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Airway Bacterial Load and Inhaled Antibiotic Response in Bronchiectasis

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Author´s contributions:

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All authors participated in study design, data analysis, and interpretation of the data.

All authors were involved in writing and revising the manuscript before submission.

This article has an online data supplement, which is accessible from this issue's table of content online at **www.atsjournals.org**

At a Glance Commentary (200/200)

Scientific knowledge on the subject:

Multiple inhaled antibiotic trials in bronchiectasis have not met their primary endpoints. To date the reason for heterogeneous responses to inhaled antibiotics have not been identified. Airway bacterial load is a key component of bronchiectasis pathogenesis and has been previously associated with increased airway inflammation and poor clinical outcomes, especially in those patients with high bacterial load. We hypothesized that higher bacterial load would be a stable trait associated with worse airway inflammation and worse quality of life, and therefore patients with the highest bacterial load would benefit most from inhaled antibiotic treatment.

What this study added to the field:

This is the first study to identify an inhaled antibiotic response phenotype based on baseline bacterial load. Patients with high bacterial load had higher airway inflammation and worse quality of life but responded with a mean in quality of life that exceeds the minimum clinically important difference with aztreonam treatment. The percentage of patients with a clinically meaningful improvement was higher with aztreonam compared with placebo at the end of both treatment cycles only in patients with baseline high bacterial load. The potential for bacterial load to guide antibiotic therapy for patients with bronchiectasis should be prospectively tested.

ABSTRACT (248/250)

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Rationale: The principal underlying inhaled antibiotic treatment in bronchiectasis is that airway bacterial load drives inflammation, and therefore antibiotic treatment will reduce symptoms.

Objective: We performed 3 studies to 1) determine the relationship between bacterial load and clinical outcomes, 2) assess the stability of bacterial load over time and 3) test the hypothesis that response to inhaled antibiotics would be predicted by baseline bacterial load.

Methods: Study 1+2: Prospective studies including adults with bronchiectasis. Study 3: Post-hoc analysis of a randomized trial of inhaled aztreonam. A priori patients were divided into low ($<10^5$ colony forming units (cfu)/g), moderate (10^5-10^6cfu/g) and high bacterial load ($\geq 10^7$cfu/g) using quantitative sputum culture.

Measurements and main results: Bacterial load was a stable trait associated with worse quality of life and more airway inflammation in studies 1, 2 and 3. In study 3, patients with high bacterial load showed an improvement in the primary endpoint (Quality of Life Bronchiectasis Respiratory Symptoms Score at week 4) in favor of aztreonam (mean difference of 9.7 points (95% confidence interval 3.4-16.0), p=0.003). The proportion of patients who achieved an increase above the Minimum Clinically Important Difference was higher in the aztreonam group at week 4 (63% vs 37%,p=0.01) and at week 12 (62% vs 38%,p=0.01) only in high bacterial load patients.

Conclusions: Improvement of quality of life with inhaled aztreonam was only evident in patients with high bacterial load. Bacterial load may be a useful biomarker of severity of disease and treatment response.

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INTRODUCTION

Bronchiectasis is a common chronic respiratory disease characterized by bronchial dilatation leading to daily productive cough and recurrent respiratory infections (1). A renewed interest in the disease has been seen in recent years, resulting in more clinical research with the aim of developing new treatments (2). Several clinical trials have attempted to adapt inhaled antibiotic treatments which are licensed for cystic fibrosis. The principle underpinning inhaled antibiotic treatment in bronchiectasis is that decreases in bacterial load will in turn reduce symptoms and the frequency of exacerbations (3, 4). However, most trials have not reached their primary endpoints in bronchiectasis. Two large phase 3 randomized trials of inhaled aztreonam concluded no improvement in quality of life (5). Multiple other trials involving nebulized colistin (6), dry powder ciprofloxacin (7, 8) and nebulized liposomal ciprofloxacin (9) have also not consistently reach primary endpoints. The new European Respiratory Society (ERS) guidelines found no strong clinical evidence in support of inhaled antibiotic therapy as a treatment (10). Despite extensive subgroup analyses, the reasons for inconsistency between bronchiectasis inhaled antibiotics trials have not been explained.

Airway bacterial load is a key component of bronchiectasis pathogenesis (11). Patients are chronically infected with a variety of bacterial pathogens, resulting in a vicious cycle of infection and inflammation with persistent respiratory symptoms and further airway damage (12). Previous studies have demonstrated a direct relationship between airway bacterial load and both airway and systemic inflammation (13, 14). Multiple studies across several diseases have suggested an "inflammatory threshold"

above 10^7 colony forming units (cfu)/gram where patient have more inflammation, worse symptoms and more exacerbations (15–17). Both systemic and inhaled antibiotic treatment reduces bacterial load, with greatest benefits in terms of reduced airway and systemic inflammation in the patients with the highest bacterial load (15). However, no recent clinical trials focused on inhaled antibiotics have considered whether inhaled antibiotic response is predicted by baseline bacterial load (5–8).

Therefore, we hypothesized that higher bacterial load would be associated with worse airway inflammation and quality of life and therefore patients with the highest bacterial load would benefit most from inhaled antibiotic treatment.

METHODS

Study design

This research is composed of three components. Study 1 examines the relationship between bacterial load and quality of life and airway inflammation in patients with bronchiectasis. Study 2 evaluates the stability of bacterial load in two prospective cohorts: one in patients receiving systemic antibiotic treatment for exacerbations, and a second cohort evaluating day to day variability in clinically stable patients. Study 3 describes a post-hoc analysis of patients enrolled in two randomized double blind trial of inhaled aztreonam to test the hypothesis that response would be predicted by bacterial load in bronchiectasis. All studies described here were approved by the relevant research ethics committees (approval numbers 11/AL/0286, 12/ES/0059, 16/ES/0047).

Study 1

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This was a prospective study of 189 bronchiectasis patients. Inclusion and exclusion criteria, recorded data and inflammatory markers evaluated are described in on-line supplement.

Sputum was processed for qualitative and quantitative bacteriology as previously described (15, 18). A priori patients were divided into low bacterial load ($<10^5$ cfu/g), moderate bacterial load (10^5-10^6 cfu/g) and high bacterial load ($\geq 10^7$ cfu/g).

Study 2

Two prospective studies were performed to demonstrate the repeatability of a high bacterial load phenotype in bronchiectasis. We hypothesized that groups of patients with different baseline levels of bacterial load would maintain similar bacterial load levels throughout follow-up, including after recovery from antibiotic treatment. First, 26 patients with bronchiectasis were followed-up for one year, and sputum analyses were performed at baseline, after receiving systemic antibiotic treatment due to an acute exacerbation (19) and after at least three months of recovery. In a second study of 10 stable patients with bronchiectasis (5 with idiopathic/post-infective and 5 with CF bronchiectasis) patients were followed-up for 14 days, and sputum analyses were performed at days 1,3,5,7 and 14. Further details are provided in the on-line supplement.

Study 3

We conducted a secondary analysis of two randomized double-blind, placebo-controlled phase 3 trials of Aztreonam for inhalation solution (AZLI) in bronchiectasis

(AIR-BX1 and AIR-BX2) (5). The original informed consent form for this study contained an optional consent for future exploratory analysis of participants data. Only data from patients that had given written consent for future re-use of their data were included in this analysis. A summary of these trials is provided in the on-line supplement. Patients received two 4-week cycles of double-blind inhaled treatment with AZLI 75 mg or placebo given three times a day, separated by 4 weeks off-treatment.

