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Intermittent inhaled tobramycin and systemic cytokines response in CF patients with *Pseudomonas aeruginosa*

Abstract

Introduction: CF pulmonary guidelines recommend alternate therapy (one month on, one month off) with inhaled tobramycin for chronic *Pseudomonas aeruginosa* colonization in cystic fibrosis (CF). Tobramycin-inhaled powder (TIP™) is increasingly replacing time-consuming nebulizer therapy. It is unclear whether laboratory parameters change during the month off period compared with the month on therapy.

Purpose: Our aim was to assess whether spirometry, lung clearance index and circulating inflammatory markers differ between on/off treatment periods.

Materials and methods: A prospective pilot study evaluating CF patients treated with TIP, on two consecutive months (on/off) therapy. The evaluations were performed at the end of a month off therapy (1-2 days before the initiation of TIP) and after 28 days of treatment with TIP (1-2 days after the end of the treatment cycle).

Results: Nineteen CF patients (10 males) with a mean age of 18.7 ± 9.7 years and BMI (body mass index) of 19.62 ± 3.53 kg/m² were evaluated. After a month off treatment with TIP, spirometry parameters and lung clearance index remained unchanged. IL-6 increased significantly ($p=0.022$) off treatment. There was a non-significant change in the other inflammatory cytokines off therapy [hs-CRP, IL-8, TNF- α , α 1-antitrypsin (α 1AT) and neutrophilic elastase].

Conclusions: The results of lung function parameters support the relative stability of CF patients during the month off therapy; however, the difference in serum IL-6 raises the possibility of ongoing higher degrees of inflammation during the month off therapy with TIP. The small sample size and the multiple parameters evaluated preclude firm conclusions; therefore, larger multicenter studies are needed to assess the on/off treatment strategy.

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Cystic fibrosis (CF) is the most common fatal genetic disease in Caucasians (1,2). There are over 2,000 known mutations in the gene encoding for the cystic fibrosis transmembrane conductance regulator (CFTR). The CFTR defect in the airways leads to decreased muco-ciliary clearance, accumulation of thick and sticky mucus, persistent bacterial colonization and chronic inflammatory response (3). Despite substantial improvements in clinical management, chronic lung disease remains the major cause of morbidity and mortality in patients with CF (4–6). About 80% of adults with CF have chronic *Pseudomonas aeruginosa* (PA) colonization has been clearly associated with a more rapid decline in lung function (7), and multiple strategies for prevention and early treatment have been studied (8). It was previously associated with 50% death within five years; however, intensive early eradication therapy, intravenous courses of antibiotics and maintenance therapy have changed the prognosis (9).

Tobramycin solution for inhalation (TIS-TOBI[®] 300 mg/5 ml) was designed to deliver high concentrations of the anti-pseudomonal agent directly to the site of infection, with the dual aims of improving efficacy and reducing toxicity (10). Studies have indicated that TIS, in cycles of one month on and one month off, resulted in improved lung function, reduced respiratory exacerbations (11–13) and reduced mortality (14). Tobramycin inhaled powder (TIP[™] 112 mg twice daily) is increasingly replacing time-consuming nebulizer therapy. The delivery time is less than one-third the time required for nebulization; thus, it is expected to improve adherence to therapy (15). TIP was found to achieve higher sputum concentrations than TIS, and to be of equivalent efficacy when compared with TIS (16).

Airway infection and inflammation are the hallmarks of CF. Furthermore, the presence of PA and other pathogens in the lower airways of infants is hypothesized to increase the host inflammatory response, thereby accelerating the process of airway damage (17). This response is characterized by a marked influx of neutrophils into the lung and elevation of inflammatory mediators (18).

