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Azithromycin to prevent *Pseudomonas aeruginosa* ventilator-associated pneumonia by inhibition of quorum sensing: a randomized controlled trial

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Abstract *Purpose:* Anti-virulence strategies have not been evaluated for the prevention of bacterial infections. Prolonged colonization of intubated patients with *Pseudomonas aeruginosa* isolates producing high-levels of the quorum sensing (QS)-regulated virulence factor rhamnolipids has been associated with ventilator-associated pneumonia (VAP). In this pathogen, azithromycin reduces QS-regulated virulence. We aimed to assess whether azithromycin could prevent VAP in patients colonized by rhamnolipids producing isolates. *Methods:* In a randomized, double-blind, multicenter trial, intubated colonized patients received either 300 mg/day azithromycin or placebo. Primary endpoint was the occurrence of *P. aeruginosa* VAP. We further identified those patients persistently colonized by isolates producing high-levels of rhamnolipids and therefore at the highest risk to develop VAP linked to this QS-dependent virulence factor. *Results:* Ninety-two patients were enrolled; 43 azithromycin-treated and 42 placebo patients were eligible for the per-protocol analysis. In the per-protocol population, the occurrence of *P. aeruginosa* VAP was reduced in the azithromycin group but without reaching statistical significance (4.7 vs. 14.3 % VAP, $p = 0.156$). QS-dependent virulence of colonizing isolates was similarly low in both study groups, and only five patients in each arm were persistently colonized by high-level rhamnolipids producing isolates. In this high-risk subgroup, the incidence of VAP was reduced fivefold in azithromycin versus placebo patients (1/5 vs. 5/5 VAP, $p = 0.048$). *Conclusions:* There was a trend towards reduced incidence of VAP in colonized azithromycin-treated patients. In addition, azithromycin significantly prevented VAP in those patients at high risk of rhamnolipid-dependent VAP, suggesting that virulence inhibition is a promising antimicrobial strategy.

Keywords *Pseudomonas aeruginosa* · Ventilator-associated pneumonia · Virulence · Quorum sensing · Rhamnolipids

Introduction

Multi-drug-resistant bacteria represent a major medical threat, contributing to the deaths of increasing numbers of patients worldwide. To face this serious challenge, alternative strategies aiming at the inhibition of bacterial virulence have been proposed [1]. Due to the absence of selective pressure, such strategies are believed to minimize the risk of emergence of resistant clones [2, 3]. To our knowledge, however, no clinical study has demonstrated the feasibility of such an approach.

Pseudomonas aeruginosa is a leading cause of ventilator-associated pneumonia (VAP) [4–6]. This pathogen frequently develops resistance to available antimicrobial agents [7–9]. Improved treatments and/or preventive measures are urgently required since attributable mortality of this condition remains high [6]. Whereas almost all cases of *P. aeruginosa*-associated VAP are preceded by colonization of the respiratory tract, only 10–20 % of colonized patients eventually evolve to VAP [4]. Quorum sensing (QS) is a complex signaling network that allows coordinated gene expression according to cell density [1] and regulates many major virulence factors in *P. aeruginosa* [10]. As a consequence, QS is an ideal target for anti-virulence strategies. Macrolides, like azithromycin, are neither bactericidal nor bacteriostatic for *P. aeruginosa* at clinically achievable concentrations and are therefore unlikely to select resistant clones. However, at these concentrations, azithromycin inhibits QS in vitro [11–14]. Furthermore, we have shown that azithromycin reduces the expression of QS-circuit and target genes *in patient* [15]. Azithromycin has a favorable profile in terms of pharmacokinetics and of metabolism. Lung tissue distribution is high, enabling to reach QS-inhibitory concentrations (2–4 μg/ml) in bronchial mucosa, epithelial lining fluid, and sputum after a single 500-mg dose [16]. No clinical trial has previously investigated a virulence inhibition strategy for the prevention of bacterial infections. Thus, we decided to conduct a proof-of-concept study, designed as a multicenter, randomized, double-blind, placebo-controlled trial to evaluate azithromycin for the prevention of *P. aeruginosa* VAP in colonized mechanically ventilated patients.

