THE LANCET Respiratory Medicine

Supplementary appendix

This appendix formed part of the original submission and has been peer reviewed. We post it as supplied by the authors.

Supplement to: Kerem E, Konstan MW, De Boeck K, et al, for the Cystic Fibrosis Ataluren Study Group. Ataluren for the treatment of nonsense-mutation cystic fibrosis: a randomised, double-blind, placebo-controlled phase 3 trial. *Lancet Respir Med* 2014; published online May 16. http://dx.doi.org/10.1016/S2213-2600(14)70100-6.

Supplementary Appendix

Patient Eligibility

Inclusion criteria

All patients had submitted a signed informed consent/assent; were ≥6 years of age; had body weight ≥16kg; exhibited abnormal NPD total chloride conductance (a less electrically negative value than 5 mV for total chloride conductance [Δchloride-free+isoproterenol]); had sweat chloride >40 mEq/L; had documentation of the presence of a nonsense mutation in at least 1 allele of the CFTR gene; verification that a blood sample had been drawn for sequencing of the CFTR gene; had ability to perform a valid, reproducible spirometry test that demonstrated an FEV_1 ≥40% and ≤90% of predicted for age, gender, and height[16,17]; resting oxygen saturation measured by pulse oximetry ≥92% room air; VivoMetrics documentation showing completed 24-hour LifeShirt cough frequency assessment; confirmed screening laboratory values within pre-specified central laboratory ranges for hepatic, adrenal, and renal organ systems, as well as serum electrolytes and pregnancy test; willingness to abstain from sexual intercourse or employ contraception during the study; willingness and ability to comply with scheduled visits, drug administration plan, study restrictions, and study procedures.

Exclusion criteria

Patients were excluded from study participation if they had a known hypersensitivity to ataluren; exposure to another investigational drug or any change in a chronic treatment or prophylaxis regimen for CF within 4 weeks of starting study treatment; ongoing participation in any other clinical trial; systemic aminoglycoside antibiotics treatment within 2 weeks before the date of

baseline NPD assessment; intravenous antibiotic treatment within 3 weeks prior to start of study treatment; ongoing immunosuppressive (non-corticosteroid), warfarin, phenytoin, or tolbutamide therapy; history of solid organ or hematological transplantation; major complications of lung disease within 8 weeks prior to start of study; pulmonary exacerbation or acute respiratory tract infection within 3 weeks before randomization; known portal hypertension; positive hepatitis B, hepatitis C, or human immunodeficiency virus test; pregnancy or breast-feeding; current smoker or smoking history of ≥10 pack-years (number of cigarette packs/day × number of years smoked); prior or ongoing medical condition that in the investigator's opinion, could have adversely affected the safety of the patient, or could impair study results assessment; and history of Grade ≥3 creatinine elevation due to aminoglycoside nephrotoxicity. Patients had the right to withdraw from the study at any time and for any reason. Patients could also be withdrawn based on worsening of their condition, investigator judgment, study protocol compliance, toxicity, broken blind, study discontinuation, and if ataluren became commercially available, in which case the patients would be transitioned to commercial drug supply.

Baseline Characteristics by Stratification Factors

Three stratification factors (use of inhaled aztreonam and/or aminoglycoside antibiotics [yes vs no], baseline age [<18 years vs ≥18 years], and baseline % predicted FEV_1 [<65% vs ≥65%]) were included to balance allocation of patients into treatment groups by these parameters which had potentially important effects. The allocation of patients into each strata is summarized in Supplementary Table 1.

