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Original Article

Role of Tris-CaEDTA as an adjuvant with nebulised tobramycin in cystic fibrosis patients with *Pseudomonas aeruginosa* lung infections: A randomised controlled trial

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ABSTRACT

Background: We tested if disrupting iron utilisation by *P. aeruginosa* by adding the Tris-buffered chelating agent CaEDTA to nebulised tobramycin would enhance bacterial clearance and improve lung function in CF patients.

Methods: In this double-blind, randomised controlled trial, 26 episodes (25 patients) with *P. aeruginosa* infection admitted to two CF centres for treatment of an acute pulmonary exacerbation were randomly assigned to receive either 75 mg CaEDTA in Tris-buffered saline or placebo (Tris-buffered saline) nebulised in combination with 250 mg tobramycin twice daily for six weeks followed with four week safety follow-up. Primary endpoints were safety, tolerability, and bacterial density of *P. aeruginosa*. A secondary endpoint was lung function.

Results: The study drug was well tolerated with adverse events comparable in both groups. The mean (SD) reduction in sputum *P. aeruginosa* count ($\log_{10}$ CFU/g) in the CaEDTA vs placebo group was 2·05 (2·57) vs 0·82 (2·71) at two weeks relative to admission ($p = 0{\cdot}39$). The mean improvement in $ppFEV_1$ was 16 vs 5 ($p = 0{\cdot}16$); 11 vs 2 ($p = 0{\cdot}28$); and 6 vs 2 percentage points ($p = 0{\cdot}47$) at two, six, and ten weeks in CaEDTA and placebo groups, respectively.

Conclusions: In this pilot study in CF patients, an increase in the reduction of sputum density of *P. aeruginosa* and an increase in $ppFEV_1$ was observed in the group of patients who received Tris-CaEDTA added to inhaled tobramycin compared to the group who received inhaled tobramycin alone, although these differences were not statistically significant. The treatment was also shown to be safe.

1. Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) lung infection is a leading cause of morbidity and mortality in cystic fibrosis (CF) [1]. The organism opportunistically infects the compromised CF airway, adapts to the environment, and develops number of sophisticated defence mechanisms against both the host and antibiotics [2]. Once established, the infection becomes virtually impossible to eradicate with existing antibiotic therapies and new approaches are needed [3].

P. aeruginosa has an absolute requirement for iron, which plays a vital role in growth, multiplication, and biofilm formation [4,5]. Patients with CF have elevated levels of sputum iron, and *P. aeruginosa* has well-developed iron-scavenging systems for obtaining iron from the CF airway environment [6,7]. Other divalent cations such as zinc and magnesium also play an important role in survival and resistance mechanisms through their role in metalloproteinase enzyme activity and stabilisation of lipopolysaccharide in the bacterial outer membrane [8].

Ethylene diamine tetra-acetate (EDTA) and the calcium salt (CaEDTA) are chelating agents with several antimicrobial properties, mediated by their ability to bind various divalent cations including iron, zinc and magnesium thus rendering them unavailable for use by microorganisms [8]. This phenomenon has been called "nutritional immunity" [9,10] and has been repeatedly shown *in vitro* [11,12] and *in vivo* in animals with chronic *P. aeruginosa*-associated otitis, sinusitis and endometritis [13,14] to weaken bacterial defence mechanisms and consequently increase bacterial killing by the concomitant antibiotic. Tromethamine, or Tris

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(hydroxymethyl)aminomethane is an FDA approved buffering agent that has been shown to synergistically increase the anti-microbial effects of EDTA and CaEDTA [14,15].

These data supported our contention that inhaling Tris-buffered CaEDTA added to tobramycin as the concomitant antibiotic could conceivably improve bacterial killing in chronically infected human CF lungs. Orally administered CaEDTA is negligibly absorbed from the enteral route, and intravenous CaEDTA, although safe and FDA-approved for treatment of lead poisoning, has poor lung bioavailability despite high systemic concentrations - making neither of these administration routes suitable for treating lung infections.

The inhalation route for EDTA/CaEDTA is not new. Since the 1960's a number of studies tried inhaling these chelators for decorporation of excess toxic metal ions in exposed lead smelter workers [16,17]. Despite the massive doses (up to 2·4 gram/day) used in these studies, no adverse effects - local or systemic - were reported. Beasley [18] initially reported an increased risk of bronchospasm in extremely sensitive asthmatics who had demonstrated paradoxical bronchoconstriction following inhalation of a bronchodilator containing excipients chlorhexidine and EDTA although a subsequent study in standard asthmatics showed no adverse effects to inhaled EDTA alone [19]. Bronchospasm has not been reported when the calcium form of EDTA (CaEDTA) has been inhaled. CaEDTA is approved by the FDA for intravenous use to reduce lead levels in patients with lead poisoning (acute and chronic) and lead encephalopathy [20].