Methods

Study population

This pilot, randomized, placebo-controlled, double-blind study (ANB 006#2001, ClinicalTrials.gov ID#NCT00 610623) was designed to assess the efficacy of azithromycin as a quorum-sensing inhibitor in preventing the occurrence of *P. aeruginosa* pneumonia in ventilated patients with documented colonization in bronchial aspirates at study entry. We obtained approval for this multicenter European study by the respective local ethics committees and national agencies. Written consent from all patients or their legal representatives was obtained according to legal and ethical considerations. The study took place between November 2002 and November 2005. Twenty-one European centers participated in this trial; eight in France, four in Spain, two in Belgium, three in Poland, two in Serbia, and two in Switzerland. We screened mechanically ventilated patients for respiratory tract colonization by *P. aeruginosa* on alternate days. Eligible patients were adults between 18 and 75 years of age, hospitalized in the ICU under mechanical ventilation expected to be required for at least 3 days, with a reasonable surviving chance (Apache score between 10 and 25), and proven to be colonized by *P. aeruginosa*. Neutropenic patients and patients treated with immunosuppressive drugs were not eligible. Patients with ongoing *P. aeruginosa* infection, having received macrolides or antibiotics active against the colonizing *P. aeruginosa* isolate during the last 14 days were excluded. Patients with proven colonization by *P. aeruginosa* were randomized (D-1) and received either placebo or 300 mg per day iv azithromycin in a double-blind fashion for a maximum of 20 days (D1 to Dx). The 300-mg dose was selected taking into account a bioavailability of 37 % of the usual 500-mg oral dose, the need for reaching steady state at lung level within 3 days, and safety considerations.

During the study, the administration of antibiotics proven to be inactive against the isolated *P. aeruginosa* strains was allowed without restrictions. The administration of antibiotics with intrinsic activity against *P. aeruginosa* strains was only allowed if considered as mandatory. The prophylaxis study period was restricted to a maximum of 20 days, and reasons for early discontinuation included extubation, death, serious adverse events, or suspected *P. aeruginosa* VAP. The diagnosis of *P. aeruginosa* VAP was based on the clinical picture, X-ray scan, a pulmonary infection score (CPIS) $\geq$6, as well as a quantitative culture of a bronchoalveolar lavage fluid (BAL) yielding $>10^4$ CFU/ml *P. aeruginosa*, in the absence of other pathogenic bacterial species [17, 18]. An independent panel of three experts blinded to treatment confirmed the *P. aeruginosa* VAP cases.

Clinical sample collection

Starting the first day of proven colonization (D-1), daily tracheal aspirates (usually 0.3–5 ml) and *P. aeruginosa* strains were collected during the entire study period. Samples were frozen at −80°C on site within 15 min, and sent on dry ice to the reference research laboratory at the University Hospital Geneva, where all analyses were performed in a blind fashion.

Study design and statistical analysis

The study was initiated and financially supported by Anbics Corporation and a grant from the Swiss Ministry of Technology (Bundesamt für Berufsausbildung und Technologie, Kommission für Technologie und Innovation, KTI). The study objective as defined per protocol was to include ten *P. aeruginosa* VAP cases confirmed by the independent expert panel. This was based on the assumption of a 10 % occurrence of pneumonia in patients colonized by *P. aeruginosa* in the placebo group, with a total patient number between 100 and 200 assessable patients. The statistical objective defined in the study protocol (10 % occurrence of VAP in placebo group, two-sided, $p = 0.10$; Fisher's exact test) required one *P. aeruginosa* VAP on azithromycin versus nine on placebo to reach significance ($p = 0.016$), or two *P. aeruginosa* VAP on azithromycin versus eight on placebo for a trend ($p = 0.092$). Because Anbics Corporation was dissolved prematurely, patient recruitment had to be stopped before reaching ten confirmed VAP cases. The investigators continued the laboratory and statistical analysis of all clinical samples, supported by grants from the Swiss National Science Foundation. The analysis was performed on the per-protocol basis. Using the placebo group of the present trial, we have described patients persistently colonized with *P. aeruginosa* strains producing elevated levels of the QS-dependent virulence factor rhamnolipids as a particular risk group to develop QS-related VAP [19, 20]. Therefore, we performed a second analysis in this sub-population. Two-sided Fisher's exact tests were used to test differences between group rates. Odds ratios and 95 % confidence intervals were also calculated for all parameters. Two-sided independent-sample *t* tests were used to test differences between group-specific means, assuming equal variances.

