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Decreased Lymphocytic Bronchitis Severity in the Era of Azithromycin Prophylaxis

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Background. Large-airway lymphocytic inflammation (LB), assessed on endobronchial biopsies, has been associated with acute cellular rejection and chronic lung allograft dysfunction (CLAD). Azithromycin (AZI) prophylaxis has been used to prevent airway inflammation and subsequent CLAD, with inconsistent results. We hypothesized that AZI prophylaxis would be associated with reduced LB, changes in bronchoalveolar lavage (BAL) immune cell populations, and improved CLAD-free survival. **Methods.** We compared frequencies of LB from endobronchial biopsies before (N = 1856) and after (N = 975) protocolized initiation of AZI prophylaxis at our center. LB was classified as none, minimal, mild, or moderate by histopathologic analysis. LB grades were compared using ordinal mixed-model regression. Corresponding automated BAL leukocyte frequencies were compared using mixed-effects modeling. The effect of AZI prophylaxis on CLAD-free survival was assessed by a Cox proportional hazards model adjusted for age, sex, ethnicity, transplant indication, and cytomegalovirus serostatus. **Results.** Biopsies in the pre-AZI era had 2-fold increased odds (95% confidence interval, 1.5-2.7; $P < 0.001$) of higher LB grades. LB was associated with BAL neutrophilia in both eras. However, there was no difference in risk for CLAD or death between AZI eras (hazard ratio 1.3; 95% confidence interval, 0.7-2.0; $P = 0.45$). **Conclusions.** Decreased airway inflammation in the era of AZI prophylaxis may represent a direct effect of AZI therapy or reflect other practices or environmental changes. In this cohort, AZI prophylaxis was not associated with improved CLAD-free survival.

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Chronic lung allograft dysfunction (CLAD) limits long-term survival after lung transplant (LTx).[1] CLAD is a syndrome defined as an irreversible loss of lung function, measured by 1-s forced expiratory volume (FEV_1), in the absence of select alternate explanations, such as airway stenosis or chronic pleural effusion.[2] However, the airway or parenchymal fibrosis associated with CLAD may result from multiple distinct pathologies, including antibody or T cell-mediated rejection.

One proposed therapy for the prevention and treatment of CLAD is azithromycin (AZI). This macrolide antibiotic inhibits the bacteria ribosomal 50S subunit but also has anti-inflammatory characteristics leading to its use in chronic inflammatory lung conditions like Chronic Obstructive Pulmonary Disease, pulmonary graft-versus-host disease after stem cell transplant, and cryptogenic organizing pneumonia.[3] AZI diminishes interleukin (IL)-17 stimulated production of IL-8 in human airway smooth muscle cells, reduces other cytokines, and promotes gut motility, all of which may reduce CLAD development.[4-6]

Multiple case series reported stabilization of lung function after AZI treatment for bronchiolitis obliterans syndrome (BOS). In these recipients, increased neutrophils in bronchoalveolar lavage (BAL) fluid, were associated with recovery following AZI therapy, leading to the distinction between neutrophilic reversible allograft dysfunction and fibroproliferative. An analogous syndrome of lymphocytic bronchitis has been identified as a precursor and risk factor for CLAD, associated with BAL neutrophils, and reversal following AZI treatment.[7-9]

A CLAD prevention study of 83 LTx recipients randomized to AZI or placebo at hospital discharge found decreased BAL neutrophils, improved FEV_1, and better CLAD-free survival in

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the AZI group, even when followed out for 7 y.[10] A separate trial randomized 46 participants with decreased FEV_1 to AZI or placebo and found that those who completed the protocol had an improvement in lung function. However, there was no statistically significant effect in the intention to treat analysis, and long-term follow-up of these recipients showed no difference in lung function or survival between these 2 groups.[11,12]

Based on these findings, several centers implemented standardized AZI prophylaxis. In one center, such prophylaxis was associated with improved overall survival and a trend toward decreased CLAD risk.[13] Thus, we hypothesized that implementation of protocolized AZI prophylaxis would decrease lymphocytic bronchitis and improve CLAD-free survival.