Two previous human studies have assessed the role of inhaled EDTA/CaEDTA in treating *P. aeruginosa* lung infections. In a study by Hillman et al. in 1984 in four ventilated non-CF patients with refractory *P. aeruginosa* lung infection, addition of nebulised CaEDTA to intravenous penicillin resulted in eradication of the infection within 48 h [21]. In another study by Brown et al. in CF patients using inhaled Tris/EDTA in combination with oral tetracycline successfully eradicated *P. aeruginosa* in the pilot study but failed to show efficacy in the main study [22].

Given the compelling evidence above and despite the negative study by Brown et al. we designed this study to test the hypothesis that addition of CaEDTA as an adjuvant to inhaled tobramycin would result in improved reduction of *P. aeruginosa* from CF airways and improve clinical outcomes compared to treatment with inhaled tobramycin alone.

2. Materials and methods

2.1. Study sites and registration

This randomised double-blind, placebo-controlled, parallel study was conducted in two hospitals in Perth, Australia: Princess Margaret Hospital for Children and Sir Charles Gairdner Hospital. The study protocol was approved by the institutional ethics committee/review boards at both sites (# 2013073EP, SCGH-2014-076) and informed consent was obtained from the parent and/or patient prior to commencing the study. The study was conducted from March 2014 through February 2016.

2.2. Participants

Inclusion criteria for subjects enroled in this study were: CF patients older than six years of age; admission to hospital for intravenous antibiotic treatment of a pulmonary exacerbation as determined by the treating physician (no validated criteria for "pulmonary exacerbation" were used); ppFEV$_1$ $\geq$ 25 points; and patients who had repeatedly been sputum culture positive for *P. aeruginosa* previously with at least one positive culture in the previous 12 months. We were unable to use the Leeds criteria [23] for chronic colonisation in this study as our centres do not use cough swabs to assess lower airway colonisation and this meant some patients had not had more than one sputum tested in the previous 12 months. Exclusion criteria were: known hypersensitivity to the investigational product or its components; participation in another study with an investigational drug within two months of the planned first dose of our investigational product; known history of substance abuse; pregnant or lactating; clinically significant medical condition other than CF or CF-related conditions that would compromise the safety of the patient or the quality of the data. Females of child-bearing potential were required to use an acceptable method of contraception for the duration of the trial.

2.3. Randomisation

Prior to commencement of the study, the pharmacist prepared a permuted-block randomisation schedule and prepared drug doses. The statistician held the full randomisation code, so that all investigators, study staff and participants remained blinded with a process of un-blinding established in the event of an emergency.

2.4. Study drug

The study drug was supplied in blinded 1·5 ml prefilled syringes. The active drug contained CaEDTA 75 mg in Tris-buffered solution while the placebo consisted of Tris-buffered 0·9% saline. Active drug or placebo, when added to tobramycin (Tobra-Day® 250 mg in 2·5 ml), resulted in a 4 ml solution at pH 7·1 to be inhaled using a PARI LC SPRINT® nebuliser. The dose and concentration of CaEDTA were based on safety and tolerability data obtained from previously published studies using inhaled CaEDTA [22].

2.5. Study design

All patients received standard treatment of their pulmonary exacerbation as per their treating physician. In addition, patients were randomised 1:1 to receive either active drug or placebo. First dose of the study drug was given within the first 72 h of initiation of intravenous antibiotics. The study consisted of three phases (i) The 0–2 week inpatient phase (while on intravenous antibiotics) when participants received study drug four times daily – twice combined with inhaled tobramycin, and twice with 0·9% normal saline; (ii) the 2–6 week outpatient phase during which the participants received study drug together with inhaled tobramycin twice daily; and (iii) The safety phase between 6 and 10 weeks, when no study drug or tobramycin was given. Compliance was determined based on the number of doses recorded as "taken" in the participant diary against the number of doses prescribed, and expressed as a percentage.

2.6. Study visits

Study subjects were screened on admission for eligibility criteria and informed consent was obtained. Subsequent visits were conducted on day one, two weeks (completion of the intravenous treatment course); six weeks (at the end of the 4-week outpatient phase); and ten weeks (safety visit, four weeks after ceasing the study drug). Two additional telephone visits were conducted at one week and four weeks to ensure safety and compliance, details in supplement Table S1.

