) $\leq 1,000$.

Participants were randomized one-to-one to azithromycin or placebo, as outlined elsewhere.¹⁸ Clinical evaluations, physical examination, and spirometry were performed at days 0 (randomization), 28, 84, and 168. Blood samples to monitor complete and differential blood counts, liver function, and creatinine were obtained at screening and days 28 and 168. Serum and plasma samples were obtained at these time points, frozen at -80°C , and shipped to the core laboratory in Denver, Colorado, for analysis of inflammatory markers as detailed here. Data reported in this study are based on analyses of these samples.

Plasma and serum aliquots (depending on the analyte) were analyzed in the Cystic Fibrosis Foundation Mediator Core Laboratory in Aurora, Colorado, using validated assays for the following protein biomarkers: serum high-sensitivity C reactive protein (hsCRP) via immunonephelometry (Siemens Healthcare Diagnostics); plasma calprotectin via enzyme-linked immunosorbent assay (ALPCO Diagnostics); serum myeloperoxidase (MPO) via enzyme-linked immunosorbent assay (CardioMPO, Cleveland HeartLab, Inc); serum amyloid A (SAA); serum granulocyte colony-stimulating factor (G-CSF), ICAM-1, and IL-6 (Luminex platform, Milliplex; EMD Millipore Corp). All assays were performed in duplicate, and mean values were used for analysis.¹⁵⁻¹⁷

Statistical Analysis

Due to the skewed distribution of the inflammatory markers, logarithmically transformed values were used. Values below the limit of detection (LOD) were assigned a value of one-half the lower LOD, and values above the upper LOD were assigned the upper LOD value. Means and corresponding 95% CIs were summarized and graphically displayed at each visit and for changes over time. Two-sample *t* tests evaluated at a two-sided .05 level of significance were used to test for differences between treatment groups in the 28- and 168-day change from baseline in each inflammatory marker.

Associations between baseline measurements of inflammatory markers and changes in weight (kg and percentile) and lung function (FEV₁ L and % predicted) over the 168-day study were evaluated by Spearman correlation test with corresponding 95% CIs. Associations between the 28- and 168-day changes in inflammatory markers and the 28- and 168-day changes in clinical parameters during the course of the trial were similarly summarized. Proportions of subjects with inflammatory markers above the upper limit of the normal range were summarized by treatment group and study visit. Analyses were performed using R statistical package version 2.14.0 (R Foundation for Statistical Computing).

RESULTS

Of the 324 participants screened for the AZ0004 study, 260 (80%) were randomized and treated; 131

were randomized to azithromycin and 129 to placebo.¹⁵ Of the 260 participants, all had inflammatory marker laboratory results for MPO, hsCRP, ICAM-1, IL-6, G-CSF, calprotectin, SAA, and ANC, but a small fraction of test results could not be used due to hemolysis. Baseline characteristics were similar between treatment groups, including baseline weight (kg and percentile), lung function (FEV₁ L and % predicted), and inflammatory markers (Table 1).¹⁸

Concentrations for ICAM-1 and IL-6 were below the detection limit of the respective assays in the majority of participants and did not demonstrate any significant differences between treatment groups at the time points measured (data not shown). All other inflammatory markers were detectable in > 95% of participants. The 28- and 168-day changes in inflammatory markers from baseline are shown by treatment group in Table 1. Compared with placebo, the azithromycin group experienced a greater decline from baseline at day 28 in all inflammatory markers ($P < .05$) except G-CSF ($P = .08$). This treatment effect was sustained at day 168 in ANC, calprotectin, and SAA ($P < .05$) (Fig 1).

The percentage of patients with measurements exceeding the upper limit of normal range is shown by visit in Table 2. Twenty-four of the 125 azithromycin group participants (19%) had calprotectin values

above the normal range at baseline and only 12 of 118 azithromycin group participants (10%) had values above the normal range at day 28, but these proportions were not significantly different. A similar reduction in calprotectin levels was not seen in placebo participants. Similar trends were observed in SAA, hsCRP, and ANC.