Supplementary Table 1: Patient Distribution by Study Stratification Factors and Genotype (ITT)

Stratification Factor	Treatment Arm	
	Ataluren	Placebo
	N=116	N=116
Chronic inhaled antibiotic use, n (%)		
Yes	64 (55.2%)	63 (54.3%)
No	52 (44.8%)	53 (45.7%)
Age group, n (%)		
<18 y	38 (32.8%)	37 (31.9%)
≥18 y	78 (67.2%)	79 (68.1%)
Baseline % predicted FEV_1, n (%)		
≥40 to <65%	64 (55.2%)	74 (63.8%)
≥65 to ≤90%	52 (44.8%)	42 (36.2%)
Premature stop codon type		
UGA	110 (94.8%)	106 (91.4%)
UAG	12 (10.3%)	11 (9.5%)
UAA	11(9.5%)	14 (12.1%)

Abbreviation: FEV_1 = forced expiratory volume in 1 second

Types of inhaled antibiotics used were primarily aminoglycosides (tobramycin in all cases), colistin, and aztreonam (Supplementary Table 2). The treatment arms were balanced with regard to inhaled antibiotic use overall and generally balanced by type.

Supplementary Table 2: Inhaled Antibiotic Use at Randomization (ITT Population)

Characteristic	Treatment Arm	
	Ataluren	Placebo
	N=116	N=116
Any antibiotic(s)[a]	64 (55.2%)	63 (53.4%)
Aminoglycoside (tobramycin)	44 (37.9%)	42 (35.6%)
Colistin	30 (25.9%)	22 (18.6%)
Aztreonam	10 (8.3%)	8 (6.8%)

[a] A patient was considered to be using inhaled antibiotics at baseline even if the patient was in the "off" portion of an intermittent cycling regimen. Patients may have been using >1 antibiotic at baseline.

The subgroups of patients not using chronic inhaled tobramycin and those using chronic inhaled tobramycin at baseline had generally similar demographics at baseline (Supplementary Table 3).

Supplementary Table 3: Patient Demographics by aminoglycoside use (ITT Population)

Baseline Characteristic	Aminoglycosides = No		Aminoglycosides = Yes	
	Ataluren	Placebo	Ataluren	Placebo
	N=72	N=74	N=44	N=42
Age, years				
Mean (SD)	24.3 (11.31)	23.7 (10.20)	20.5 (7.54)	22.3 (7.56)
Median	22.5	22.0	20.5	22.0
Range	6, 49	8, 53	6, 48	8, 45
Sex, n (%)				
Male	35 (48.6%)	32 (43.2%)	25 (56.8%)	26 (61.9%)
Female	37 (51.4%)	42 (56.7%)	19 (43.2%)	16 (38.1%)
Body weight, kg				
Mean (SD)	53.2 (14.16)	55.7 (13.68)	53.9 (13.73)	56.5 (12.30)
Median	53.5	56.8	55.1	58.0
Range	22, 105	28, 93	21, 80	24, 92
% predicted FEV_1				
Mean (SD)	61.6 (13.11)	60.5 (15.68)	62.8 (14.52)	59.8 (14.32)
Median	62.9	59.7	63.9	58.3
Range	39.4, 89.7	36.2, 88.3	38.4, 90.3	40.1, 92.6
Sweat chloride				
Mean (SD)	98.3 (15.66)	96.9 (16.59)	103.3 (10.91)	96.0 (14.86)
Median	101.0	100.5	102.5	98.0
Range	22.5, 116.0	22.0, 117.5	82.5, 128.0	49.0, 116.0

A total of 34 patients discontinued participation prior to completion of 48 weeks of blinded treatment. The reasons for these discontinuations are summarized in Supplementary Table 4.

Supplementary Table 4: Reasons for Study Discontinuation (All Patients)

Reason	Treatment Arm	
	Ataluren	Placebo
	N=120	N=116
Adverse Event	7 (5.8%)	2 (1.7%)
Lost to Follow-up	1 (0.8%)	0