2.7. Study assessments and endpoints

The objectives of the study were to compare the safety and efficacy of Tris-CaEDTA in combination with inhaled tobramycin vs inhaled Tris-buffered tobramycin alone. Primary endpoints were tol-

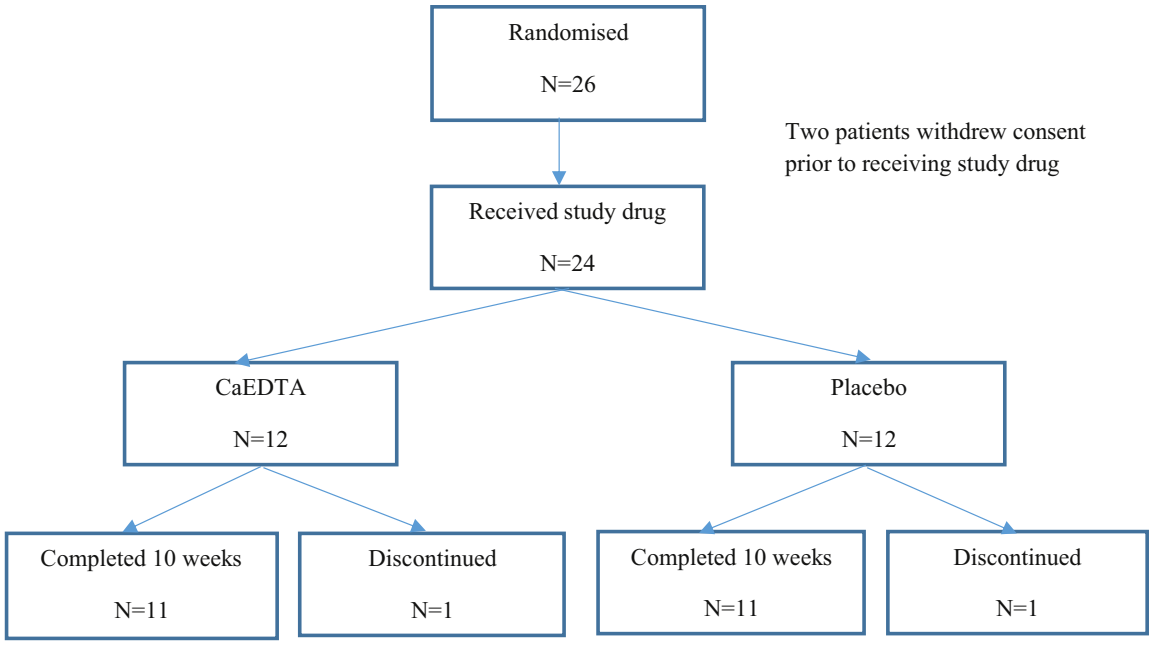

Fig. 1. Participant flow chart. Total of 26 episodes (25 patients) were randomised to participate in the study. Two patients (one from each group) withdrew consent before receiving the first dose of the study drug and hence excluded from all safety and efficacy analysis. Further two patients (one from each group) discontinued from the study before completing the full ten week study protocol, one of them was re-enroled into the same treatment arm.

erability and safety as measured by: clinical status; pre- and post-study drug lung function (measurements taken prior to first dose of study drug, 30 min, one hour and two hours after first dose); and blood tests (full blood count, blood urea nitrogen, serum creatinine, liver function tests, iron indices, calcium, magnesium, and phosphorus). The primary efficacy endpoint of the study was reduction in sputum bacterial load of *P. aeruginosa* as measured by colony forming unit (CFU) count, and the secondary efficacy endpoint was improvement in lung function as measured by $ppFEV_1$. Sputum was collected by induced sputum technique as previously described [24]. Details of sputum processing in supplement 2.

2.8. Statistical analysis

Efficacy analyses were reported in the per-protocol population, defined as participants who completed $\geq$ 75% of doses with not more than three consecutive missed doses. Test of normality was determined using Shapiro-Wilk test. When data is normally distributed, values were presented as means and standard deviations and student's *t*- test was used to determine statistical significance. When data were not normally distributed, results were presented as medians and interquartile ranges and Mann-Whitney test was applied to determine statistical significance. The study was initially designed to recruit 32 patients (16 in each group) which would have 80% power of detecting a 2·6 $\log_{10}$ CFU/g difference in the bacterial load at 0·05 level of significance. Due to slow recruitment with only 26 episodes recruited in 20 months, the study was discontinued on recommendation from the Data Safety Management Committee and biostatistician.

2.9. Role of the funding source

The study sponsors had no role in study design, in the collection, analysis, or interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

3. Results

3.1. Study population

Of the twenty six episodes (25 patients), two patients withdrew prior to receiving the study drug and were not included in the analysis. Two further patients discontinued from the study due to personal reasons (one on Day 7 and one on Day 2), one of whom re-enroled later and was assigned to the same study arm although counted only once in analysis. Twenty-two patients completed the study procedures through to ten weeks (Fig. 1). The safety data were analysed from all patients who received at least one dose of the study drug while efficacy data were analysed from the available data from 11 patients in the CaEDTA group and 11 patients in the placebo group who received at least 75% of the study drug.