The primary endpoint was change in the QoL-B-RSS between baseline and week 4. Minimum Clinical Important Difference (MCID) was considered an increase of 8 points (20). Secondary outcomes were changes in the QoL-B-RSS at week 12, 6 minute walk distance at week 4, EQ-5D questionnaire at week 4 and frequency of exacerbations during the study. Bacterial load data throughout the study was used to further demonstrate the repeatability of bacterial load over time.

Bacterial load in cfu /g of target Gram-negative pathogens was measured in sputum at baseline by a central laboratory. Based on our own previous work (15, 17), we divided patients a priori into 3 groups based on their quantitative bacterial culture; low bacterial load ($<10^5$ cfu/g), moderate bacterial load (10^5-10^6 cfu/g) and high bacterial load ($\geq10^7$ cfu/g).

Statistical analyses

Statistical analysis was performed using the SPSS 22.0 software. Categorical variables are presented by frequencies and percentages, and differences were analysed using the χ^2 test or Fisher exact test when required. Continuous variables are presented as mean and standard deviation (SD), or median and interquartile range (IQR) when data was not distributed normally. Differences in continuous variables

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were analysed using Student t and ANOVA tests, or their corresponding non-parametrical tests when required. Pearson correlation was used to examine the relationship between linear variables. We defined statistical significance as a two-tailed p<0.05.

RESULTS

Study 1

One hundred eighty-nine clinically stable patients were recruited for the study. **Table 1** shows the demographic characteristics of the study population. Mean age ($\pm$ SD) was 64 ($\pm$ 12 years old), 56% were female and mean FEV1 was 72 ($\pm$ 25) % of predicted. Most patients were classified as Idiopathic (46%) and post-infective (18%). *P. aeruginosa* was present in the sputum of 34 (18%) patients, and mean QoL-B-RSS at baseline was 56 ($\pm$22) points.

Mean bacterial load was 6.6 ($\pm$1.7) log 10 cfu/g. Fifty-two patients (28%) had low bacterial load, 51 patients (26%) had moderated bacterial load and 86 patients (46%) had high bacterial load. Patients with high bacterial load were older, had lower FEV_1, and high percentage of *P. aeruginosa*. No other differences among groups were found (**Table 1**).

Bacterial load was weakly associated with QoL-B-RSS (r=-0.20, p=0.004) and disease severity (r=0.38, p<0.001 with BSI score and r=0.34, p<0.001 with FACED score). Compared to those with moderate and low bacterial load, patients with high bacterial load had worse QoL-B-RSS (50 ($\pm$21) vs 59 ($\pm$24) vs 61 ($\pm$22) points, p=0.01) and more severe disease (9.3 ($\pm$4.2) vs 7.7 ($\pm$4.6) vs 5.2 ($\pm$3.1), p<0.001 points in BSI score). (**Figure 1**).

Patients with high bacterial load had higher sputum neutrophil count (median (IQR) of 9.2 (5.2-15.7) vs 5.2 (2.5-9.2) vs 3.3 (1.8-6.6) 10^6cells/g, p=0.007) and higher sputum myeloperoxidase activity (1.89 (0.58-6.34) vs 0.97 (0.06-2.21) vs 0.12 (0.01-1.02) U/mL , p<0.001) (**Figure 1**). In addition, higher CXCL8 (38.3 (27.5-53.0) vs 34.1 (18.2-44.7) vs 33.7 (21.1-44.2) ng/mL) and TNF-α (1010.5 (418.8-2941.8) vs 520.0 (77.8-1400.6) vs 829.2 (177.8-3414.2) ng/mL) concentrations were observed in the patients with high bacterial loads, though the differences between the groups were not statistically significant (p=0.08, 0.2).

Study 2

Twenty-six patients with bronchiectasis were included in the exacerbation study. Patient characteristics are shown in **table E1** in the online supplement. Fourteen patients (54%) had high bacterial load at baseline, while 7 patients (27%) had moderate and 5 (19%) had low bacterial load.

Mean bacterial load decreased in all groups after antibiotic treatment (p<0.001). However, during the follow up, it increased again in all groups (p<0.001) (**Figure 2A**). Eleven out of 14 patients with high bacterial load at baseline (78%) also had high bacterial load during follow up.

Ten clinically stable patients were included in the stability study. Demographic characteristics of the patients are described in the **table E2** in the online supplement. Eight out of the 10 patients (80%) maintained similar levels of bacterial load during the follow-up, while only n=2 patients (20%) experienced significant changes (**figure 2B**).

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No differences between patients with CF and idiopathic/post-infective bronchiectasis were found.

Study 3

440 patients out of 540 (81%) had consented to have their data reanalyzed (n=181 from the AIR-BX 1 and n=259 from AIR-BX2). There were no significant demographic differences between those consenting to reanalysis and the full cohort of all subjects in the original trials (**table E3** in the on-line supplement).

Table 2 shows the main clinical characteristics patients included in the study. Mean age (±SD) was 64 (± 13) years old, 69% were female and mean FEV1 was 62 (±20) % of predicted. *P. aeruginosa* was present in the sputum of 357 patients (80%), and mean QoL-B-RSS at baseline was 56 (±18) points. No differences among baseline characteristics of patients from AIR-BX1 and AIR-BX 2 were found.

A total of 421 patients had documented baseline bacterial loads, with a mean of 5.1 (±2.3) log10 cfu/g. Bacterial load was weakly, and negatively, associated with baseline FEV_1 % predicted (r=-0.19, p=0.0003) and baseline QoL-B-RSS (r=-0.11, p=0.03). Baseline bacterial load was significantly higher in patients with *P. aeruginosa* (mean 5.6 (±2.1) log10cfu/g vs 3.5 (±1.8) log10cfu/g, p<0.001) when compared to those patients with other Gram-negative bacteria on sputum at baseline. 147 patients (35%) had low bacterial load, 172 patients (41%) had moderate bacterial load and 102 patients (24%) had high bacterial load. No differences among clinical characteristics according to bacterial load were found, except that patients with high bacterial load had higher percentage of *P. aeruginosa* (see **table E4** in the on-line supplement).

369 patients with documented baseline bacterial load (88%) completed the double blind treatment period.

The consistency of sputum bacterial load within the 3 groups over time demonstrated in study 2 was confirmed in the AIRBX studies. Figure 3A shows sputum bacterial load in patients treated with aztreonam demonstrating that the mean bacterial load remained consistent between the high, moderate and low groups in two pre-treatment visits (screening- visit 1 and baseline- visit 2). Bacterial load was reduced with aztreonam treatment at day 14 on-treatment- visit 3, and day 28 on-treatment-visit 4. Following drug discontinuation bacterial load returned to a mean level equivalent to the group baseline. Similar behavior was observed over the second treatment cycle (figure 3A). In placebo patients clear differences between the three groups were observed at each time point indicating that bacterial loads remain stable in these groups (figure 3B).

Primary endpoint

Comparing AZLI vs placebo, there was no improvement in QoL-B-RSS at week 4 with AZLI treatment for patients with low bacterial load (mean difference 1.6, 95% Confidence Interval -4.9 – 8.30, p= 0.6) and moderate bacterial load (mean difference 0.9, 95CI -4.8 – 6.8, p= 0.8). However, there was a clear difference in favor of AZLI treatment above the MCID in patients with baseline high bacterial load (mean difference 9.7, 95CI 3.4 – 16.0, p=0.003) (**Figure 4A**). All mean differences refer to a comparison in the change in QOL-B between aztreonam and placebo treatment. An increase in QoL-B RSS of greater than or equal to 8 points is regarded as clinically meaningful. We therefore analysed the proportion of responders stratified by baseline bacterial load. There was no significant difference in the percentage of responders in

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patients with low or moderate bacterial load. In patients with high bacterial load, 63% achieved a QOL-RSS increase above the MCID compared to 37% of patients in the placebo group (p=0.01) (**Figure 4B**).