MATERIALS AND METHODS

Study Design, Participants, and Setting

We performed a retrospective, single-center, preintervention, and postintervention observational cohort study. All study participants provided written informed consent and this study was approved by the University of California, San Francisco internal review board (no. 13-10738). All consenting participants transplanted between May 1, 2005, and December 31, 2015, were included. December 31, 2015, was used as the end date for data collection as there was a clinical protocol change where endobronchial biopsies were no longer performed routinely but were limited to cases with clinical suspicion of airway pathology. BAL was collected from the right middle lobe or lingula using up to 5 sequential 20 mL aliquots of saline. We implemented AZI prophylaxis on January 1, 2011, defining the pre-AZI era as May 1, 2005, until December 31, 2010, and the AZI era as January 1, 2011, until December 31, 2015. Median duration of follow-up for this cohort was 2560 d (7 y).

Predictor and Outcome Variables

Participants were retrospectively assigned to "Pre" or "During" AZI prophylaxis eras based on the date of transplant. The AZI era was defined as transplants occurring before or after January 1, 2011. Recipients in the pre-AZI era who survived to 2011 were initiated on prophylactic AZI, at which point they were censored for follow-up. Initiation of AZI was protocolized to start at 30 d after transplant. AZI was prescribed at 250 mg 3 times weekly. Standard posttransplant maintenance immunosuppression regimen did not differ between eras and included tacrolimus, prednisone, and mycophenolate mofetil. Induction therapy shifted slightly with the decreased use of antithymocyte globulin in favor of basiliximab. For each participant, the date of initiation of AZI prophylaxis was abstracted from medical records.

Pathology reports with "endobronchial biopsy" were obtained by CoPath query (Cerner, 2800 Rockcreek Pkwy, Kansas City). Lymphocytic bronchitis was quantified on endobronchial biopsies as none, minimal, moderate, or severe chronic inflammation, as part of routine clinical care. These categories correspond to the International Society for Heart and Lung Transplantation grades of lymphocytic bronchiolitis, as previously described.[7]

Automated cell counts were performed on BAL collected for microbiologic analysis and cytology.[14] Additional participant characteristics were obtained from United Network for Organ Sharing (UNOS) records.

Spirometry data were extracted from electronic medical records. CLAD was defined as an irreversible drop in FEV_1 by 20% for at least 3 wk from the posttransplant baseline. Restrictive allograft syndrome (RAS) was defined to be FEV_1/FVC ratio <0.8, all other participants were deemed to have BOS as their CLAD phenotype. Participants were right censored at the time of the last spirometry.

Statistical Approach

We visualized differences in large-airway lymphocytic inflammation (LB) grade across the pre-AZI and during-AZI eras by box and whisker plot. To determine if LB severity changed over the era, we used cumulative link mixed model with LB grade as the ordinal outcome, and era as the predictor. 95% confidence intervals (CIs) were calculated by bootstrapping. Participant identifier was included as a random effect to adjust for multiple samples per participant.

To determine if era influenced lymphocytic bronchitis and BAL cell populations, we used linear mixed-effects models. Cell counts were log-transformed to account for their log-normal distribution.

We visualized time to CLAD, death, or retransplantation with Kaplan-Meier plots. The hazard of CLAD, death, or retransplantation between eras was determined with Cox proportional hazard models. As a sensitivity analysis, we evaluated AZI prophylaxis as abstracted from medical records as a time-dependent covariate. Furthermore, the subdistribution hazards of CLAD (censored on death), RAS, and BOS in relation to AZI exposure, as a time-dependent covariate, were determined with the Fine-Gray method. Participants were right censored at the time of most recent spirometry or at the start of the AZI era, for participants in the pre-AZI cohort. All models were adjusted for donor and recipient age, gender, ethnicity, procedure type, cytomegalovirus status, lung allocation score (LAS), and UNOS indication group.