3.2. Demographic details and clinical characteristics

The baseline demographics at enrolment showed the groups were generally matched regarding age, gender, BMI and previous hospital admissions (Table 1). However, notable differences were detected between the two groups in mean $ppFEV_1$ levels at enrolment, sputum load of *P. aeruginosa* at enrolment, median duration of intravenous (IV) antibiotics and weight. The mean (SD) $ppFEV_1$ levels in the CaEDTA and placebo groups at enrolment were 53 (27) vs 72 (23), and the mean (SD) drop in this $ppFEV_1$ compared to their best recorded measurement in the previous 12 months was 21 (23·5) vs 11·5 (8·9) ($p = 0·23$) respectively. The mean (SD) $\log_{10}$ *P. aeruginosa* (CFU/g) was 4·5 (2·4) vs 3·8 (2·5) in the CaEDTA and placebo group respectively ($p = 0·52$). The median (IQR) duration of intravenous antibiotics was 14 (2) vs 16 (4), in CaEDTA and placebo group respectively ($p = 0·008$). Azithromycin prophylaxis was noted in five patients in CaEDTA group and eight patients in placebo group. Details of antibiotic use in supplement 8 Table S7. *Aspergillus* was isolated in four patients in CaEDTA group and five patients in placebo group with *Staphylococcus* aureus isolated in one patient in CaEDTA group and three patients in placebo group.

Table 1
Demographic and clinical characteristics of patients included in per protocol efficacy analysis.

	CaEDTA group $N = 11$	Placebo group $N = 11$	Total $N = 22$
Age, years; median (range)	17 (7–46)	16 (7–35)	16 (7–46)
Age group; *n* (%)			
6–13 years	3 (27·3%)	4 (36·3%)	7 (31·8%)
14–17 years	3 (27·3%)	3 (27·3%)	6 (27·2%)
>18 years	5 (45·4%)	4 (36·3%)	9 (41%)
Male gender; *n* (%)	8 (72·7%)	6 (54·5%)	14 (63·6%)
Pancreatic insufficient; *n* (%)	11 (100%)	9 (81·8%)	20 (90·9%)
Weight, kg; median (IQR)	71 (29–73)	49 (38–73)	62 (20–77)
BMI; mean (SD)	20.50 (4.57)	19.35 (3.28)	19.93 (3.93)
Duration of intravenous antibiotics; median (IQR)*	14 (2)	16 (4)	14.8 (2.25)
IV antibiotics used			
Ticarcillin+Clavulinic acid	3	3	6
Ceftazidime	1	2	3
Meropenem	0	3	3
Piperacillin+Tazobactam	6	2	8
Cefepime	1	1	2
Oral antibiotic use in outpatient phase; number	5	4	9
Azithromycin prophylaxis; number of patients	5	8	13
Hospital admissions in previous 12 months; mean (SD)	1·2 (1·2)	1·0 (1·0)	1·2 (1.1)
$ppFEV_1$ at admission; mean (SD)	53 (27)	72 (23)	62 (26)
Best FEV_1 in 12 months (baseline); mean (SD)	75.5 (27.1)	84.3 (24.6)	79.9 (25.7)
$ppFEV_1$ lost; mean (SD)	21 (23.5)	11.5 (8.9)	16.2 (18.0)
P. aeruginosa density (log_{10}CFU/g sputum) mean (SD)	4·5 (2·4)	3·8 (2·5)	4·17 (2·4)
Aspergillus positive (number)	4	5	9
Staphylococcus aureus positive (number)	1	3	4

* Statistically significant at 0.05 level of significance.

Table 2
Adverse events.

	Inpatient phase		Outpatient phase		Safety phase	
Adverse event category	Ca-EDTA	Placebo	CaEDTA	Placebo	CaEDTA	Placebo
Haemoptysis	1[a]	1[b]		1[b]		
Epistaxis				1		
Increase in cough				2		1
Sore throat		1	1			2
Nausea/vomiting		1		1[c]	1	
Abdomen pain	1	1	1			1
Rashes		2[d]		1		1
Headache	1	2	2	1	1	1
Jaundice or deranged LFT'S	1	1[e]				

[a] One patient in CaEDTA group had two episodes of haemoptysis.
[b] Same patient in Placebo group had small haemoptysis during inpatient and outpatient phase.
[c] Two episodes in the same patient.
[d] Rashes in both patients were deemed to be from other medications (ceftazidime and vancomycin).
[e] ALT ≥3 time upper limit of normal is regarded as abnormal.