The response in patients with high bacterial load was consistent and unaffected by patients characteristics, with mean differences favoring AZLI treatment in all subgroups including FEV1 (≥50% or <50%), QoL-B-RSS (≥60 or <60 points), sex and patients with *P. aeruginosa*.

In the original AIR-BX1 and AIR-BX2 studies (5), AIR-BX2 found a significant effect of AZLI on QoL-B-RSS (mean difference 4.6, 95CI 1.1 – 8.2, p=0.01), while AIR-BX1 did not (mean difference 0.8, 95CI -3.1 – 4.7, p=0.6). We hypothesized this difference may have been due to lower numbers of patients with high bacterial load in AIR-BX1 vs AIR-BX2 and indeed as shown in **Figure 5A**, more than double the number of high bacterial load patients were seen in AIR-BX 2 (75/259 (29%) vs 27/181 (15%), p<0.001). When effects in high bacterial load subjects were compared between trials there was a consistent treatment response regardless of other baseline characteristics. (**Figure 5B**).

Analysis of the 12 week secondary endpoints supported the primary analyses with no benefit in patients with low and moderate bacterial load (mean difference of -1.6 (95%CI -11.8 – 8.5, p=0.7) and 1.7 (95CI -4.7 – 8.1, p=0.6), respectively), but a mean difference of 5.5 (95CI -1.7 – 12.7, p=0.1) in those with high bacterial load. The percentage of patients with an improvement in QOL-B RSS above the MCID was 62% for aztreonam vs 38% for placebo at the 12 week visit (p=0.01) for patients with high bacterial load with no difference in responders for low and moderate groups.

Secondary endpoints

For the secondary endpoints of reduction in CFU at week 4, aztreonam reduced bacterial load by 2 log units (95% CI 1.84-2.14,p<0.0001) compared to placebo in the high bacterial load group. This effect was superior that seen in the moderate bacterial load group (mean difference 0.98 log units 95% CI 0.92-1.03,p<0.0001). The effect was lowest in the group with lower bacterial load, 0.47 log units 95% CI 0.29-0.65,p<0.0001) (figure 3A/B). There were no overall improvements in the EQ5D or 6-minute walk distance between the groups and no benefit of aztreonam treatment on exacerbation frequency in the original AIR-BX analysis.

Patients with higher bacterial load had the higher numerical improvement in 6-minute walk distance at week 4, 18m (95% CI -5 to 41.3, p=0.1), compared to a deterioration of -12m in the moderate group (95% CI -27.9 to 4.0, p=0.1). The low bacterial load group improved by 14m (95% CI -4 to 34,p=0.1). There were 155 exacerbations during the study in 131 patients. Analysis of exacerbation frequency was limited by the small number of events in each subgroup, but no impact of aztreonam of exacerbations was observed in the high (IRR 1.02 95% CI 0.80-1.29,p=0.9), moderate (IRR 1.13 95% CI 0.96-1.34,p=0.1) or low (IRR 0.92 95% CI 0.76-1.12,p=0.4) bacterial load group. No between group differences were observed in the EQ5D at any timepoint.

DISCUSSION

In our study, we demonstrated that high airway bacterial load is associated with worse quality of life and increased airway inflammation in bronchiectasis. In addition, high bacterial load remained elevated over time during clinical stability and returned to high levels after suppression with antibiotic treatment. This led us to reanalyze two previously published negative randomized controlled trials (5) , where

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for the first time we have demonstrated a consistent predictor of inhaled antibiotic response by showing that patients with elevated baseline bacterial loads had a clear improvement in respiratory symptoms when treated with aztreonam compared to placebo. These results were consistent both in terms of the mean improvement in quality of life, but also in the proportion of patients achieving a clinically meaningful response at 4 and also 12 weeks. These findings suggested that bacterial load is a key component in the heterogeneous response to inhaled antibiotic treatment in bronchiectasis.

To the best of our knowledge, this is the first study to describe an inhaled antibiotic response phenotype. Most of the recent clinical trials on inhaled antibiotics in bronchiectasis have not reached their primary outcomes and identifying the response phenotype has been identified a key research priority (7, 8, 21). Haworth et al (6) allocated patients with chronic *P. aeruginosa* infection to either nebulized colistin or placebo, and the study did not meet its primary endpoint of time to first exacerbation. The international trials RESPIRE 1 (7) and RESPIRE 2 (8) and ORBIT 3 and ORBIT 4 (9) have assessed different formulations of inhaled ciprofloxacin in patients with and without *P.aeruginosa* infection, and primary endpoints (time to first exacerbation, frequency of exacerbations) were reached in some trials (7, 9) but not met in replicate trials (8, 9). Clinical parameters such as lung function or microbiology were not sufficient in any of these trials to define response populations emphasizing the need for a biomarker such as bacterial load. In AIR-BX1 and AIR-BX2 (5), the primary outcome (changes in QoL-B-RSS) was reached in AIR-BX2 but not in AIR-BX1, even in AIR-BX2 the difference was below the MCID. We demonstrate that the difference between AIR-BX1 and AIR-BX2 studies may be explained by the much lower

bacterial load in AIR-BX1 and that responses were quite consistent when controlling

for bacterial load. It would be important to now apply this knowledge to other

inconsistent trial populations.

Airway bacterial load was shown to be important on clinical outcomes in

bronchiectasis (13–15, 17). In our study, we demonstrated that high bacterial load is

associated with severity of disease (both BSI and FACED scores), worse quality of life

(QoL-B-RSS) and increased airway inflammation. The "threshold" of $\geq 10^7$ cfu/gr to

detect patients with high bacterial load was previously related to airway inflammation,

neutrophil elastase activity and increased exacerbations in bronchiectasis (15, 17). In

COPD, this cut-off has been related to airway inflammation (16), which has been

associated with poor outcomes in several studies (22, 23). Therefore, patients with a

bacterial load higher than this threshold could be the most suitable to receive

treatments such as inhaled antibiotics.

Some clinical trials in bronchiectasis have evaluated baseline bacterial load.

Interestingly, two small randomized controlled trials which included patients with the

highest bacterial load showed positive results (18, 24). Patients who received inhaled

tobramycin had a mean baseline bacterial load of 7.1 ($\pm$1.4) log10 cfu/g, and

experienced an improvement in their medical condition (24). In the most positive

study published on inhaled antibiotics in bronchiectasis to date, patients who received

nebulized gentamicin had a baseline bacterial load of 8.0 (7.6 – 8.2) log10 cfu/g, which

is the highest baseline bacterial load included in all studies. After 12 months of

treatment, these patients experienced an improvement in quality of life and a

decrease in exacerbation frequency (18). On the other hand, patients included in AIR-

BX studies (5) presented the lowest baseline bacterial load (5.1 ($\pm$2.3) log10cfu/g).

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RESPIRE 1 and RESPIRE 2 did not evaluated baseline bacterial load (7, 8). Limiting study inclusion criteria to include only those patients with high bacterial load may enrich for those patients more likely to respond to therapy. As high bacterial load patients are a subgroup, it is possible this could make trials more difficult to recruit, but this decrease in eligible patients may be offset by a greater treatment effect and if bacterial load is the key treatable trait, it is possible that other inclusion and exclusion criteria could be relaxed.