RESULTS

A total of 331 LTx recipients with 2319 biopsies were included in this study. Participant characteristics were largely similar for the 153 recipients (1344 biopsies) in the pre-AZI era compared with the 178 recipients (975 biopsies) in the AZI era (Table 1). No recipients in the pre-AZI ear received AZI prophylaxis before 2011, whereas all recipients in the AZI prophylaxis era were started on AZI within 12 mo of their transplantation with a median time to initiation of 30 d (mean 68 d). Recipient age was older during the AZI era as compared with the pre-AZI era ($P = 0.03$). Most transplants were performed for category D (pulmonary fibrosis) with a trend toward increase in the AZI era ($P = 0.06$). Number of biopsies per participant in the pre-AZI era (8.8) was higher compared with the number of biopsies per participant in the during-AZI era (5.5) ($P < 0.001$). Participants' characteristics, including ethnicity and cytomegalovirus status, were otherwise similar between the eras.

To assess the impact of protocolized AZI prophylaxis on lymphocytic bronchitis severity, we compared the distribution of pathology grades between eras. As shown in Figure 1, moderate and minimal lymphocytic bronchitis was less common in the AZI era, although no lymphocytic bronchitis was more common. Using ordinal mixed-model regression, odds

TABLE 1.

Participant characteristics by AZI era.

Characteristic	Pre-AZI	During-AZI	*P*
Participants, N	153	178	
Biopsies, N	1344	975	
Age, mean (SD)			
Recipient	53.19 (12.05)	56.05 (11.74)	0.03
Donor	32.33 (13.89)	32.22 (13.64)	0.94
Male gender, N (%)			
Recipient	89 (58.2)	103 (57.9)	1
Donor	80 (53.3)	111 (64.9)	0.06
UNOS lung disease group			0.06
A—obstructive	35 (22.9)	29 (16.3)	
B—pulmonary vascular	10 (6.5)	4 (2.2)	
C—cystic fibrosis	10 (6.5)	19 (10.7)	
D—restrictive	98 (64.1)	126 (70.8)	
Recipient ethnicity, N (%)			0.74
White	115 (75.2)	130 (73.0)	
Asian	19 (12.4)	24 (13.5)	
Black	8 (5.2)	15 (8.4)	
American Indian/Alaska Native	10 (6.5)	8 (4.5)	
Multiracial	1 (0.7)	1 (0.6)	
Donor ethnicity, N (%)			0.60
White	71 (47.7)	85 (50.9)	
Asian	46 (30.9)	48 (28.7)	
Black	19 (12.8)	15 (9.0)	
American Indian/Alaska Native	10 (6.7)	17 (10.2)	
Multiracial	3 (2.0)	2 (1.2)	
Cytomegalovirus donor/recipient, N (%)			0.90
N/N	22 (18.2)	27 (16.1)	
N/P	23 (19.0)	34 (20.2)	
P/N	30 (24.8)	47 (28.0)	
P/P	46 (38.0)	60 (35.7)	
Procedure, N (%)			0.32
Double			
Double	136 (90.7)	158 (92.4)	
Heart/lung			
Heart/lung	4 (2.7)	1 (0.6)	
Single			
Single	10 (6.7)	12 (7.0)	
LAS, mean (SD)	51.16 (20.33)	58.93 (22.67)	0.001
Number of biopsies per participant, mean (SD)	8.78 (4.56)	5.48 (3.56)	<0.001

UNOS lung disease groups: A—chronic obstructive pulmonary disease, B—pulmonary arterial hypertension, C—cystic fibrosis, D—pulmonary fibrosis.
AZI, azithromycin; LAS, lung allocation score; N, number of events; P, probability; UNOS, United Network for Organ Sharing.

of observing the same or lower grade of lymphocytic bronchitis of 1.8 (95% CI, 1.3-2.4)-fold ($P < 0.001$). After adjusting for characteristics in Table 1, the AZI era was associated with a 1.6 (95% CI, 1.2-2.3)-fold odds of same or lower pathology grade ($P = 0.004$).