Associations between baseline measurements of the inflammatory markers and 28- and 168-day changes in lung function and weight are shown in Fig 2. Except for ANC, which was negatively correlated with 168-day changes in weight (kg), no significant correlations were observed in this group of children with CF who had preserved lung function (mean [SD] baseline FEV₁ 97.7 [16.40] for the azithromycin group and 99.6 [13.65] for the placebo group).

Associations between 28-day changes in the measurements of inflammatory markers and 28-day changes in weight and lung function are shown in Fig 3A. Changes in hsCRP, calprotectin, and SAA at day 28 were negatively correlated with changes in FEV₁ (L) and FEV₁ (% predicted), as well as both absolute and relative changes in weight ($P < .05$). In contrast, no significant associations were observed for MPO, G-CSF, and ANC.

Associations between 168-day changes in the measurements of inflammatory markers and 168-day changes in weight and lung function are shown in

Table 1—Baseline Concentrations and Changes in Inflammatory Markers in Patients Receiving Azithromycin or Placebo Over the 168-d Study Period

Variable	Azithromycin Group	Placebo Group A	Treatment Effect (95% CI)	P Value
Absolute neutrophil counts ^a				
Baseline	0.64 (0.19)	0.63 (0.18)	...	...
Day 28-baseline	−0.10 (0.22)	0.01 (0.20)	−0.10 (−0.15 to −0.05)	.0002
Day 168-baseline	−0.05 (0.23)	0.01 (0.20)	−0.06 (−0.12 to −0.01)	.0233
MPO, ^a pmol/L				
Baseline	2.87 (0.33)	2.82 (0.27)	...	...
Day 28-baseline	−0.10 (0.28)	0.03 (0.32)	−0.13 (−0.20 to −0.05)	.0015
Day 168-baseline	−0.03 (0.30)	0.04 (0.27)	−0.07 (−0.14 to 0.00)	.0601
hsCRP, ^a mg/L				
Baseline	−0.42 (0.73)	−0.47 (0.67)	...	...
Day 28-baseline	−0.24 (0.67)	0.02 (0.70)	−0.26 (−0.44 to −0.09)	.0033
Day 168-baseline	−0.08 (0.61)	0.04 (0.73)	−0.12 (−0.29 to 0.05)	.1779
Calprotectin, ^a μg/mL				
Baseline	0.35 (0.33)	0.33 (0.31)	...	...
Day 28-baseline	−0.16 (0.35)	0.01 (0.37)	−0.17 (−0.27 to −0.08)	.0003
Day 168-baseline	−0.06 (0.31)	0.03 (0.37)	−0.09 (−0.18 to 0.00)	.0455
G-CSF, ^a pg/mL				
Baseline	1.61 (0.37)	1.67 (0.38)	...	...
Day 28-baseline	−0.09 (0.36)	−0.01 (0.37)	−0.08 (−0.18 to 0.01)	.0777
Day 168-baseline	−0.09 (0.33)	−0.01 (0.39)	−0.07 (−0.17 to 0.02)	.1178
Serum amyloid A, ^a ng/mL				
Baseline	3.70 (0.75)	3.74 (0.66)	...	...
Day 28-baseline	−0.24 (0.81)	0.01 (0.68)	−0.25 (−0.44 to −0.06)	.0112
Day 168-baseline	−0.10 (0.68)	0.09 (0.73)	−0.18 (−0.36 to 0.00)	.0495

Data given as mean (SD) unless otherwise indicated. G-CSF = granulocyte colony-stimulating factor; hsCRP = high-sensitivity C reactive protein; MPO = myeloperoxidase.

^aData are log transformed.

FIGURE 1. Changes in absolute neutrophil counts, serum amyloid A, and calprotectin during the 168-d study period. A, Absolute neutrophil counts. B, Calprotectin. C, Serum amyloid A. Absolute neutrophil counts, serum amyloid A, and calprotectin decreased significantly from baseline in the azithromycin group after 28 d, while no significant changes were observed in the placebo group. The asterisk shows significant differences between the azithromycin and the placebo group. Similar trends were observed at day 168, but the differences were less pronounced and reached the level of statistical significance only for calprotectin and serum amyloid A.