The stability over time of high bacterial load suggests this microbiological characteristic meets the criteria to be considered as a phenotype, as it is measurable, consistent over time and linked to relevant outcomes (25). In our study, we demonstrated that most of the patients with high bacterial load did not experience changes in a day to day comparison and were recovered after receiving antibiotic treatment. Findings in our single centre UK studies were then confirmed in the AIR-BX studies where remarkable consistency in the 3 bacterial load groups was observed over the entire treatment period. The mean bacterial load remained consistent throughout the study in the placebo group, with only modest reductions at some timepoints likely due to antibiotic treatment of exacerbations. In the aztreonam group, consistent with data we obtained with exacerbation treatment, aztreonam produces a reduction in CFU/g during treatment but the mean level of bacterial load returned to the baseline level after discontinuation of treatment. These findings suggested that bacterial load is a consistent trait that will be associated with poor outcomes without treatment making it a key "treatable trait" according to recent concepts (2). The three studies included in our analysis are heterogeneous in terms of the patient population included, as studies 1 and 2 included a broad representative population of

bronchiectasis patients while study 2B included patients with idiopathic/post-infective

bronchiectasis and CF. Finally, the AIR-BX studies included only patients with chronic

Gram-negative airway infection. The heterogeneity of these populations may be

viewed as a limitation but we regard it as a strength because we have identified

consistent results across multiple different patient populations and a total sample size

of 646 patients. This replication and validation of our findings suggests they are robust

and likely to be reproduced in future studies.

Our study has limitations. First, we performed a post-hoc analysis and

therefore results need to be confirmed in further prospective studies. Second, not all

subjects included in AIR-BX1 and AIR-BX2 studies were available for reanalysis,

although no differences were observed between available and unavailable patients.

Third, the results of our study are based on airway bacterial load determined by

culture. This bacterial load measurement is restricted to a set of pre-defined Gram-

negative pathogens. In the future, it is likely that molecular diagnostic tests will replace

or at least supplement data obtained from culture. It must be emphasised that the

results of our study may not be directly transferable to molecular assays which

measures, for example, 16s rRNA load, since this would quantify all organisms

including "pathogens" and "non-pathogens". Targeted PCR directed against, for

example, P. aeruginosa or a limited set of organisms is possible and widely performed

in research studies. These also currently have limitations such as inability to

differentiate between viable and non-viable bacteria. Therefore a future study is

required to evaluate the relationship between culture based and molecular based

assessments of bacterial infection and response to antibiotic therapy."Fourth, the AIR-

BX studies were not long enough or sufficiently powered to look at exacerbations or

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other efficacy endpoints such as 6-minute walk distance or EQ5D. Bacterial load is

known to predict exacerbations (15), and future studies should determine if inhaled

antibiotics also decrease exacerbations in high bacterial load patients.

In conclusion, we have shown that bacterial load is potentially a key

bronchiectasis treatable trait that predicts response to inhaled antibiotics.

Table 1. Patient demographics of all study population and divided according to

bacterial load

	All n=189	Low bacteria load n=52	Moderate bacterial load n=51	High bacterial load n=86	p value
Age, years Mean (SD)	64.7 (11.8)	60.0 (14.3)	65.3 (11.3)	67.2 (9.6)	0.002
Female sex	107 (56.6)	30 (57.7)	29 (56.9)	48 (55.8)	0.9
FEV1 % predicted Mean (SD)	71.8 (25.1)	75.03 (28.1)	77.5 (23.9)	66.6 (23.1)	0.02
BMI Mean (SD)	26.1 (5.5)	26.5 (5.08)	25.2 (6.3)	26.5 (5.3)	0.3
*Pseudomonas aeruginosa**	34 (18)	3 (5.8)	6 (11.8)	25 (29.1)	0.001
Haemophilus Influenzae	77 (41)	18 (35)	17 (33)	42 (49)	0.1
Moraxella catarrhalis	35 (19)	5 (10)	8 (16)	22 (26)	0.053
Staphylococcus aureus	33 (17)	5 (10)	7 (14)	11 (13)	0.8
Streptococcus pneumoniae	14 (7)	8 (15)	2 (4)	4 (5)	0.035
Enterobacteriaceae	42 (22)	12 (23)	10 (20)	20 (23)	0.9
Smoking status					
Never	115 (60.8)	33 (63.5)	28 (54.9)	54 (62.8)	0.4
Ex-smoker	67 (35.4)	19 (36.5)	20 (39.2)	28 (32.6)	
Current	7 (3.7)	0	3 (5.9)	4 (4.7)	
Aetiology					
Idiopathic	87 (46)	23 (44.2)	20 (39.2)	44 (51.2)	0.3
Postinfective	35 (18.5)	11 (21.2)	11 (21.6)	13 (15.1)	0.5
Previous ABPA	25 (13.2)	4 (7.7)	5 (9.8)	16 (18.6)	0.1
Asthma	7 (3.7)	2 (3.8)	3 (5.9)	2 (2.3)	0.5
COPD	7 (3.7)	1 (1.9)	4 (7.8)	2 (2.3)	0.1
Rheumatoid arthritis	5 (2.6)	1 (1.9)	1 (2.0)	3 (3.5)	0.8
Immunodeficiency	8 (4.2)	5 (9.6)	2 (3.9)	1 (1.2)	0.05
Sarcoidosis	2 (1.1)	2 (3.8)	0	0	0.07
IBD	6 (3.2)	1 (1.9)	4 (7.8)	1 (1.2)	0.08

Data are presented as n(%) unless otherwise indicated.
FEV1=forced expiratory volume in 1 s; BMI=body mass index kg/m2; 6MW=6-minute walk test; QOL-B-RSS=Quality of Life-Bronchiectasis Respiratory Symptoms scores; SD=standard deviation; ABPA=allergic bronchopulmonary aspergillosis; COPD=chronic obstructive pulmonary disease; IBD=inflammatory bowel disease. *for cultured organisms numbers may not add up to 100% due to culturing of more than one organism per sputum sample. In addition, other organisms not included in the above groups were isolated including: *Acromobacter xyloxidans* (1), *Aeromonas hydrophilia* (2), *Burkholderia cepacia* (1), *Pasteurella multocida* (2), *Haemophilus parainfluenzae* (3), *Pseudomonas fluorescens* (1) and *Stenotrophomonas maltophilia* (3),

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Table 2. Patient demographics of all population and from AIR-BX1 and AIR-BX2 studies

included in the reanalyses.

	All n=440	AIR-BX1 n=181 (40.9%)	AIR-BX2 n=259 (58.5%)	p value
Age, years Mean (SD)	63.8 (12.8)	64.8 (11.5)	63.1 (13.7)	0.1
Female sex	306 (69%)	126 (69%)	180 (69%)	0.9
FEV1 % predicted Mean (SD)	62.2 (20.3)	60.8 (19.8)	63.2 (20.7%)	0.2
6MWT distance Mean (SD), m	422 (120)	421 (114)	423 (124)	0.8
QOL-B-RSS Mean (SD)	55.9 (18.5)	54.6 (19.2)	56.7 (18.03)	0.2
Pseudomonas aeruginosa	357 (80%)	146 (80%)	211 (81%)	0.8
Treatment group	219 (49.4%)	89 (49.2%)	130 (50.2%)	0.8
Placebo group	221 (50.2%)	92 (50.8%)	129 (49.8%)	0.8

Data are presented as n(%) unless otherwise indicated.
FEV1=forced expiratory volume in 1 s; 6MW=6-minute walk test; QOL-B-RSS=Quality of Life-
Bronchiectasis Respiratory Symptoms scores; SD=standard deviation.