Based on prior observations that LB is associated with BAL neutrophilia and neutrophilic reversible allograft dysfunction, we examined BAL cell type distributions between LB grades. As shown in **Figure 2A**, higher grades of lymphocytic bronchitis were associated with an increased frequency of BAL neutrophilia. We found a 3.1% increase in BAL neutrophils for each incremental grade of LB (95% CI, 2.6-3.7%, adjusted $P < 0.001$).

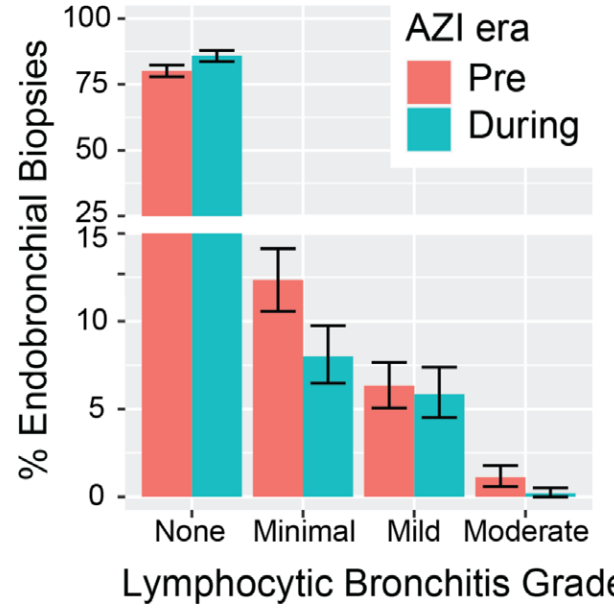

FIGURE 1. Rate of LB and severity by AZI eras. The proportion of endobronchial biopsies in each severity grade is shown stratified by era with 95% CIs. Biopsies in the era of AZI prophylaxis had a 1.8 (95% CI, 1.3-2.4)-fold ($P < 0.001$) adjusted odds of having the same or lower grade of lymphocytic bronchitis. AZI, azithromycin; CI, confidence interval; LB, large-airway lymphocytic inflammation.

We next assessed the effect of the AZI era and lymphocytic bronchitis on other BAL cell populations, as captured by clinical cell counts and flow cytometry. We found that lymphocytic bronchitis was associated with decreased BAL T cells, monocytes, and NK cells. Interestingly, compared with the pre-AZI era, recipient BAL in the AZI era had more neutrophils across all LB grades (adjusted effect 0.23, 95% CI, 0.13-0.32, $P = 0.007$). Overall lymphocyte count was similar between eras, but as described above, the relative ratios of different cell types were different (**Figure 2B**).

To determine the impact of AZI prophylaxis on long-term outcomes, we examined the time to CLAD, death, or retransplantation between the 2 eras. As shown in Figure 3, there was no significant difference in CLAD-free survival in unadjusted analysis (log-rank $P = 0.70$). In a Cox proportional hazards model including characteristics in Table 1, the hazard ratio (HR) for CLAD, death, or retransplantation was 1.15, with 95% CI, 0.66-2.00 ($P = 0.61$). Similarly, there was no statistically significant effect of AZI era on adjusted subdistribution HRs for CLAD, censored on death (HR 1.21; 95% CI, 0.66-2.21) or overall survival (HR 1.42; 95% CI, 0.97-2.01). We then examined whether there was an effect of AZI on the development of RAS using a Fine-Gray subdistribution analysis (HR 1.05; 95% CI, 0.59-1.86). This same model was applied to assess the development of BOS (HR 1.125; 95% CI, 0.67-1.88).

DISCUSSION

In this single-center retrospective cohort study, we found a decrease in the incidence and severity of lymphocytic bronchitis in the era of AZI prophylaxis. However, despite this effect, there was no difference in CLAD-free survival or overall survival, even when CLAD was separated into obstructive versus restrictive phenotypes.