Results

Study group description

Out of 92 randomized patients, seven had to be excluded for major protocol violations including absence of *P. aeruginosa* colonization at study entry, treatment with macrolides or agents active against the colonizing *P. aeruginosa* isolates, and premature death (Fig. 1). Forty-two placebo and 43 azithromycin patients were included in the per-protocol analysis. The treatment groups were well balanced for race, age, gender, and severity-of-disease (measured by Apache II score) (Table 1). The most frequent medical reasons for requiring mechanical ventilation were surgical complications, deterioration of underlying diseases (congestive heart and/or acute respiratory failure) and acute respiratory distress syndrome, which were all equally distributed in both study groups. Twenty-three azithromycin and 28 placebo patients received a systemic antibiotherapy during the study period ($p = 0.270$, Table 2). Antibiotics devoid of activity against *P. aeruginosa* were administered to 21 azithromycin and 25 placebo patients ($p = 0.386$). Agents active against *P. aeruginosa* (ciprofloxacin, aminoglycosides, piperacillin/tazobactam, ceftazidime, aztreonam or carbapenems) were administered to seven azithromycin and eight placebo patients ($p = 0.783$, Table 2) for non-respiratory tract infections, which occurred mainly during the last days of the study. In six of these patients (three in each group), this treatment was active against the colonizing *P. aeruginosa* isolates. None of them developed *P. aeruginosa* VAP and only one placebo patient belonged to the high-risk group for developing QS-related VAP (as defined by persistent colonization with isolates producing high levels of the QS-dependent virulence factor rhamnolipids, see below). The colonizing isolates of the nine other patients (four azithromycin and five placebo) were found to be resistant to the antibiotic used. Azithromycin exposure did not lead to an MIC increase comparing the initial and last *P. aeruginosa* isolates (data not shown). As compared to the placebo group, no increased incidence of infections, whether due to *P. aeruginosa* or other bacteria, was detected at other sites in the azithromycin-treated group. We observed no significant differences in the distribution of *P. aeruginosa* genotypes or virulence determinants (production of the QS-dependent virulence factors elastase and rhamnolipids, presence of the *Pseudomonas* pathogenicity islands PAI-1 and PAI-2, and the phospholipase gene *ExoU*) between the two study groups (Table 3, for details see online supplementary data). We have recently shown that patients can be classified into three risk-categories for VAP according to the production of rhamnolipids of their colonizing isolates [20]. Whereas 11 placebo [20] and seven azithromycin-treated (Supplementary Fig. 1B and C) patients were persistently colonized by isolates producing low levels, 13 placebo and 18 treated patients were colonized by isolates producing intermediate levels, and five patients in each study group were persistently colonized by isolates producing high levels of rhamnolipids (Table 3).

Primary clinical endpoints

In the intention-to-treat analysis, the mean duration of treatment was 9.5 days for the azithromycin-treated and 10.8 days for the placebo groups (Table 4). Eight azithromycin and ten placebo patients were treated for the entire study period of 20 days ($p = 0.604$). Reasons for early discontinuation were similar between the treatment groups when assessing for serious adverse events, non-pseudomonal pneumonia, extubation, and death unrelated to *P. aeruginosa* VAP (Table 4). Concerning the primary study end-point on the per-protocol population,