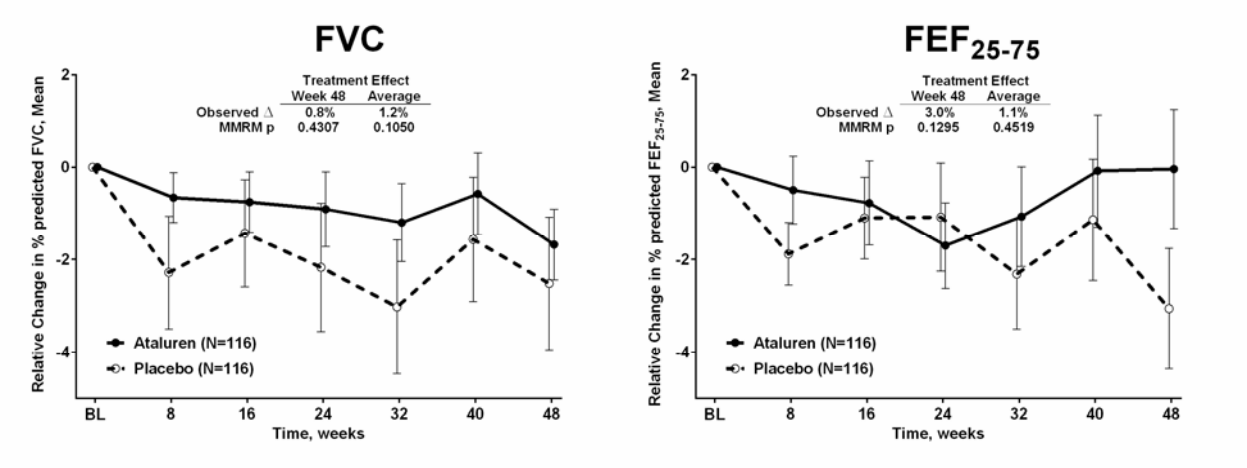
Withdrew Consent	9 (7.5%)	9 (7.6%)
Investigator Decision	1 (0.8%)	0
Protocol Noncompliance	1 (0.8%)	1 (0.8%)
Other	1 (0.8%)	2 (1.7%)

Additional spirometry data

Changes in % predicted forced vital capacity (FVC) as a secondary endpoint, and changes in % predicted forced expiratory flow between 25-75% (FEF_{25-75}), as a tertiary endpoint, were analyzed via repeated-measures analysis of co-variance (RANCOVA) in combination with an unpaired t-test comparing ataluren with placebo at Week 48.

The pattern of changes in % predicted FVC and % predicted FEF_{25-75} were similar to that observed for % predicted FEV_1, ie, a smaller decline over 48 weeks was seen in ataluren-treated patients compared to placebo (Supplementary Figure 1).

Supplementary Figure 1: Mean Changes in % predicted FVC and FEF_{25-75} by Visit (ITT Population)



The plotted values represent observed data (±SEM).

The p-values were obtained from mixed-model repeated measures (MMRM) analyses. Covariates were baseline % predicted FVC (or FEF_{25-75}), treatment, visit, treatment-by-visit interaction, baseline FVC (or FEF_{25-75})-by-visit interaction, and the stratification factors of baseline inhaled antibiotics (yes vs no), baseline age (<18 vs ≥18 years), and baseline % predicted FEV_1 (40 to <65% vs ≥65 to 90%).

Abbreviations: FEF_{25-75} = forced expiratory flow between 25% and 75% of expiration, FEV_1 = forced expiratory volume in 1 second, FVC = forced vital capacity, ITT = intent-to-treat, MMRM = mixed-model repeated-measures

Additional pulmonary exacerbation data

Pulmonary exacerbation was defined in a number of ways in this study (Supplementary Table 5). Fuchs' criteria defines a pulmonary exacerbation as an event requiring treatment with parenteral antibiotics for any four of the following 12 symptoms[20]: change in sputum; new or increased hemoptysis; increased cough; increased dyspnea; fatigue; temperature >38°C; anorexia; sinus pain; change in sinus discharge; change in physical examination of the chest; decrease in pulmonary function by 10 percent or more from a previously recorded value; or radiographic changes indicative of pulmonary function. Modified Fuchs' criteria, the primary definition used in this study, is primarily a symptom based definition and comprises the same Fuchs signs and symptoms criteria without the requirement for treatment with antibiotics. In contrast, the expanded Fuchs' criteria requires the use of any form of antibiotic treatment (inhaled, oral, or intravenous) in addition to the presence of at least 4 of 12 Fuchs' signs and symptoms. The investigator assessment of the occurrence of a pulmonary exacerbation was recorded in respiratory events forms, which also documented the signs and symptoms associated with the event. The investigator determination was based on the individual physician's clinical judgment and did not require meeting any specific criteria.