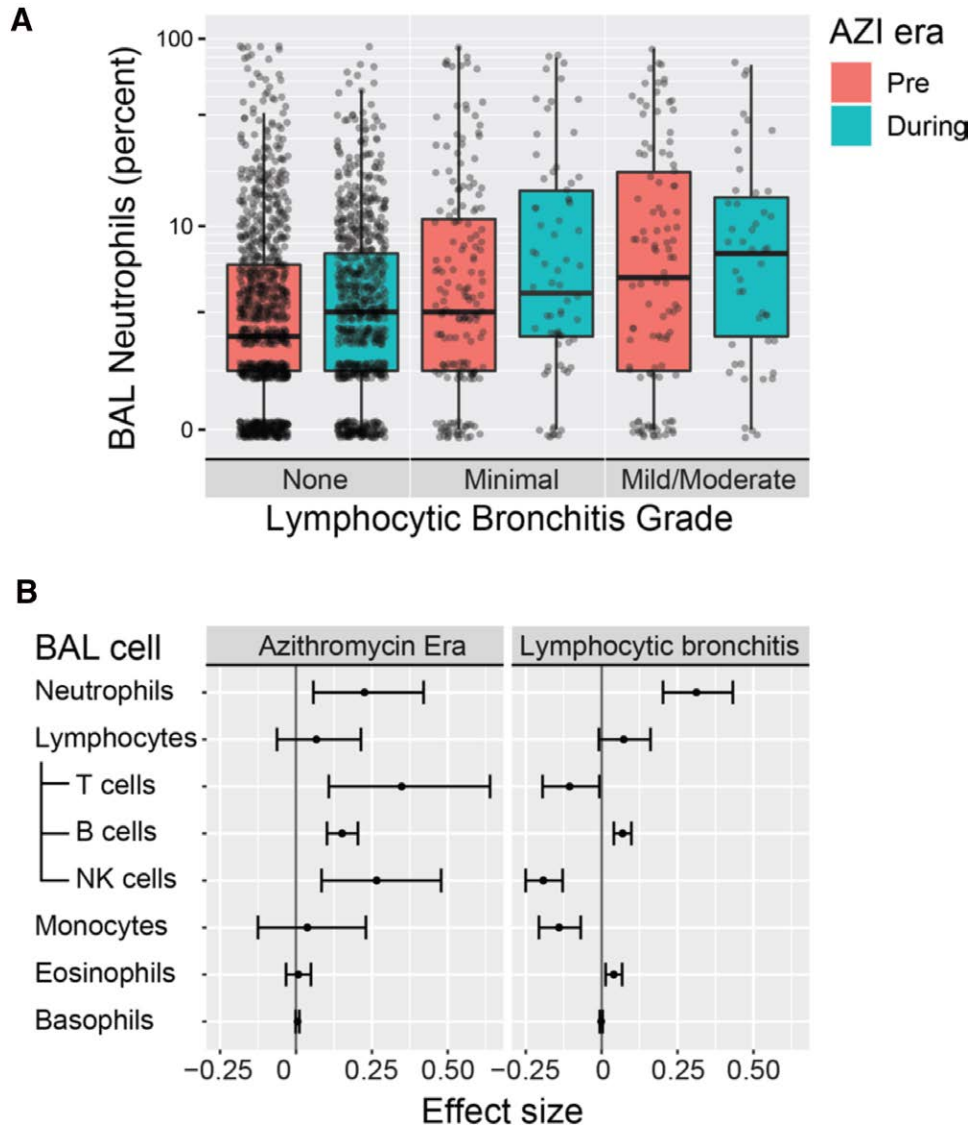

FIGURE 2. AZI era and lymphocytic bronchitis impacts on BAL cell distributions. A, BAL neutrophil percent is shown stratified by lymphocytic bronchitis grade and AZI era with datapoints plotted over Tukey box and whiskers plot. B, Effect estimates on BAL cell count frequencies with 95% CIs are shown for AZI and lymphocytic bronchitis grade, as calculated using mixed models adjusted for participant characteristics. AZI, azithromycin; BAL, bronchoalveolar lavage; CI, confidence interval.