3.3. Safety analysis

3.3.1. Tolerability and adherence

Both the placebo and Tris-CaEDTA compounds were well tolerated with 94% and 90% adherence in the first two weeks (inpatient phase) and 91% and 80% adherence between 2 and 6 weeks (out-patient phase) respectively. Lung function ($ppFEV_1$) was measured prior to the administration of the first dose of the study drug and repeated at 30 min, one hour, and two hours post-dose. There was no significant fall in the lung function (defined as drop in $ppFEV_1$ ≥15% from pre-dose) following inhalation of Tris-CaEDTA or placebo in any of the subjects (details in supplement 3 Table S2). There was no wheeze, chest tightness, discomfort, or alteration in taste noted in any of the patients.

3.3.2. Adverse events

Most adverse events were mild, and none were specifically attributable to the study drug (Table 2). Three patients experienced transient small haemoptysis, two in the placebo group and one in the CaEDTA group, the latter requiring interruption of study drug for 12 h. One patient in the placebo group had increased cough in the outpatient phase resulting in discontinuation of the study drug after taking 77% of the scheduled doses. Headache was the most common reporting complaint (four patients in each group), but severity was mild, and episodes were short-lived.

3.3.3. Blood test safety analysis

No significant or clinically relevant differences were noted in renal function, hepatic function, iron, calcium, or magnesium levels between the two groups. Renal function was monitored by serum creatinine levels, abnormal if outside reference intervals [25]. Details of serum creatinine for each visit for individual patients in Supplement 4 Table S3. One patient in the placebo group had serum creatinine above the reference interval at 6 weeks but returned to normal reference range at 10 weeks (25 µmol/L at admission, 77 µmol/L at 6 weeks and 43 µmol/L at 10 weeks). Effects on hepatic function was monitored by alanine aminotransferase (ALT) levels (abnormal if ≥3 times the upper limit of normal) [26]. Details in Supplement 5 Table S4. One patient in the placebo group had abnormal ALT which returned to normal at subsequent visit.

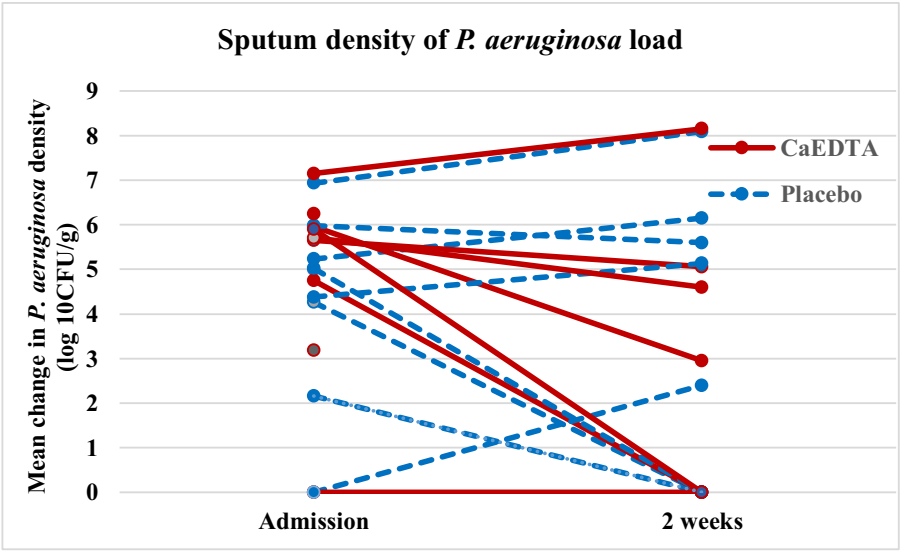

Fig. 2. Change in sputum *P. aeruginosa* relative to admission. Mean (SD) reduction in the sputum *aeruginosa* (log_{10} CFU/g) in the CaEDTA vs. placebo groups respectively was 2·05 (2.57) vs. 0·82 (2·71) at two weeks relative to admission (paired values for *P. aeruginosa* for 7 patients in CaEDTA group and 8 in placebo group, $p = 0·39$). Due to clinical improvement many patients were unable to provide enough sputum specimens and hence analysis limited to 2 weeks.

One patient in the CaEDTA group had abnormal ALT, but no result was available at subsequent visit due to sampling difficulties. No evidence of any potential chelating effects of Tris-CaEDTA on serum iron, calcium, or magnesium were found by comparing the mean change in levels from admission to two weeks, six weeks, and ten weeks between the two groups. Details in supplement 6 Table S5.