The new era of CF treatment includes a growing list of medications. Since it is becoming more difficult to demonstrate efficacy with therapies in CF, the use of systemic markers to assess treatment efficacy has been suggested. Several studies investigated the impact of antibiotic treatment on inflammatory markers in patients with PA infection, mostly in the bronchoalveolar lavage fluid or sputum (12,19,20). Blood biomarkers have the advantage of being available, minimally invasive and reflective of the entire lung. Serum inflammatory markers such as α 1-antitrypsin (α 1AT), IL-6 and IL-8 and

α 1AT neutrophilic elastase (α 1AT-NE) complex levels were found to be inversely correlated with FEV1 (21,22). Plasma C-reactive protein and neutrophil elastase complexes correlated most strongly with clinical measures at exacerbation onset, and decreased after antibiotic therapy (23). Overall, systemic measures of inflammation have been more consistent (24) than studies relying on sputum (25), including their response to treatment (26,27). The on/off treatment strategy presents an opportunity to define the effect of TIP therapy on systemic measures of inflammation and lung function parameters.

In a multicenter trial evaluating a panel of 15 plasma proteins in 103 CF patients, few inflammatory proteins were able to capture the response to treatment and were suggested to predict non-responders (23). These markers may also predict lung injury and may be useful in monitoring disease progression (28). TIS was previously demonstrated to have a modulatory effect on inflammation (12). TIP is now increasingly replacing nebulizer therapy. There are major differences between inhalation of a nebulized antibiotic and a dry powder inhaler (DPI), which may affect deposition and efficacy. We are not aware of a study evaluating the effect of TIP on modulation of inflammation. The possibility of clarifying which patients should be treated with on/off inhaled therapy and who should be switched to continuous alternating regimens is fascinating. Thus, the aim of this pilot study was to assess the differences between the month on and off therapy with TIP with regard to lung function tests and circulating inflammatory markers in CF patients colonized with PA. We hypothesized that the month on TIP would result in lower circulating inflammatory markers and may lead to better lung function.

Materials and methods

Participants

This was a single center prospective study. The institutional board reviewed and approved the study, and informed consent was obtained from subjects or their legal guardians prior to recruitment. The study population consisted of patients aged >6 years diagnosed with CF, chronically colonized with PA and receiving TIP (112 mg BID 28 days on and off) as a standard of care. Patients had to be able to properly perform pulmonary function tests and TIP inhaler technique. Chronic colonization with PA was defined as ≥ 3 positive cultures in the previous year.

Prior to the evaluations, short-acting and long-acting bronchodilators were not permitted for 6 h and 24 h,

respectively. All patients were instructed to take Pulmozyme inhalation daily at 13:00 PM in order to avoid possible increase in serum elastase levels after inhalation. Exclusion criteria included the following: an acute respiratory exacerbation requiring additional anti-pseudomonal agents (during the visit or the preceding week); treatment with continuous alternating regimen of inhaled antibiotics; systemic corticosteroids over the month prior to inclusion; and breast-feeding/pregnant females. Acute respiratory exacerbation was defined as a recent change in at least two of the following: change in sputum volume or color; increased cough; dyspnea; malaise; fatigue or lethargy; anorexia or weight loss; and decrease in pulmonary function by $\geq 10\%$ or radiographic changes. The evaluations were performed at two time points within a month interval: at the end of a month off therapy (1-2 days before the initiation of TIP); and after 28 days of treatment with TIP (1-2 days after the end of the treatment cycle). Evaluations included the following: spirometry; lung clearance index (LCI) measurements; a sputum culture and circulating markers of inflammation (serum hs-CRP [high sensitivity C-reactive protein], IL-6, IL-8, TNF- α , $\alpha 1$ AT and neutrophilic elastase).

Demographic variables were extracted anonymously from the patients' files, including age, gender and CF-related conditions (pancreatic insufficiency and CF-related diabetes).

Spirometry

Spirometry was performed in accordance with the ATS/ERS (American Thoracic Society/European Respiratory Society) Task Force, using a KoKo spirometer (n-Spire Healthcare, Longmont, CO, USA) (29). Results were expressed as absolute values and percent predicted derived from Polgar and Quanjer (30).