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Figure legends

Figure 1. Association between low ($<10^5$ cfu/g) , moderate (10^{5-6} cfu/g) and high

($\geq10^7$cfu/g) bacterial load with Quality of Life-Bronchiectasis-Respiratory Symptoms

(QoL-B-RSS) (Panel A), Bronchiectasis Severity Index (BSI) (Panel B), Forced expiratory

volume in 1 second (Panel C), Neutrophil count in sputum (Panel D) and

Myeloperoxidase (MPO) activity in sputum(Panel E). p value is from comparison of all

groups (ANOVA tests for A-C, and Kruskall-Wallis test for C and D)

Figure 2. Mean bacterial load of patients with bronchiectasis at baseline, after

receiving systemic antibiotic and after at least 3 month of follow-up (Panel A), and

bacterial load at days 1,3,5,7 and 14 during clinically stability (Panel B). Patients are

divided into high (green), moderate (orange) and low (blue) baseline bacterial load.

Figure 3. Changes in quantitative bacteriology during the study. The top panel shows

the active treatment group (aztreonam) and the lower panel shows subjects treated

with placebo. The yellow highlighted period indicates visits that took place during

active treatment (visits 3, 4 and 6). Visit labels are as follows: visit 1= screening, visit 2=

baseline, visit 3= 14 days on-treatment, vist 4= 28 days on treatment, visit 5= 28 days

off-treatment, visit 6= 28-days on-treatment during the second cycle, visit 7= final visit,

off treatment.

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Figure 4. Changes in Quality of Life-Bronchiectasis-Respiratory Symptoms (QoL-B-RSS) from baseline to week 4 in bronchiectasis patients divided into high, moderate and low bacterial load. Data are presented as mean difference (boxes) and 95% confidence interval (plots) (Panel A). Percentage of patients with response in Quality of Life-Bronchiectasis-Respiratory Symptoms Score (QoL-B-RSS) above the Minimum Clinically Important Difference according to bacterial load at week 4 (Panel B).

Figure 5. Percentage of patients with high bacterial load included in AIR-BX1 and AIR-BX2 studies. p value is from t-student test (Panel A). Changes in Quality of Life-Bronchiectasis-Respiratory Symptoms (QoL-B-RSS) from baseline to week 4 in patients with high bacterial load, divided into studies (AIR-BX1 and AIR-BX2), lung function (FEV1 $\geq$50% or <50% of predicted value), sex (male of female), baseline quality of life (QoL-B-RSS $\geq$60 or <60 points) and the presence of *P. aeruginosa*. Data are presented as mean difference (boxes) and 95% confidence interval (plots).
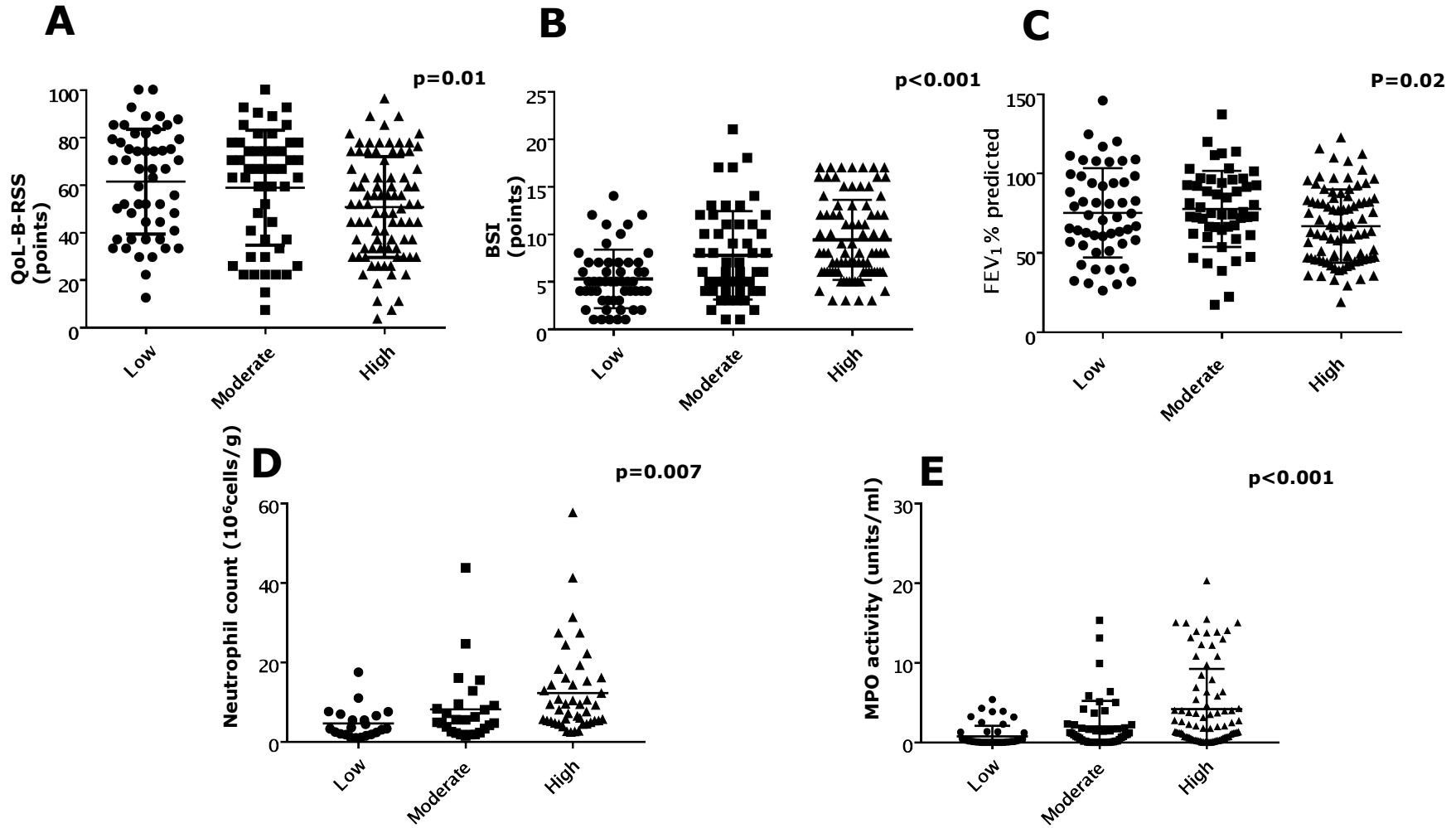
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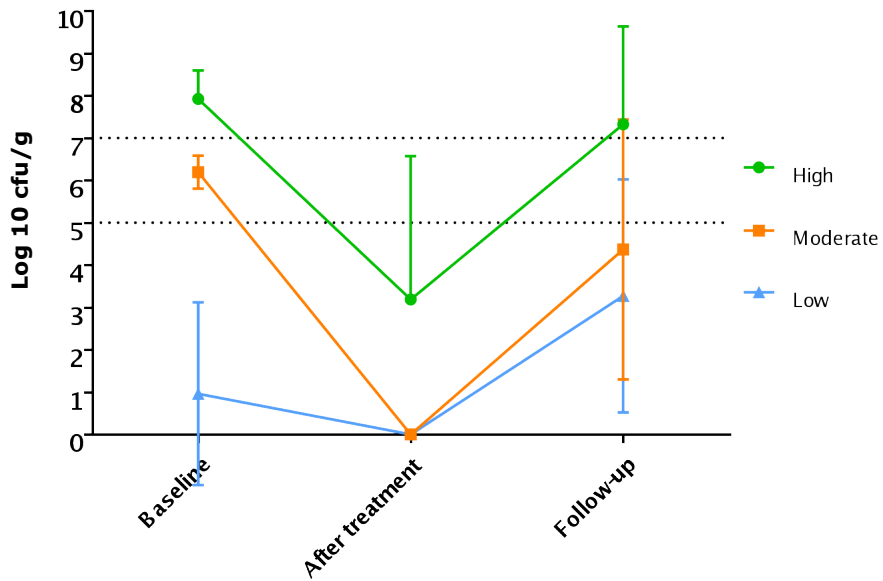Murris M, Cantón R, Torres A, Dimakou K, De Soyza A, Hill AT, Haworth CS,