3.4. Efficacy analysis

3.4.1. Change in sputum P. aeruginosa load (CFU/g sputum)

Of the 22 patients who completed all the study procedures through to ten weeks, two patients who had negative cultures throughout the study and one patient unable to produce sputum were excluded from the *P. aeruginosa* sputum analysis. Patients with negative cultures on follow up were treated as zero for bacterial load, while those unable to provide an adequate sputum sample were considered as missing information. Mean (SD) reduction in the *P. aeruginosa* sputum count (log_{10} CFU/g) in the CaEDTA vs placebo groups respectively was 2·05 (2.57) vs 0·82 (2·71), paired values for seven patients in CaEDTA group and eight patients in placebo group, $p = 0·39$, (Fig. 2). Three patients in each group had sputum cultures negative for *P. aeruginosa* at two weeks. In the active group, CFU count at two weeks decreased in 6 out of 7 patients (86%) while in the placebo group, the CFU count decreased in 5 out of 8 patients (62%). Analysis of CFU count was not performed at six and ten weeks as too many patients were too well to produce sputum or had negative sputum cultures. Details in supplement 7 Table S6.

3.4.2. Change in lung function ($ppFEV_1$)

Of the 22 patients who completed the study through to ten weeks, one patient was not adherent to the study drug after discharge from the hospital (two weeks) and hence excluded from lung function data analysis after two weeks. One patient with poor effort at lung function at ten weeks did not meet ATS criteria and hence was excluded from efficacy analysis. Mean (SD) improvement in the $ppFEV_1$ relative to admission in the CaEDTA and placebo groups respectively was 16 (22) vs 5 (10·6) at two weeks, ($n = 11$ in each group, $p = 0·16$), 11 (20·24) vs 2 (13·7) at six weeks, ($n = 10$ in each group, $p = 0·28$), and 6 (13·93) vs 2 (9·61) points at ten weeks ($n = 9$ in CaEDTA group and 11 in placebo group, $p = 0·47$), (Fig. 3). The differences in $ppFEV_1$ between active and placebo groups at two, six and ten weeks were not statistically significant.

3.4.3. Time to next exacerbation

In the two years subsequent to screening, median (IQR) number of days until the next admission for treatment of a pulmonary exacerbation was 390 (566) vs 212 (465) days for participants in the CaEDTA group and placebo groups respectively, $p = 0·4$, (Fig. 4).

4. Discussion

Although an increased reduction in sputum density of *P. aeruginosa* (log_{10} CFU/g) was seen after two weeks of treatment in the group receiving nebulised Tris-buffered CaEDTA and tobramycin (2·05) compared to the group receiving Tris-buffered tobramycin alone (0·82), this did not reach statistical significance. As a pilot study, the small sample size meant this result was not expected to reach statistical significance. Nevertheless, the result suggests a microbiological signal which is in keeping with other studies mentioned earlier demonstrating the potential of CaEDTA to increase the efficacy of the concomitant antibiotic against *P. aeruginosa* [12,13,27]. These studies also identify several of the mechanisms whereby CaEDTA exerts this effect including its ability to enhance breakdown of biofilm - one of bacteria's most potent protective mechanisms against antibiotics [5]. In addition, (Ca)EDTA has been shown to disrupt the lipopolysaccharide layer in the *P. aeruginosa* outer membrane resulting in increased cell wall permeability and antibiotic penetration into the bacterial cell [8]. Another potential mechanism is CaEDTA's zinc-chelating ability which has been shown to neutralise metallo-beta-lactamase (MBL) produced by some strains of *P. aeruginosa* [28]. Although this will not directly influence tobramycin efficacy, it will enhance killing by beta-lactam antibiotics which are commonly used as the second intravenous antibiotic together with tobramycin for the treatment of *P. aeruginosa* infection. The study by Aoki et al. [28] in mice infected with an MBL-producing *P. aeruginosa* convincingly shows that CaEDTA delivered to the airway concomitantly with an intravenously administered beta-lactam antibiotic (imipenem) effectively cleared the infection compared to intra-