Lung Clearance Index (LCI)

Multiple breath washout (MBW) measurements were performed using the Easy-One Pro, MBW Module (NDD Medical Technologies, Zurich, Switzerland) as first described by Fuchs *et al.* in 2008 (31). WBreath software (NDD Medical Technologies) was used for data acquisition, storage and analysis.

As recommended, the minimum re-equilibration time between trials was the time needed for washout in the previous trial (32). An increased LCI (>7) indicated more FRC turnovers required for the washout, reflecting ventilation inhomogeneity (32–34).

Sputum

Sputum samples were examined for cultures as soon as possible, within 2 hours of collection. Sputum volume was measured, then diluted 1:1 with Sputasol (Thermo Scientific-SR0233), and plated on McConkey, Chocolate, UriSelec 4, Sabouraud dextrose and Mycobacteria agar media, in a quantitative manner. Bacterial identification was performed by MALDI-TOF MS and biochemistry tests.

Circulatory inflammatory markers

Venous blood was collected, processed and stored at -80°C . Serum high-sensitivity C-reactive protein (hs-CRP) was determined using a highly sensitive CRP assay. The sensitivity of the assay ranged between 0.5-240 mg/L. IL-6, IL-8, TNF- α , $\alpha 1$ AT and neutrophilic elastase were measured by commercial kits as follows: Human IL-6 quantikine ELISA Kit (R&D Systems- D6050), Human IL-8 Quantikine ELISA Kit (R&D Systems-D8000C), Human TNF- α Quantikine HS ELISA (R&D Systems-HSTA00D), Human α -1 Antitrypsin ELISA Kit (SERPINA1) (Abcam-ab189579) and Human PMN-Elastase Platinum ELISA Kit (Affymetrix eBioscience-BMS269), respectively. High-sensitive ELISA allows the measurement of multiple cytokines in small volume samples.

Statistical analysis

The primary outcome parameter was serum hs-CRP on compared with off therapy. All the other parameters, including spirometry, LCI and the other inflammatory markers, were considered as secondary outcome parameters.

Sample size was calculated according to CRP using Win Episcopo 2.0. Assuming that baseline CRP after one month off treatment would be 10 ± 4 mg/l and, at the end of TIP, would decrease by 20%, 35 CF patients were required.

Statistical analysis was performed by SPSS program version 21. Descriptive statistics in terms of means, medians, percentiles and ranges were used for the parameters. The normality of the quantitative parameters was tested by the Kolmogorov-Smirnov test. As a result of this test, parametric and non-parametric tests were used. Paired tests (by Wilcoxon signed rank test) were used for differences between pre and post measurements and $p < 0.05$ was considered as significant. Pearson correlation was used for the relation between the different circulatory inflammatory markers and pulmonary function parameters (spirometry values and LCI).

FIGURE 1. Individual changes of IL-6 on/off treatment therapy with TIP™

Results

Nineteen CF patients (10 males) with a mean age of 18.7 ± 9.7 years, median age 16.2 (range 8.4–41.7) years and BMI (body mass index) of 19.62 ± 3.53 kg/m² were evaluated. Table 1 shows the comparison of parameters evaluated at the end on and end off TIP therapy. As can be seen, there were no differences in pulmonary function parameters (spirometry and LCI) between the on/off TIP periods.

Interestingly, although the patients were treated for chronic colonization with *P. aeruginosa*, cultures taken at baseline were PA positive only in eight patients. The other 11 patients had their last positive culture within three months before baseline evaluations. There was no difference in the time elapsed since the first colonization of PA in both sub-groups; mean 54.9 ± 61.9 , median 32.5 (range 10–188) months, and mean 55.7 ± 35.9 , median 46.0 (range 20.5–112) months, in those whose baseline culture was PA-positive and -negative, respectively ($p=0.48$). Most cultures were positive for more than one organism. Five patients had co-infection with *Staphylococcus aureus*, two patients had *Mycobacterium simiae*, two patients had *Aspergillus fumigatus*. Additionally, *Streptococcus viridans* ($n=1$), *Streptococcus pneumoniae* ($n=1$), *Enterobacter* ($n=1$) and *Escherichia coli* ($n=1$) were found.