Supplementary Table 5: Definitions of Pulmonary Exacerbation

Definition*	Presence of ≥4 of 12 Fuchs' Signs and Symptoms	Use of IV Antibiotics	Use of Any Antibiotics (oral, inhaled, or IV)
Fuchs	Yes	Yes	NA
Modified Fuchs (primary definition)	Yes	No	No
Expanded Fuchs	Yes	No	Yes
Investigator assessment	No	No	No

*Indicates criteria that must be met in order for an event to be counted as an exacerbation

Ataluren showed positive trends in all three Fuchs-related definitions of pulmonary exacerbation, but the results were not statistically significant (Supplementary Table 6).

Supplementary Table 6: Summary of Pulmonary Exacerbation Rate Over 48 Weeks for Different Definitions of Pulmonary Exacerbation (ITT)

Definition	Treatment Arm		Rate Ratio
	Ataluren	Placebo	
Modified Fuchs'			
Estimated rate (95% CI)	1.42 (1.05, 1.79)	1.78 (1.38, 2.17)	0.77 (0.57, 1.05)
p-value[a]	--	--	0.0992
Expanded Fuchs'			
Estimated rate (95% CI)	1.32 (0.96, 1.68)	1.60 (1.22, 1.98)	0.79 (0.57, 1.10)
p-value[a]	--	--	0.16
Fuchs'			
Estimated rate (95% CI)	0.98 (0.65, 1.30)	1.13 (0.80, 1.44)	0.86 (0.31, 1.14)
p-value[a]	--	--	0.12
Investigator judgment			
Estimated rate (95% CI)	2.26 (1.83, 2.69)	2.28 (1.88, 2.86)	0.94 (0.74, 1.18)
p-value[a]	--	--	0.52
Patient self-report			
Estimated rate (95% CI)	1.98 (1.50, 2.46)	1.79 (1.39, 2.21)	1.13 (0.79, 1.62)
p-value[a]	--	--	0.51

[a] The p-values were obtained from a negative binomial regression analysis. Covariates were treatment and stratification factors of chronic inhaled antibiotic use (yes vs no), age (<18 vs ≥18 years), and baseline % predicted FEV_1 (≥40 to <65% vs ≥65 to ≤90%).

Abbreviations: CI = confidence interval, FEV_1 = forced expiratory volume in 1 second, ITT = intent-to-treat population

The time to first and second pulmonary exacerbation based on modified Fuchs' criteria was analyzed in the intent-to-treat population. The median (CI) time to first pulmonary exacerbation in the ataluren arm was 197 (147 to 314) days vs 172 (112 to 230) days in placebo (p=0·3861). The median time to second pulmonary exacerbation data could not be calculated. The 25% quartile (CI) time to second pulmonary exacerbation was 273 (221 to NA) days in the ataluren arm vs 195 (138 to 310) days in placebo (p=0·1290).

Tertiary and other endpoints

Sweat Chloride

At baseline, mean sweat chloride concentration was 100·1 mmol/L for ataluren and 96·6 mmol/L for placebo (Supplementary Table 7). The mean change in sweat chloride concentration from baseline to Week 48 was -1·3 mmol/L for ataluren and -0·6 mmol/L for placebo. Thus, the difference between ataluren and placebo in mean change in sweat chloride concentration from baseline to Week 48 was 0·7 mmol/L (p=0·9919).