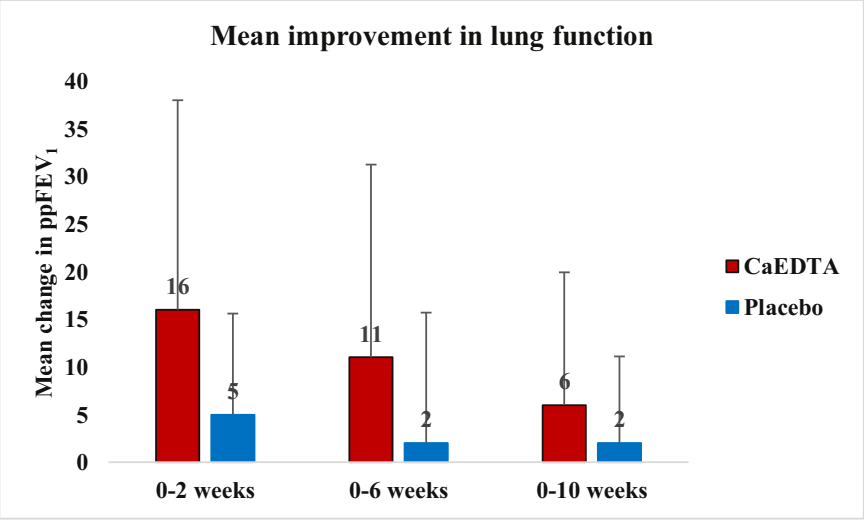

Fig. 3. Change in $ppFEV_1$ relative to admission. Mean improvement in $ppFEV_1$ was 16 vs 5 at two weeks ($n = 11$ in each group, $p = 0{\cdot}16$), 11 vs 2 at 6 weeks ($n = 10$ in each group, $p = 0{\cdot}28$), and 6 vs 2 points at ten weeks ($n = 9$ in CaEDTA group and 11 in placebo group, $p = 0{\cdot}47$) in CaEDTA and placebo groups respectively.

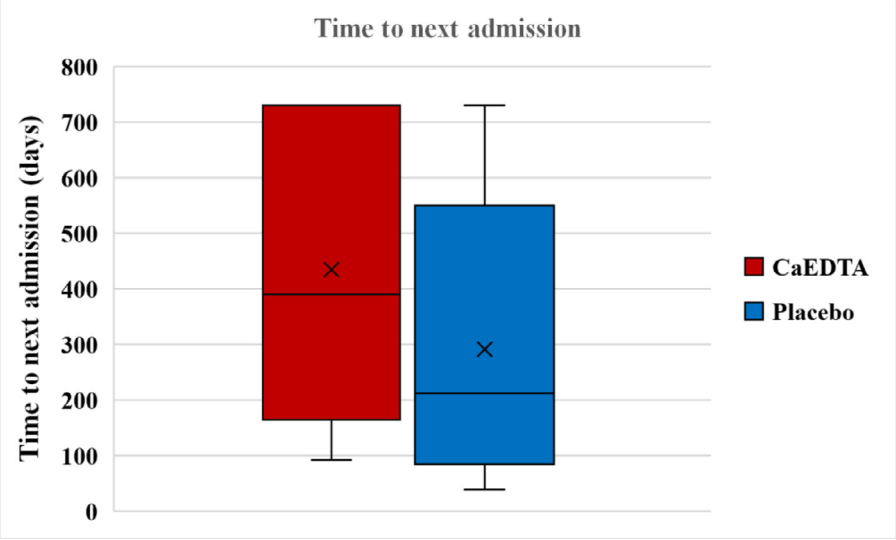

Fig. 4. Time from screening to next exacerbation. Median (IQR) number of days until the next admission was 390 (566) vs 212 (465) days in CaEDTA and placebo groups respectively, $p = 0.4$.The line in the bar graph represents median while the "X' represents mean.

venous imipenem without inhaled CaEDTA. While MBL-producing *P. aeruginosa* strains are not thought to be common in CF patients, this property of CaEDTA does have the potential to improve treatment outcomes when present.

Additional clinical evidence that inhaled CaEDTA can provide adjuvant activity to systemically administered antibiotic treatment is found in the study by Hillman et al. in ventilated patients with recalcitrant *P. aeruginosa* infection. Using continuous nebulised CaEDTA 0·6 gm/day for 3 days together with daily intra-tracheal CaEDTA 0·2 gm/day along with intravenous penicillin achieved clinical recovery and complete clearance of *P. aeruginosa* within 48 h [21]. In contrast, the study by Brown et al. in 1985 using 50 mM Tris buffered edetate sodium plus oral tetracycline in 10 CF patients failed to show improvement in lung function or sputum load of *P. aeruginosa* [22]. The lack of improvement in this study could be explained by several factors including: infrequent administration of the drug, inadequate drug delivery, and relatively poor antipseudomonal effect of oral tetracycline.

The observed increase in $ppFEV_1$ at two weeks in the CaEDTA group (16%) compared to the placebo group (5%) while impressive, was not statistically significant, and again the small sample size prevents us from drawing any conclusions from this result. In addition, the demographic differences between the two groups could also have influenced this result. Intuitively, the larger mean drop in $ppFEV_1$ from best $ppFEV_1$ in the year prior to enrolment in the CaEDTA group compared to the placebo group might suggest more room for improvement in the active group, however, the lower mean best $ppFEV_1$ in the CaEDTA group (75%) compared to the placebo group (84%) suggests more severe underlying disease in this group and this would be expected to limit improvement following treatment. A larger study with appropriate sample size is needed to resolve this issue.

The small sample size did not allow propensity matching within the groups to strengthen the results. Nevertheless, the observed increase in $ppFEV_1$ of 16% in the CaEDTA group, as well as the 11% difference compared to the placebo group, are

higher than most studies report following a course of treatment [29].