Plasma cytokines

As can be seen, no significant differences were found in serum hs-CRP, IL-8, TNF- α , α 1AT and neutrophilic elastase on and off treatment. IL-6 increased significantly off therapy. The individual changes in the levels of IL-6 are presented in Figure 1. We assessed correlation of circulatory cytokines with pulmonary function parameters. Off treatment, we found

positive correlation between IL-6 and LCI ($r=0.55$; $p=0.027$) and negative correlation between α 1AT and both LCI and FEF25-75 ($r=-0.54$, $p=0.028$ and $r=-0.517$, $p=0.031$, respectively).

Discussion

This study is the first to assess the difference between consecutive months of on/off treatment with TIP using systemic inflammatory markers in combination with spirometry and LCI measurements. After a month off treatment with TIP, spirometry parameters and lung clearance index remained similar to the month on therapy. While hs-CRP, IL-8, TNF- α , α 1AT and neutrophilic elastase were similar on and off treatment, IL-6 increased significantly off therapy. The results of lung function parameters support the relative stability of CF patients during the month off therapy with TIP; however, the change in circulatory IL-6 raises the possibility of an ongoing higher degree of inflammation.

CF pulmonary guidelines recommend inhaled tobramycin on alternate months for patients with chronic PA, irrespective of the severity of the lung disease (35). However, there are evolving practices by clinicians of adopting a continuous alternating regimen in clinical practice (e.g., a month of TOBI alternating with a month of Coliracin or Aztreonam). This practice is based on the clinical impression that the month off has disadvantages over the month on therapy (36). Theoretically, on/off treatment evaluations may help in identifying patients who deteriorate over the month off treatment. Recently, it was found that CF patients with more severe disease are more likely to receive continuous alternating inhaled antibiotics (37). In another double-blind study, continuous alternate therapy was found to be clinically favorable compared to an intermittent regimen. However, most patients were on continuous alternating regimen, and the study was underpowered (38).

In our study, spirometry values were similar on/off therapy. One previous study evaluated the response of lung function tests to inhaled tobramycin (either TIS or TIP). Only in patients who received inhaled tobramycin for the first time (treatment-naïve), was there a significant improvement in FEV1 (39). In our study, four patients were treatment naïve to tobramycin; however, the small number of patients precluded sub-group analysis. Notably, most of our patients were relatively healthy (FEV1 only mildly decreased), and the results may be different with a more affected population.

When evaluating lung clearance index (LCI), a marker of deranged ventilation, we hypothesized that TIP may reach the peripheral small airways, leading to improved LCI on

TABLE 1. Parameters assessed on/off treatment with TIP™

P value	Off TIP	On TIP	Parameter
0.27	75.00 (64.00-82.00)	75.00 (62.00-81.00)	FEV1 %
0.29	2.30 (1.24-2.73)	2.18 (1.30-2.60)	FEV1 (L)
0.18	79.00 (73.00-98.00)	83.00 (67.00-96.00)	FVC %
0.19	2.75 (1.93-3.53)	2.55 (1.89-3.20)	FVC (L)
0.41	39 (68-84)	39 (66-81)	FEF25-75 %
0.38	2.2 (1.1-3.3)	2.36 (1.19-3.18)	FEF25-75 (L)
0.35	152.00 (139.00-193.00)	152.00 (131.00-231.50)	LCI %
0.33	8.71 (7.88-11.35)	8.48 (7.43-13.15)	LCI
0.57	5.28 (2.84-12.9)	4.59 (2.94-13.60)	hs-CRP (n=14)
0.02	0.41 (0.23-1.57)	0.19 (0.07-0.7)	IL-6 (pg/mL)
0.12	5.10 (2.60-13.70)	3.82 (2.41-7.83)	IL-8 (pg/mL)
0.62	11.56 (6.17-18.59)	7.38 (5.56-22.50)	TNF- α (pg/mL)
0.14	1.17 (0.56-4.10)	1.98 (0.63-4.23)	α 1AT (mg/dL)
0.17	23.93 (18.47-27.9)	27.62 (23.73-30.33)	Neutrophilic elastase