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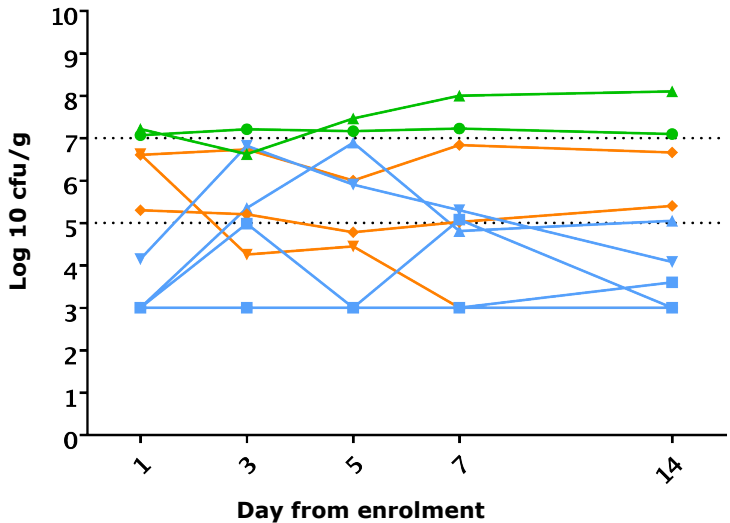
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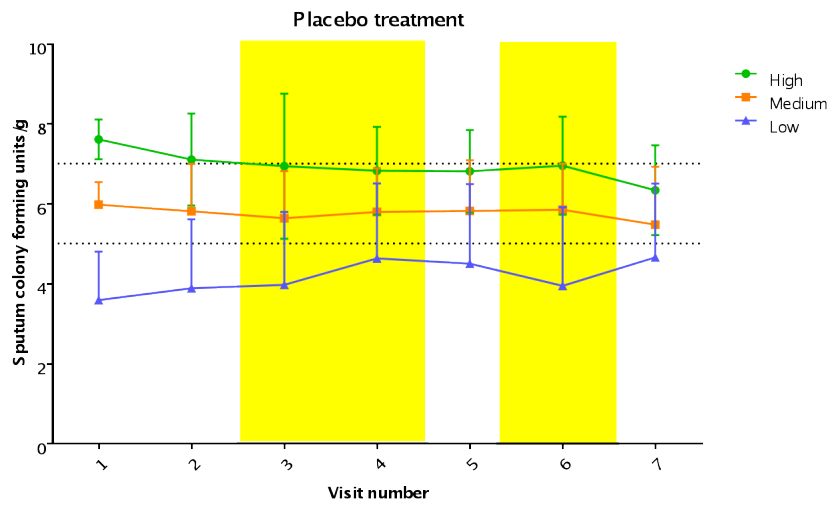
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A

Bacterial load

High

Moderate

Low

-10   -5   0   5   10   15   20

Favour placebo

FavourAztreonam

Changes in QoL-B-RSS
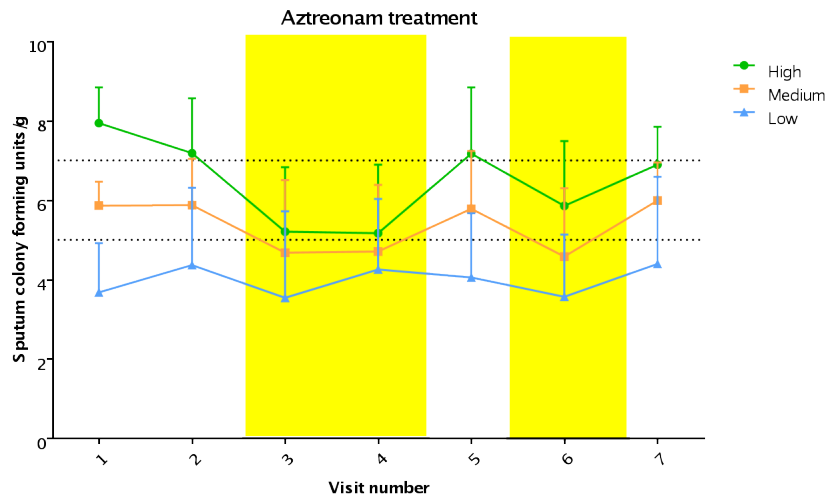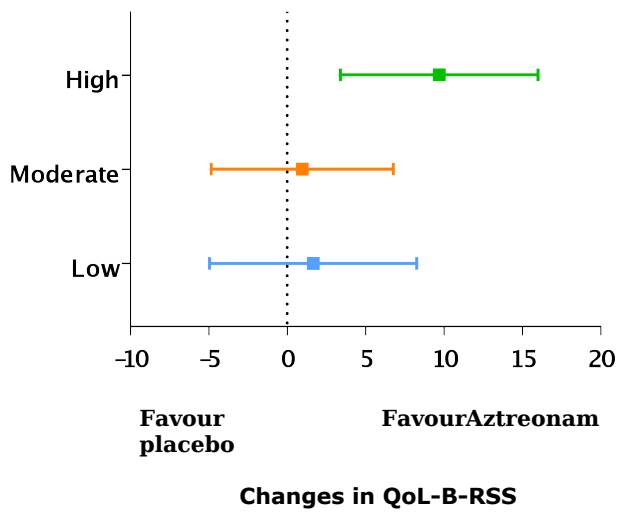
B