Fig 3B. Except for weight (%), the associations remained significant for calprotectin; FEV₁(L) and weight (%) remained significantly correlated with 168-day change in hsCRP. The 168-day change in ANC was significantly correlated with changes in lung function, but not in weight; the change in G-CSF was significantly correlated with the change in weight (%) only.

DISCUSSION

To the best of our knowledge, this is the first large study to assess the utility of a panel of systemic inflammatory markers in an interventional trial of patients with CF who have preserved lung function. Patients treated with azithromycin exhibited significant reductions in inflammatory markers after 4 weeks that persisted (albeit to a lesser degree) to week 24. Reduction in inflammatory markers were correlated to

improvements in lung function and weight gain, providing indirect evidence that these changes were associated with clinically meaningful outcomes. These associations of changes in inflammatory markers with changes in lung function were seen despite the findings of the overall trial, which did not show a significant effect of azithromycin on lung function, but did show significant weight gain in the azithromycin group.¹⁸

Azithromycin has demonstrated clinical benefits in both children and adults with CF,^{3,20-25} but its mechanism of action is unclear. Potential beneficial effects include antimicrobial effects, effect on sputum rheology, and direct effects on airway inflammation. Multiple in vitro studies have demonstrated that azithromycin can reduce production of proinflammatory cytokines and clinical effects could be observed in patients with bronchiolitis obliterans who do not usually have chronic bacterial infection.²⁶⁻²⁸ In the current trial, no changes were observed in microbiology except for an increase in *Staphylococcus aureus* and *Haemophilus influenzae* resistance to macrolides, common CF pathogens were not eradicated by azithromycin, and placebo participants did not have increased acquisition of CF pathogens. However, since most of the patients were unable to produce sputum, these data are based on qualitative, rather than quantitative, airway cultures. While this does not fully exclude the possibility that azithromycin had antimicrobial effects, these findings make it more likely that the observed effects are driven by the impact of azithromycin on airway inflammation, as suggested in previous studies.²⁰⁻²⁵

Azithromycin has been associated with reductions in CRP concentrations that were not observed in untreated patients.^{20,23,25} While patients enrolled in these previous trials had more significant lung disease compared with participants in the current study, reductions in CRP were also observed in patients with relatively mild disease. Interestingly, the effects on inflammatory markers were found to be more pronounced after 28 days compared with 168 days of treatment. This may suggest that treatment efficacy of azithromycin may decrease over time and raises the question of whether long-term continuous therapy, rather than intermittent therapy, is the optimal way to treat patients with CF. However, a study comparing clinical, rather than surrogate, outcomes for both treatment approaches would be required to test this hypothesis.

Systemic inflammatory markers in CF have largely been studied to diagnose and track patients during pulmonary exacerbations. These studies have shown that CRP is elevated in only a subgroup of patients, but previous studies may be limited by the use of CRP rather than hsCRP as an outcome parameter.²⁹⁻³² Similarly, studies in patients with pulmonary exacerbations

Table 2—Absolute Number and Percentage of Tests Above Upper Limit of Normal Range

Variable	Baseline		Day 28		Day 168	
	Azithromycin	Placebo	Azithromycin	Placebo	Azithromycin	Placebo
ANC	11/131 (8.4)	8/128 (6.3)	3/121 (2.5)	8/125 (6.4)	11/123 (8.9)	14/122 (11.5)
MPO	38/126 (30.2)	29/125 (23.2)	23/122 (18.9)	30/125 (24.0)	34/123 (27.6)	23/118 (28.3)
hsCRP	16/126 (12.7)	9/126 (7.1)	7/119 (5.9)	12/125 (9.6)	11/123 (8.9)	... 70/120
G-CSF	67/126 (53.2)	77/125 (61.6)	53/121 (43.8)	68/125 (54.4)	52/122 (42.6)	... (58.3)
Calprotectin	24/125 (19.2)	24/126 (19.0)	12/118 (10.2)	23/125 (18.4)	19/123 (15.4)	25/118 (21.2)
Serum amyloid A	32/126 (25.4)	29/124 (23.4)	18/121 (14.9)	30/125 (24.0)	30/123 (24.4)	39/120 (32.5)

All data given as No. patients/total No. for whom the inflammatory marker measurement was made (%). ANC = absolute neutrophil count. See Table 1 legend for expansion of other abbreviations.