Results are expressed as median and 25-75 interquartile range.

treatment compared to off treatment. LCI has been recognized as an outcome measure for CF clinical trials (40–43), but the effect of inhaled antibiotics on LCI was not reported. The results showed similar LCI values at the off compared to on treatment periods, supporting the relative stability of CF patients on alternate inhaled therapy.

As mentioned earlier, we evaluated several circulatory markers. One marker, α 1AT, was found to interfere with inflammatory responses, to lower levels of IL-8, and to protect from cell death. Genetic deficiency of α 1AT is associated with early onset emphysema. It has been suggested that in CF, α 1AT has an important role in protecting the lower respiratory tract from neutrophilic elastase induced injury; inhalations of α 1AT has been shown to reduce inflammation (44). In a recent study, α 1AT levels were found to have changed following IV antibiotics course (23). In our small study, α 1AT levels were similar in on/off therapy. IL-8, IL-6 and TNF- α were suggested as useful, noninvasive outcome measures for assessing the response to antibiotic therapy in CF. A previous study on/off inhaled TOBI treatment resulted in a marked reduction in IL-8 and IL-6 sputum levels, without a change in neutrophilic elastase (12). In a more recent study (21), among all the cytokines measured, only IL-6 was found

to decrease after 14 days of IV antibiotic therapy, and not in all patients.

In our study, IL-8, TNF- α and neutrophilic elastase levels were similar on and off treatment, while IL-6 increased significantly off therapy. We found a positive correlation between LCI and IL-6, and a negative correlation between α 1AT and both LCI and FEF25-75. We could not find correlations between the other cytokines and parameters of lung function tests. This may suggest that LCI could be more sensitive than spirometry for detecting changes in inflammatory markers following inhaled therapy; however, the small sample size precludes any firm conclusions.

The strength of our study is its design; addressing an important clinical question in the management of CF patients. We assumed that determination of the short-term effects of TIP could assist in understanding its long-term effects. The prospective nature and the use of inflammatory markers provide a sensitive measure to compare the on and off treatment periods.

Our study has several limitations. The major limitation is the small study population size. Study enrollment was limited because of the evolving practices of a continuous alternating regimen in clinical practice. We found only a few patients who

met the inclusion criterion of alternate inhaled therapy with TIP. Of >300 patients treated in other CF centers, we were unable to find any patients who were treated with an on/off antibiotic regimen and who met all the other inclusion criteria. Consequently, the study was underpowered. The small sample size (19 patients) and the multiple parameters (including six cytokines) evaluated could lead to statistical errors. Although, Bonferroni correction controls the probability of a type-1 error (α error), this correction ordinarily comes at the cost of increasing the probability of a type-2 error (β error); therefore, these parameters require evaluation in a larger sample size. Another limitation of the study was the treatment duration prior to recruitment, which varied significantly among patients.

In conclusion, this pilot study shows that lung function parameters remain similar in the month off therapy in stable CF patients. The higher circulating IL-6 at the end of the month off therapy may suggest a higher degree of inflammation. In this era of evolving new therapies (including antibiotics) for CF patients, determination of inflammation could help in personalized medicine, clarifying which patients should be treated with on/off inhaled therapy and who should be switched to continuous alternating regimen. The potential detractors of continuous therapy, such as increasing resistance, cumulative toxicities or cost, should be addressed. These important issues should be explored in a large multicenter study.

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