Percentages (%) of response

80

60

40

20

0

Low   Moderate   High

p =

AZLI

Placebo

Bacterial Load

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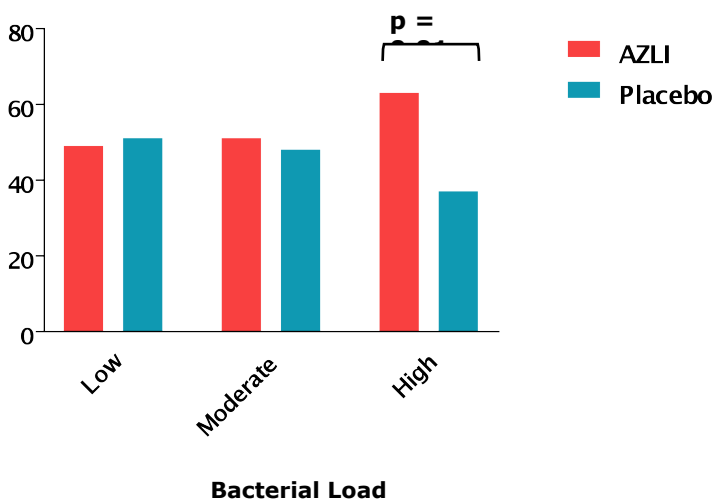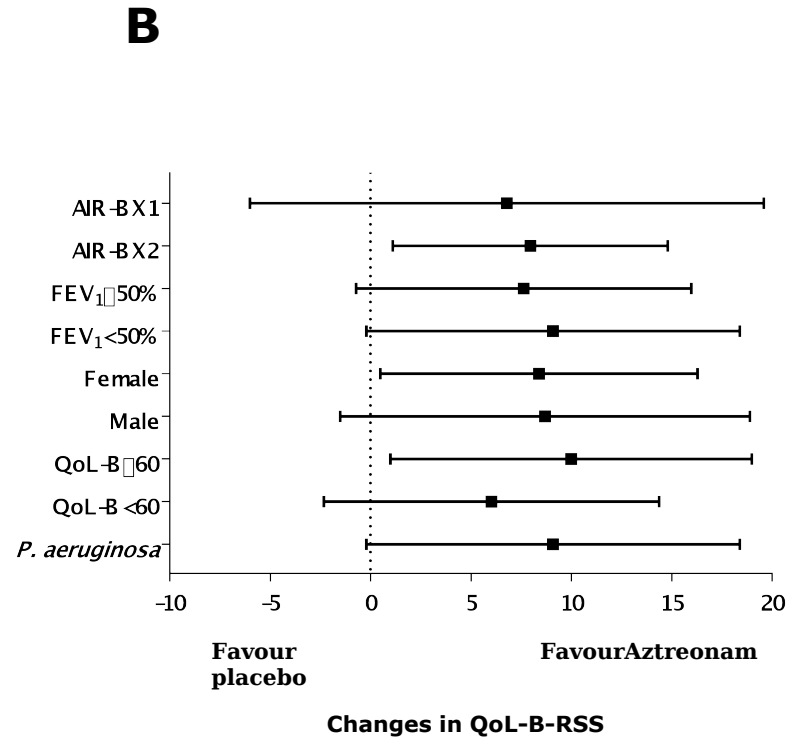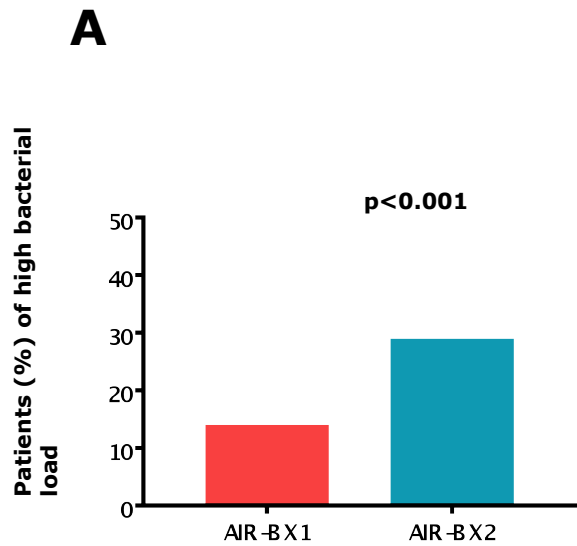
ONLINE SUPPLEMENTARY DATA

Airway bacterial load and inhaled antibiotic response in Bronchiectasis

Oriol Sibila [1,2], Elena Laserna [3], Amelia Shoemark [4], Holly R Keir [4], Simon Finch [4], Ana Rodrigo-Troyano [1,2],

Lidia Perea [2], Mike Lonergan [4], Pieter C Goeminne [5,6], James D Chalmers [4].

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METHODS:

Study 1

We performed a cross-sectional study of consecutive adult patients with bronchiectasis attending a specialist bronchiectasis clinic at Ninewells Hospital, Dundee in the UK. The study was approved by the local research ethical committee and all participants gave written informed consent. Bronchiectasis was defined as presence of bronchial dilatation on high-resolution CT scanning with compatible clinical history of cough with sputum production and/or recurrent respiratory infections [10]. The inclusion criteria were age $\geq$18 years, HRCT confirmed bronchiectasis affecting 1 or more lobes and the ability produce a spontaneous sputum sample for microbiology and biomarker measurement. Patients with cystic fibrosis, primary immunodeficiency (eg, common variable immunodeficiency), active malignant disease, active allergic bronchopulmonary aspergillosis, interstitial lung disease, active mycobacterial disease (including non-tuberculosis mycobacteria), HIV infection or long-term oral corticosteroid treatment were excluded.

Sputum was processed for qualitative and quantitative bacteriology as previously described [15]. Colony forming units (cfu) for each predominant pathogen were then identified by standard procedures and counted after 48 hours aerobic incubation on selective medium at 37ºC to determine the sputum bacterial load, expressed as log10cfu/gram (g) [18]. A priori patients were divided into low bacterial load ($<10^5$ cfu/g), moderate bacterial load (10^5-10^6 cfu/g) and high bacterial load ($\geq10^7$ cfu/g).

All patients were clinically stable as defined by the absence of an exacerbation that required antibiotic or steroid treatment within 4 weeks prior to inclusion at the time of clinical assessment. Demographic data was recorded as previously described [17]. The Bronchiectasis Severity Index (BSI) and FACED scores were also calculated [19,20].

Quality of life was determined using Quality of Life-Bronchiectasis Respiratory Symptoms score (QoL-B-RSS). Scores are 0-100, with higher scores representing fewer symptoms [21]. Spirometry was performed according to ERS/ATS recommendations for calculation of the forced expiratory volume in 1 second percentage of predicted value.

Neutrophil count in sputum was determined by cytospins. Sputum was ultracentrifuged at 50,000 G for 90 minutes. Inflammatory markers in sputum (CXCL8, TNF-α) were measured by ELISA (R+D systems, Abingdon, UK). Myeloperoxidase activity in sputum supernatants were measured by chromogenic assay as previously described [17]. We hypothesized that bacterial load would be associated with quality of life, severity of disease, forced expiratory volume in 1 second, and airway neutrophil biomarkers.

Study 2

Two prospective studies were performed to demonstrate the repeatability of a high bacterial load phenotype in bronchiectasis. These were single centre studies performed as a specialist bronchiectasis clinic at Ninewells Hospital, Dundee, UK. In the first study,26 patients with bronchiectasis (inclusion criteria as per study 1) were followed-up for one year, and sputum analyses were performed at baseline, after receiving systemic antibiotic treatment of at least 14 days due to an acute exacerbation [22] and after at least three months of recovery. Following the baseline visit patients were asked to contact the study team to report symptoms consistent with an exacerbation. If exacerbation was confirmed, using the British Thoracic Society definition of an exacerbation, the study team would prescribe antibiotics and proceed with taking an additional sputum sample for culture and biomarker measurement. Patients were asked to return to provide a further sputum sample at 14 days after antibiotic treatment and at 3 months post-treatment. The follow-up visit was deferred if the patient had

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received further systematic antibiotics (excluding stable macrolide) in the 2 weeks prior the stable visit. Sample processing for microbiology and biomarker measurement was as described for study 1 above.

In a second study of 10 stable patients with bronchiectasis (5 with idiopathic/post-infective and 5 with CF bronchiectasis) patients were followed-up for 14 days, and sputum analyses were performed at days 1,3,5,7 and 14. Patients were free from antibiotic treatment for a minimum of 28 days prior to this 14 day observation period. Quantitative sputum analyses were performed at every visit. Sputum were processed as previously described [15]. Demographic data, etiology, number of exacerbations in the previous year, lung function tests, and current inhaled or oral antibiotic treatments were recorded at inclusion. CF patients were included in the second study to validate that observations were consistent independent of aetiology. All patients were adults aged$\geq$18 years and were clinically stable at enrolment, defined by no antibiotic treatment for at least 4-weeks except for antibiotics taking prophylactically at stable dose. Patients were also asked to confirm the absence of exacerbation at the screening visit. Patients had to report daily sputum production and to be able to produce a sputum sample at the screening visit. Exclusion criteria for both groups were; inability to give informed consent; immunodeficiency, active mycobacterial infection; current smokers/ex-smokers of <1 year; active ABPA; pregnancy/breast feeding; long-term oxygen therapy and FEV_1<25% predicted. In addition specific inclusion criteria for CF subjects were: A diagnosis of CF with two disease causing CF transmembrane conductance regulator mutations;. Inclusion criteria for BE subjects: idiopathic or post-infective BE confirmed on CT; Exclusion criteria were: active sarcoidosis; alpha-1-antitrypsin deficiency; poorly controlled asthma and active malignancy. Patients attended at 9am on each study day (1,3,5,7 and 14 post enrolment) and provided a sputum sample for biomarker measurement and microbiology. Spontaneous sputum, induced sputum following induction and 24 hour sputum collection from the previous

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day were all collected as part of the study. We report only the spontaneous sputum results in this manuscript as they correspond to those obtained in study 1 and study 3. The primary objective of the study was to evaluate the day to day variability in sputum derived biomarkers including neutrophil biomarkers and airway bacterial load to facilitate power calculations for future studies.