have suggested that SAA and calprotectin may be useful markers to track inflammation in CF.^{33,34} Interestingly, the current study demonstrates that a significant proportion of clinically stable patients with preserved lung function have inflammation that can be measured systemically, which supports BAL studies demonstrating persistent neutrophilic inflammation in clinically stable patients with CF.^{35,36}

To our knowledge, only one study has explored the utility of calprotectin as an inflammatory marker and demonstrated a correlation between changes in calprotectin and improvements in FEV₁ during pulmonary exacerbations.³⁴ This finding is supported by the current data, which show that calprotectin concentrations were not only correlated with baseline weight and lung function, but that reductions in calprotectin levels over time also correlated with improvements in weight and FEV₁ after both 4 and 24 weeks of azithromycin therapy. This suggests that calprotectin may be superior to other inflammatory markers for tracking inflammation over time in patients with CF and that calprotectin could potentially be used as a marker of inflammation in interventional studies. While the functional role of calprotectin in CF is not well defined, calprotectin is produced by neutrophils and plasma levels are elevated during infection and inflammation.³⁷ Calprotectin has antimicrobial effects; its mechanism of action is proposed to be that of a chelator of manganese and zinc ions, as it inhibits growth of *S aureus* in vitro and in mice models of abscesses.³⁸ Calprotectin also has antifungal activity, most notably as the major component of neutrophil extracellular traps.³⁹ Calprotectin is also a ligand for toll-like receptor 4, can stimulate CD4⁺ cell-driven IL-17 production, and may, therefore, be important for the cross-talk between neutrophilic and lymphocytic

inflammation, which has gained more interest in CF research.⁴⁰⁻⁴²

The current study has a number of important limitations. Systemic markers of inflammation are proxy surrogates of the inflammatory process in the airways and the sensitivity and specificity for these indirect markers is still unclear. While most patients with CF and normal lung function will have ongoing airway inflammation, the majority of patients in this study had baseline measurements within the normal range (Table 2), which likely explains why the difference in proportions for these parameters failed to be significant. This would suggest that the sensitivity of markers obtained in the blood might be lower than that of markers directly obtained from the airways. While BAL studies have demonstrated that neutrophilic inflammation in BAL predicts subsequent lung function decline, similar information is not available from prospective studies for systemic inflammatory markers. In addition, clinically meaningful thresholds for changes in these parameters are yet unknown. Longitudinal data tracking patients over time will be helpful in defining the utility of these markers in the clinic, as well as in clinical trials.

In summary, we demonstrated that azithromycin therapy in patients with preserved lung function who are not infected with *P aeruginosa* is associated with a reduction in systemic markers of inflammation. These effects were most pronounced after 28 days of treatment, but persisted over the 168-day treatment period and were noted for ANC, hsCRP, and calprotectin. Reductions in calprotectin levels over time correlated with improvements in weight and lung function, suggesting that these changes are linked to clinically meaningful outcomes. These data suggest that systemic markers of inflammation have added value and should be included in future CF clinical trials.

FIGURE 2. Forest plot of correlations between baseline measures of inflammation and baseline FEV₁ in L and % predicted, as well as absolute and relative weight change. A, after 28 d of treatment. B, After 168 d of treatment. The mean and 95% CI are shown for the correlations; correlations are significant if the CI does not cross zero. ANC = absolute neutrophil count; G-CSF = granulocyte colony-stimulating factor; hsCRP = high-sensitivity C reactive protein; MPO = myeloperoxidase; SAA = serum amyloid.

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Dr Ratjen: contributed to conception and design of the study; data acquisition, analysis, and interpretation; writing or revising

FIGURE 3. Forest plot of correlations changes in markers of inflammation and FEV₁ in L and % predicted, as well as absolute and relative weight change. A, At day 28. B, At day 168. The mean and 95% CI of the correlations are shown; correlations are significant if the CI does not cross zero. See Figure 2 legend for expansion of abbreviations.

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Dr Anstead: contributed to conception and design of the study; data acquisition, analysis, and interpretation; writing or revising the final manuscript for important intellectual content; and approval of the final manuscript.

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