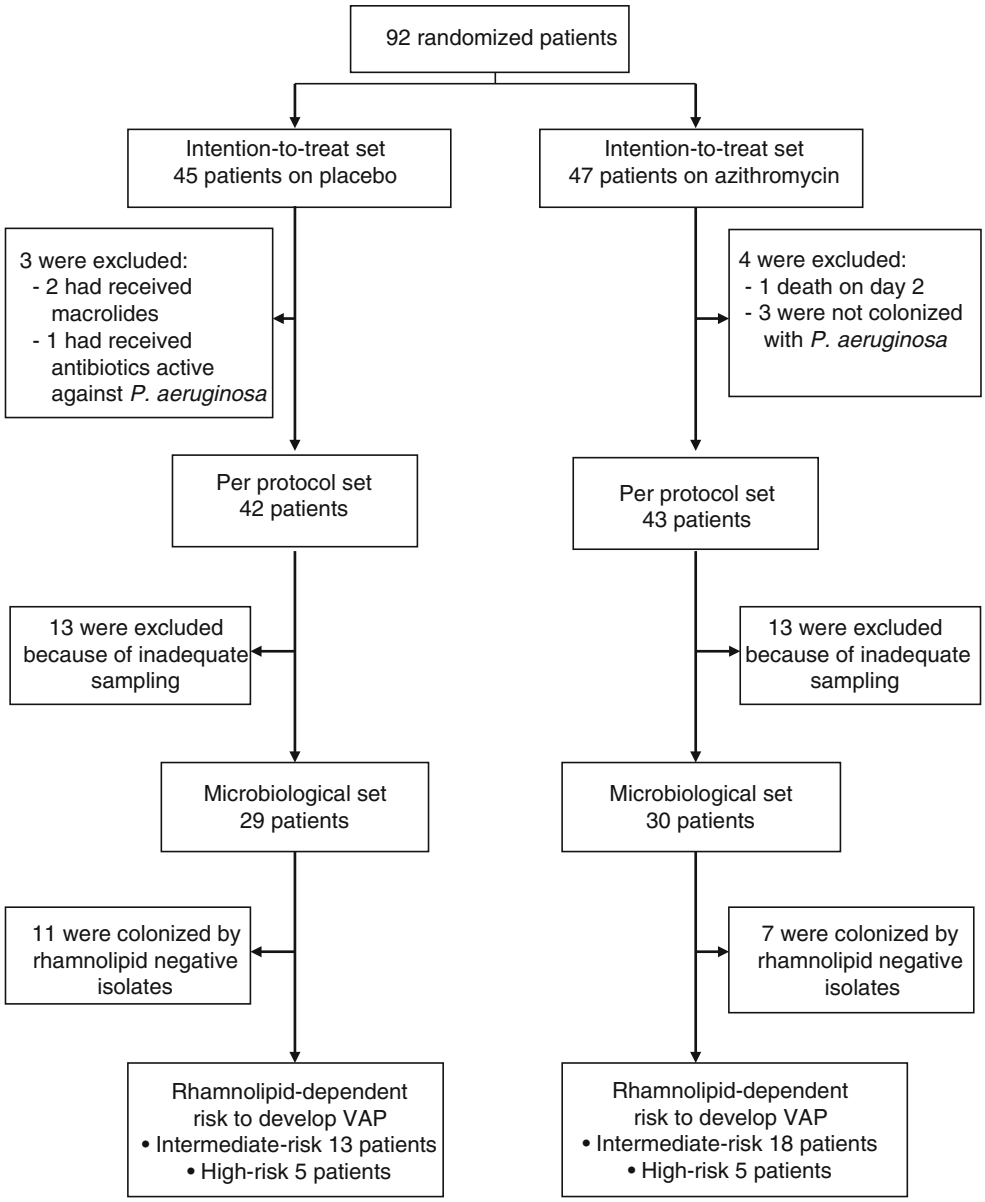

Fig. 1 Patient enrolment and follow-up

P. aeruginosa VAP occurred in 2/43 azithromycin-treated and in 6/42 placebo patients (4.7 %, respectively, 14.3 %, odds ratio; 3.42, CI 95 %; 0.65–18.00, $p = 0.156$) during the prophylaxis study window (Table 5). One patient of each study group (placebo patient #13116 [20] and treated patient #13115, supplementary Fig. 1A) colonized by ExoU-positive isolates developed *P. aeruginosa* VAP.