The hypothesis of our analysis of these two studies was that high bacterial load would be a stable characteristic of bronchiectasis and therefore patients with increased bacterial load would have consistent high bacterial load during stable state and would return to high bacterial load following antibiotic treatment.

Study 3

We conducted a secondary analysis of two randomized double-blind, placebo-controlled phase 3 trials of Aztreonam for inhalation solution (AZLI) in bronchiectasis (AIR-BX1 and AIR-BX2) [5]. These trials included patients with CT-chest scan confirmed bronchiectasis with a positive sputum or bronchoscopic culture for Gram-negative organism or treatment of exacerbation with Gram-negative coverage in the previous 5 years, positive sputum culture for target Gram-negative bacteria at screening, chronic sputum production and $FEV_1 \geq 20\%$ of predicted. Pre-defined target Gram-negative respiratory pathogens were species of *Pseudomonas*, as well as *Achromobacter*, *Burkholderia*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Moraxella*, *Proteus*, *Serratia* and *Stenotrophomonas*. Exclusion criteria were hospital admission in the previous 2 weeks, haemoptysis of more than 30 mL or antibiotic treatment in the previous 2 weeks, changes in other treatments in previous 4 weeks and current treatment for non-tuberculous mycobacteria infection. The study sponsor, Gilead, included in the

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original consent form an optional consent for patients to allow future re-analysis of their data and sharing of their data with independent researchers. Only where the investigators and patients had signed this consent could the data be made available to us for analysis.

Patients received two 4-week cycles of double-blind inhaled treatment with AZLI 75 mg or placebo given three times a day, separated by 4 weeks off-treatment. The study consisted for 7 visits, visit 1, screening, visit 2, baseline, visit 3 at 14 days after initiation of aztreonam or placebo, visit 4 at 28 days after initiation of aztreonam or placebo, visit 5 after 28 days off aztreonam or placebo (end of off-treatment period), visit 6 at the end of the second 28 day on-treatment cycle and visit 7 at the conclusion of the study when the patient was off-treatment.

The primary endpoint was change in the QoL-B-RSS between baseline and week 4. Minimum Clinical Important Difference (MCID) was considered an increase of 8 points [21]. Secondary outcomes were changes in the QoL-B-RSS at week 12, which was the end of the second cycle of double blind treatment. Additional exploratory endpoints included changes in 6-minute walk distance at 4 weeks, change in the EQ5D questionnaire at 4 weeks, change in bacterial load at 4 weeks and the frequency of exacerbations during the study.

Bacterial load in cfu /g of target Gram-negative pathogens was measured in sputum at baseline by a central laboratory.

Based on our own previous work [15,17], we divided patients a priori into 3 groups; low bacterial load ($<10^5$ cfu/g), moderate bacterial load (10^5-10^6 cfu/g) and high bacterial load ($\geq10^7$ cfu/g).

RESULTS

Table E1. Baseline characteristics of patients with bronchiectasis (n=26) included in the Exacerbation Study.

Age (years)	68 (60-73)
Gender (% female)	16 (61.5%)
MRC (dyspnea Score)	3 (2-4)
Smoking status (never)	22 (84.6%)
Aetiology	
Idiopathic	12 (46.2%)
Post-infective	6 (23.1%)
Others	8 (30.8%)
Reiff score	8 (5-10)
FEV_1 (% predicted)	65.7 (42.1-85.6)
FVC (% predicted)	75.6 (55.9-95.4)
BMI (kg/m^2)	23.6 (20.7-26.4)
Exacerbations previous year	3 (1-4)
BSI total score	12 (7-16)

Data is presented as median (IQR) or number (%).

MRC= Medical Research Council; FEV1=forced expiratory volume in 1 s; FVC=forced vital capacity; BMI=body mass index kg/m2; BSI= Bronchiectasis Severity Index.

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Table E2. Clinical characteristics of patients with bronchiectasis (n=10) included in the day to day variability study.

Age (years)	48.5 (22.7-68.5)
Gender (% female)	4 (40%)
MRC (dyspnea Score)	1 (1-2)
Smoking status (never)	9 (90%)
Aetiology	
CF	5 (50%)
Post-infective	3 (30%)
Idiopathic	2 (20%)
Inhaled antibiotic	1 (10%)
FEV_1 (% predicted)	70 (45.7-88.5)
FEV_1 (L)	2.4 (1.4-2.9)
BMI (kg/m^2)	24 (20.2-27)
Exacerbations previous year	1 (0-3)

Data is presented as median (IQR) or number (%).

MRC= Medical Research Council; FEV1=forced expiratory volume in 1 s; BMI=body mass index kg/m2.

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Table E3. Patient demographics of all patients included in AIR-BX 1 and AIR-BX 2 studies and those who had consented to have their data reanalyzed.

	AIR-BX1 n=266 (49.2%)	AIR-BX1 Reanalysis n=181 (40.9%)	AIR-BX2 n=274 (50.7%)	AIR-BX2 Reanalysis n=259 (58.5%)
Age, years Mean (SD)	64.6 (12.5)	64.8 (11.5)	62.7 (13.3)	63.1 (13.7)
Female sex	181 (68%)	126 (69%)	190 (69%)	180 (69%)
FEV1 % predicted Mean (SD)	62.4 (20.8)	60.8 (19.8)	63.6 (20.5)	63.2 (20.7%)
6MWT distance Mean (SD), m	423 (118)	421 (114)	426 (124)	423 (124)
QOL-B-RSS Mean (SD)	55.2 (19.3)	54.6 (19.2)	56.8 (18.0)	56.7 (18.03)
Pseudomonas aeruginosa	217 (82%)	146 (80%)	219 (80%)	211 (81%)
Treatment group	134 (50.3%)	89 (49.2%)	136 (49.6%)	130 (50.2%)
Placebo group	132 (49.6%)	92 (50.8%)	138 (50.3%)	129 (49.8%)

Data are presented as n(%) unless otherwise indicated.

FEV1=forced expiratory volume in 1 s; 6MW=6-minute walk test; QOL-B-RSS=Quality of Life-Bronchiectasis Respiratory Symptoms scores; SD=standard deviation.

p value >0.1 in all comparisons.

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Table E4. Patient demographics of patients included in the reanalysis according to bacterial load

	Low bacterial load (n=147)	Moderate bacterial load (n=172)	High Bacterial load (n=102)	p value
Age, years **Mean (SD)**	63.5 (12.7)	65.1 (12.3)	61.5 (14.0)	0.08
Female sex	95 (64%)	127 (73%)	71 (69%)	0.20
FEV1 % predicted **Mean (SD)**	64.3 (21.1)	62.2 (20.3)	58.7 (18.8)	0.10
6MWT distance **Mean (SD), m**	421 (126.4)	414 (110.9)	431 (128.3)	0.51
QOL-B-RSS **Mean (SD)**	58.4 (18.0)	53.9 (18.6)	53.9 (17.9)	0.06
Pseudomonas ***aeruginosa***	93 (63%)	156 (90%)	90 (88%)	0.00

Data are presented as n(%) unless otherwise indicated.

FEV1=forced expiratory volume in 1 s; 6MW=6-minute walk test; QOL-B-RSS=Quality of Life-Bronchiectasis Respiratory Symptoms scores; SD=standard deviation.