These findings were unexpected based on the randomized controlled trial data from Vos et al[15] and a prior single-center study assessing AZI prophylaxis in a nontrial setting. In that retrospective study,[13] participants who received AZI prophylaxis were transplanted more recently, less likely to receive intraoperative support, and more likely to have tacrolimus-based immunosuppression and induction with IL-2 receptor antagonists. Similarly, in this cohort, it is difficult to exclude residual confounding from our practice changes. Here, recipients in the AZI prophylaxis era were older and had higher illness severity, as determined by LAS.

We had previously identified lymphocytic bronchitis as a risk factor for CLAD and death in LTx recipients. As demonstrated here, rates of lymphocytic bronchitis declined at the end of 2015, motivating our center to stop routine endobronchial biopsies. It is possible that other practice changes in addition to AZI contributed to this decline, but the role of AZI is biologically plausible, given prior reports of resolution of lymphocytic bronchitis following AZI treatment and its role in suppressing IL-8.[16] Despite the decreased observation of lymphocytic airway inflammation on histopathology, this biologic process may contribute to CLAD pathogenesis because its detection by transcriptional signature in small airways is associated with risk of graft failure.[17] Surprisingly, AZI prophylaxis was associated with an increase in BAL neutrophil count, contrasting with prior studies.[10] This could suggest an attenuation of AZI blockade of IL-8-mediated neutrophil chemotaxis.[18] The observed effects of IL-8 may require interactions with medications, genotypes, or microorganisms not present in this cohort. In the initial randomized controlled trial, AZI therapy did not measurably impact the microbiome.[19] However, we previously observed increased rates of *Haemophilus parainfluenza* in LTx recipients BAL coincident with the initiation of AZI prophylaxis, suggesting how AZI could paradoxically worsen airway inflammation.[20]

This study has some limitations. As a single-center study, it is difficult to generalize these findings to centers where practice patterns may differ, particularly in the light of

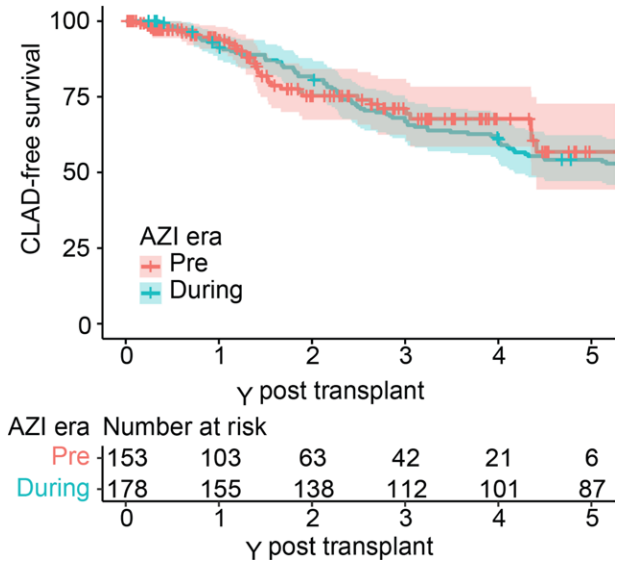

FIGURE 3. CLAD-free survival in the AZI era. A Kaplan-Meier plot shows CLAD-free survival percentage as a function of time posttransplant stratified by AZI prophylaxis era. The log-rank *P* value for differences between eras was 0.70. AZI, azithromycin; CLAD, chronic lung allograft dysfunction.

conflicting data. Although comparable in size to other AZI studies, it is possible a larger study could identify more subtle effects. The retrospective preintervention and postintervention design have inherent limitations because we cannot account for all other changes that may have occurred between these eras. We attempted to address this with multivariable modeling and time-dependent analyses but cannot exclude residual confounding. Although the frequency of biopsies was less in the AZI prophylaxis era, a decrease in not-for-cause biopsies would be expected to increase the rates of observed inflammation and would have biased these results toward the null.

In summary, following the implementation of AZI prophylaxis, we observed decreased lymphocytic bronchitis without an impact on CLAD-free survival. Although our center continues to use AXI prophylaxis, these data would support a multicenter randomized investigation of AZI prophylaxis to test its role in CLAD prevention.

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