Clinical efficacy of azithromycin in patients colonized by isolates producing the QS-dependent virulence factor rhamnolipids

It became apparent after study completion that many patients of both study arms were colonized by either completely or partially QS-deficient isolates. Therefore, many patients were at low risk of developing "QS-dependent" VAP. QS-inhibition by azithromycin was likely to be ineffective, respectively of less clinical benefit to these patients, as compared to patients colonized by fully QS-proficient isolates [19, 20]. We therefore performed a subanalysis including only those patients in whom the QS inhibition by azithromycin could be effective. First, we compared the occurrence of VAP in patients colonized by isolates producing intermediate or high levels of rhamnolipids (Table 3). In this subgroup, *P. aeruginosa* VAP occurred in 5/18 placebo and in 1/23 azithromycin-treated patients (Table 5) (odds ratio 8.46, CI 95 % 0.89–80.59, $p = 0.07$). Using the placebo group of the present study, we have previously shown that

Table 1 Baseline characteristics

Variable	Azithromycin	Placebo
Patient randomization		
Patients in the intention-to-treat group	47	45
Patients in the per-protocol group	43	42
Patient demographics		
Race		
Caucasian	45	43
Other	2	2
Age: Mean (years ± SD)	59.3 (16.98)	59.7 (15.18)
Gender		
Male	28	28
Female	19	17
Apache II score		
Mean (±SD)	14.9 (3.82)	14.3 (3.79)
Underlying diseases		
Surgical complications	16 (34 %)	18 (40 %)
Visceral	6 (13)	6 (13 %)
Cardiovascular	5 (11 %)	6 (13 %)
Pulmonary	1 (2 %)	1 (2 %)
Transplantation	0 (0 %)	1 (2 %)
Multiple or thoracic trauma	4 (9 %)	4 (9 %)
Acute respiratory distress syndrome	4 (9 %)	4 (9 %)
Aspiration	0 (0 %)	3 (7 %)
Sepsis	4 (9 %)	1 (2 %)
Deterioration of underlying diseases	17 (36 %)	13 (29 %)
Heart failure	6	2
Respiratory failure	11	11
Other	10 (21 %)	14 (22 %)

Table 2 Administration of antibiotics

Type of therapy	Azithromycin	Placebo	*p* value
Systemic antibiotherapy	23 (54 %)	28 (67 %)	0.270
Non anti-pseudomonal antibiotics	21 (49 %)	25 (60 %)	0.386
Anti-pseudomonal antibiotics	7 (16 %)	8 (19 %)	0.783
Antibiotics active against colonizing *P. aeruginosa* isolates	3 (7 %)	3 (7 %)	1.0

patients at highest risk for developing QS-related VAP are those persistently colonized by isolates producing high levels of rhamnolipids [15]. Considering only this high-risk subpopulation, *P. aeruginosa* VAP occurred in 1/5 azithromycin-treated and in 5/5 placebo patients (20 vs. 100 %, $p = 0.047$) during the prophylaxis study window (Table 5). One azithromycin-treated patient developed acute *P. aeruginosa* pneumonia outside of the prophylaxis study window (48 h after his extubation and study drug discontinuation).

Table 3 Virulence determinants of initial colonizing isolates

Variable	Azithromycin ($n = 30$)	Placebo ($n = 29$)	*p* value[a]
Genotypes			
Number of clones	22	18	NA
Clone F469	2	7	0.080
PAPI-1	3	2	1.0
PAPI-2	26	25	1.0
ExoU	9/26	11/25	0.573
Initial isolates producing reduced levels of QS-dependent virulence factors	24	22	0.761
Longitudinal production of rhamnolipids:			
High	5	5	1.0
Intermediate	18	13	0.302
Low	7	11	0.267

NA Not applicable (total number of clones)
[a] Fisher's exact test

Table 4 Reasons for discontinuation of treatment among azithromycin-treated and placebo patients