A higher than expected increase in ppFEV1 could also suggest that CaEDTA may provide additional benefits to the lung environment over and beyond *P. aeruginosa* killing. For instance, it has been shown that free iron is greatly increased in the CF lung and reducing the availability of this excess iron through CaEDTA's chelating ability may reduce production of superoxide radicals known to contribute to the damaging increased oxidant activity found in the airway of CF patients [6].

At the time when this study was performed in Australia, regular alternate month inhaled tobramycin for bacterial suppression was not standard practice. In our study, no patients were on regular alternate monthly nebulised tobramycin and in addition, none reported using inhaled tobramycin in the two weeks leading up to the study. Thirteen patients (5 in the CaEDTA group and 8 in the placebo group) were on regular oral Azithromycin prophylaxis at enrolment. All patients were advised to follow the standard recommendation at the time and discontinue Azithromycin for the duration of the study due to its recognised effect in reducing tobramycin efficacy [30] - despite the known long half-life of Azithromycin making this recommendation dubious. A full list of the antibiotics used in this study is provided in Supplement Table S7.

Finally, the longer time to next admission in the CaEDTA group compared to the placebo group (390 days vs 212 days) in this study, although not statistically significant due to small sample size provides additional support for further exploration of the potential of using Tris-CaEDTA as an adjuvant with inhaled tobramycin.

The choice of Tris-buffered saline rather than normal saline for the placebo arm of the study effectively rules out any individual Tris effect contributing to the increased bacterial killing and/or the unexpectedly large increase in $ppFEV_1$ in the active group. On the other hand, the synergy with Tris and CaEDTA is well recognised and this synergy - rather than Tris itself - may well contribute to these differences [15].

Compliance was high in both groups throughout the study period, strengthening the results. Importantly, the active drug was shown to be safe and well tolerated with no increase in adverse events compared to placebo. This provides reassurance for future studies. Blood tests showed no adverse effects on renal or hepatic function, and no effect on iron (including iron storage), calcium, or magnesium levels. The risk of intolerance or local airway toxicity due to inhaled EDTA is low, and even less so with CaEDTA where neither bronchoconstriction nor any other adverse local effects have yet been reported. This has been substantiated by our study. Systemic toxicity due to CaEDTA inhalation is also unlikely as the amount of CaEDTA absorbed from the lung ranges from ~5–10% of the inhaled dose. Furthermore, inhaled doses used up till now (including in our study) are much lower than those recommended for intravenous CaEDTA in the treatment of heavy metal poisoning. Nephrotoxicity reports are rare and likely to occur only with excessively high and/or prolonged IV dosing regimens or in patients with prior renal disease.

5. Conclusion

In this pilot study in CF patients admitted for treatment of a pulmonary exacerbation, the group of patients receiving Tris-CaEDTA added to nebulised tobramycin showed an increased reduction in the mean CFU count of *P. aeruginosa* and an increase in the mean $ppFEV_1$ compared to the group receiving inhaled tobramycin alone after two weeks of treatment. These differences were not statistically significant. The study also demonstrated the safety and tolerability of this inhaled treatment. These results support further exploration of the potential of inhaled CaEDTA as an adjuvant to antibiotic therapy in a larger clinical study where the groups are better matched.

Limitations and future directions

The small numbers in each group mean we are not able to draw any definitive conclusions from the results. Propensity matching to avoid any potential bias in outcomes due to differences in baseline disease severity could not be carried out in this study due to small sample size. Our study did not define pulmonary exacerbation. Use of Azithromycin and other concomitant oral antibiotics prior to and during the outpatient phase have the potential to affect treatment outcomes of pulmonary exacerbation and these were not controlled for in our study. In addition, and regrettably, much useful information was missed due to the difficulty in obtaining adequate sputum samples as patients improved on treatment. Future studies should aim to acquire more pK and pD data. Sputum iron (as well as zinc and magnesium) levels (bound and unbound to CaEDTA) could improve our understanding of the role of chelation in achieving the effects seen. Genetic studies could provide valuable insight into changes in resistance patterns of *P. aeruginosa* while being exposed to Tris-CaEDTA with tobramycin.

Author contributions

BC and SS designed and conceived the study and obtained funding for carrying out the clinical trial. DR as a recognised leader in the field of iron and its role in *pseudomonas aeruginosa* in CF played a significant role in providing intellectual input into the study design, outcome measures, testing and interpretation of results. BC, RP and LM recruited patients, conducted study visits, carried out laboratory procedures and collected data. IL and WD performed sputum analysis. HM and SG assisted in data collection, data analysis and manuscript preparation. RP and BC wrote the first draft of the manuscript. SS provided substantial input into the manuscript. All authors discussed the results and reviewed the manuscript before submission.

Declaration of Competing Interest

The authors have no conflict of interest to declare.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcf.2020.12.004.

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