	Azithromycin ($n = 47$)	Placebo ($n = 45$)	*p* value[a]
Mean duration of treatment (days ± SD)	9.5 (5.97)	10.8 (6.11)	NS
Patients treated for 20 days	8	10	0.604
Reason for treatment discontinuation			
Extubation	24/39	17/35	0.350
Non-pseudomonal pneumonia	5/39	9/35	0.235
Serious adverse event	3/39	1/35	0.617
Death	2/39	1/35	1.0
Other	4/39	7/35	0.330

[a] Fisher's exact test

Table 5 *P. aeruginosa* VAP in azithromycin-treated and placebo patients

P. aeruginosa VAP	Azithromycin	Placebo	Odds ratio (CI 95 %)	*p* value[a]
Per-protocol set	2/43	6/42	3.42 (0.65; 18.00)	0.156
According to rhamnolipid-dependent risk				
High and intermediate risk	1/23	5/18	8.46 (0.89; 80.59)	0.070
High risk	1/5	5/5	NA	0.048

NA not applicable
[a] Fisher's exact test

Safety and tolerance

The safety profile was assessed in 92 patients up to 7 days after completion of the prophylaxis study window. The adverse event (AE) profile was balanced between the azithromycin and placebo groups. At least one treatment-

related AE was reported for 11 versus 13 % of patients and serious treatment-related adverse events occurred in one azithromycin (bradycardia and bronchospasm) and two placebo patients (ventricular fibrillation, massive pneumonic infiltrate). In the intention-to-treat set, nine azithromycin and six placebo patients died, while in the per-protocol set, six patients of each treatment group died. None of these deaths was considered drug-related, and the majority (9/12) occurred after cessation of treatment (Table 4). No increased risk for infections and/or emergence of resistance to antibiotics was identified. A single case of pseudomembranous colitis occurred in an azithromycin-treated patient.

Discussion

This multicenter, placebo-controlled trial of 92 intubated patients colonized by *P. aeruginosa* is the first pilot trial investigating the efficacy of an anti-virulence strategy to prevent a major infection. Such new interventions are desperately needed in view of the rising concern about infections caused by multi-resistant bacteria. Unfortunately, the study population was smaller than initially planned as a result of a premature study stop due to discontinuation of financial support. Consequently, the study was not sufficiently powered to detect statistically significant differences in the incidence of VAP in the per-protocol analysis.

We used the macrolide azithromycin, known to be neither bactericidal nor bacteriostatic on *P. aeruginosa*, but which inhibits QS-dependent virulence [11, 14]. We applied special care to determine both QS-dependent and QS-independent virulence determinants of sequential isolates of both study populations since any differences could have potentially influenced the efficacy of an anti-virulence intervention. Both study populations were colonized by isolates with similar virulence profiles, however, to our surprise, many patients were colonized by QS-deficient isolates. Studies performed on the placebo group of the present trial have shown that these patients are at low risk of developing QS-related VAP [19, 20]. Obviously such patients could not be anticipated to benefit from the anti-QS activity of azithromycin, reducing further the population under survey. Added to the premature stop of patient recruitment, this might explain the absence of a more significant advantage in the per-protocol analysis, although an encouraging difference was seen (4.7 vs. 14.3 %). Moreover the complex work performed on the tracheal isolates and aspirates from the placebo group allowed us to dissect the role of QS phenotypes after completion of the study. Detailed discussions concerning the implications of genotypes and virulence phenotypes, as well as dynamics of *P. aeruginosa* populations, have been published elsewhere [15, 19–21]. It became apparent that only patients persistently colonized by isolates producing elevated levels of the QS-dependent virulence factors rhamnolipids were actually at high risk of developing VAP [20]. While we observed a trend ($p = 0.07$) towards less VAP cases in the subgroup of azithromycin-treated patients colonized by isolates producing detectable levels of rhamnolipids, a protective effect of azithromycin became apparent ($p = 0.048$) in those treated patients who were colonized by high rhamnolipid producers, potentially preventing four out of five of these high-risk patients from developing VAP.

Two patients, one in the placebo (#13116) and one in the azithromycin-treated group (#13115), developed VAP while colonized by *lasR-rhlR* double mutants. These isolates carried the *exoU* gene encoding a phospholipase, which is translocated through the type III secretion system (TTSS) and associated with increased mortality from pneumonia [22]. However, whether colonization with *exoU* isolates is a risk factor for progression from colonization to VAP is unknown, and our results suggest that this is not the case [20]. It appears that *exoU* is not associated with the risk for VAP but with its severity. Interestingly, the TTSS is negatively controlled by QS [23, 24]. Therefore, inhibition of QS by azithromycin should have potentially increased the expression of type III cytotoxins, and therefore the risk for severe VAP. In the present study, 11 placebo and nine azithromycin-treated patients were colonized by strains harboring *exoU*. As only one patient of each study group developed VAP while colonized by *exoU*-positive isolates, this cannot have accounted for the increased incidence of VAP in the placebo group.

We have previously shown that a natural cooperative selection process during *P. aeruginosa* colonization led to progressive replacement of QS-proficient by QS-deficient isolates (cheater cells) due to increase in frequency of *lasR* mutants in the placebo group [19]. Remarkably, using the patients of the present study, we showed that QS inhibition by azithromycin reduced this natural selection towards QS-deficient isolates [15]. As a consequence, treated patients were more frequently colonized at study end with QS-competent, highly virulent bacteria [15]. This potentially increased their risk of infection once the prophylaxis was stopped, as suggested by the occurrence of *P. aeruginosa* pneumonia outside of the prophylaxis study window in one treated patient. It should induce caution upon use of antivirulence strategies in general, and special care should be taken in future studies in covering for the risk of post-ventilation infection by extending prophylaxis after extubation, at least for a few days.

Our study has several limitations. (1) The study was underpowered to detect differences in the per-protocol

analysis and caution has to be taken when extrapolations are made from analyses performed on sub-groups defined after study closure. (2) As we did not measure rhamnolipid concentrations in respiratory secretions, we cannot fully exclude whether part of the beneficial effect of azithromycin could have been mediated through its immunomodulatory activities, or through modifications of the lung-resident microbial flora and not through inhibition of rhamnolipid production [25, 26]. However, one would expect that any immunosuppressant activity of azithromycin would have potentially worsened the progression to and/or severity of an acute infection such as *P. aeruginosa* VAP. This was not observed in our trial. Moreover, the resident microbial flora is more likely to protect from *P. aeruginosa* VAP, and therefore its elimination by azithromycin should have increased the risk of VAP instead of protecting from it. Furthermore, using the patients of the present trial, we have previously demonstrated that QS-gene expression, required for rhamnolipid production, was reduced in the azithromycin-treated as compared to the placebo group [15]. These results provide indirect proof that the prevention of VAP in patients colonized by QS-proficient isolates most likely resulted from inhibition of QS-gene expression, and reduction of rhamnolipid production, by azithromycin. (3) Even if the administration of antibiotics was strictly controlled during the entire study, three patients in each group received antibiotics active against their colonizing isolates. Only one of these patients was colonized by high-rhamnolipid producers and received placebo. Therefore, it is unlikely that this might have influenced the outcome of the subgroup analysis in favor of the azithromycin group. (4) We did not investigate whether azithromycin potentially modifies the antibiotic susceptibly of the gut flora. However, azithromycin did not select for increased resistance among *P. aeruginosa* isolates and did not elicit an increased incidence of infections with resistant non-Pseudomonal strains.

In conclusion, this study is the first randomized placebo-controlled trial evaluating the efficacy of an anti-virulence intervention for the prevention of a major nosocomial infection. Our results suggest that azithromycin might not prevent *P. aeruginosa* VAP in all intubated colonized patients. However, a subgroup of patients, at high risk of developing *P. aeruginosa* VAP as a consequence of their colonization by rhamnolipid-producing isolates, might benefit from the QS-blocking activity of azithromycin. These data stress the need for a large phase IIb clinical trial restricted to patients colonized by QS-proficient isolates to further establish safety and efficacy of QS inhibition by azithromycin to prevent *P. aeruginosa* VAP.

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