Supplementary Table 7: Summary of Sweat Chloride Concentration at Baseline and Week 48 (ITT Population)

Visit/Parameter, mmol/L	Treatment Arm	
	Ataluren	**Placebo**
Baseline, N	114	111
Mean (SD)	100.1 (14.22)	96.6 (15.93)
Median	101.5	100.0
Minimum, maximum	22.5, 128.0	22.0, 117.5
Week 48, N	98	102
Mean (SD)	100.1 (13.35)	96.7 (17.20)
Median	101.3	101.5
Minimum, maximum	30.0, 127.5	38.5, 126.5
Δ from baseline to Week 48, N	97	97
Mean (SD)	-1.3 (8.94)	-0.6 (10.27)
Median	-1.0	0.0
Minimum, maximum	-31.5, 16.0	-52.0, 19.0
p-value[a]	0.9919	

[a]The p-value was obtained from an MMRM analysis. Covariates were baseline sweat chloride concentration, treatment, visit, treatment-by-visit interaction, baseline sweat chloride concentration-by-visit interaction, and the stratification factors of baseline inhaled antibiotics (yes vs no), baseline age (<18 vs ≥18 years), and baseline % predicted FEV1 (40 to <65% vs ≥65 to 90%).

Abbreviations: FEV_1 = forced expiratory volume in 1 second, ITT = intent-to-treat, MMRM = mixed-model repeated measures, SD = standard deviation

Sweat Chloride Sensitivity Analysis

Analysis was also performed for change from baseline in sweat chloride concentration excluding patients with baseline <40 mmol/L in the intent-to-treat population. The overall difference in the ataluren arm vs placebo was -0·62 and was not statistically significant (p = 0·9423).

Nasal Transepithelial Potential Difference

Total chloride transport (the change induced by zero chloride plus isoproteronol) was assessed by NPD. At baseline, mean total chloride transport was similar in the ataluren and placebo arms (Supplementary Table 8). The mean change in total chloride transport from baseline to Week 48 was 0·31 mV for ataluren vs 0·14 mV for placebo, resulting in a small, non-meaningful difference between treatment arms.

Supplementary Table 8: Summary of Total Chloride Transport at Baseline and Week 48 (ITT Population)

Total Chloride Transport, Visit/Parameter, mV	Treatment Arm	
	Ataluren	**Placebo**
Baseline, N	116	116
Mean (SD)	1.58 (3.88)	1.95 (3.55)
Median	1.69	1.85
Minimum, maximum	-5.82, 11.36	-6.26, 12.33
Week 48, N	100	104
Mean (SD)	1.72 (4.19)	1.99 (4.65)
Median	1.61	2.32
Minimum, maximum	-10.65, 15.39	-8.64, 17.64
Δ from baseline to Week 48, N	100	104
Mean (SD)	0.31 (5.06)	0.14 (5.81)
Median	0.678	1.133
Minimum, maximum	-14.95, 11.48	-16.07, 19.29
p-value[b]	0.8587	

[a] The centrally read results of an unblinded reader were used for eligibility assessment of 2 patients whose centrally analyzed blinded NPD reading was subsequently found to be more electrically negative than -5 mV for total chloride conductance [Δchloride-free+isoproterenol].

bThe p-value was obtained from an MMRM analysis. Covariates were baseline total chloride transport, treatment, visit, treatment-by-visit interaction, baseline total chloride transport-by-visit interaction, and the stratification factors of baseline inhaled antibiotics (yes vs no), baseline age (<18 vs ≥18 years), and baseline % predicted FEV1 (40 to <65% vs ≥65 to 90%).

Abbreviations: FEV1 = forced expiratory volume in 1 second, ITT = intent-to-treat, MMRM = mixed-model repeated measures, SD = standard deviation

Total chloride transport responses and hyperpolarizations were observed in similar frequencies in both treatment arms (Supplementary Table 9), and among these other nasal parameters, differences between arms were very small and not statistically significant.

Supplementary Table 9: Summary of Total Chloride Transport Responders and Hyperpolarizers (ITT Population)

Visit, n (%)[a]	Treatment Arm	
	Ataluren (N=116)	**Placebo (N=116)**
Baseline		
Responder[b]	NA	NA
Hyperpolarizer[c]	3 (2.6)	3 (2.6)
Week 16		
Responder[b]	14 (13.3)	21 (18.9)
Hyperpolarizer[c]	4 (3.8)	6 (5.4)
Week 32		
Responder[b]	15 (15.3)	22 (21.4)
Hyperpolarizer[c]	5 (5.1)	12 (11.7)
Week 48		
Responder[b]	13 (13)	16 (15.4)
Hyperpolarizer[c]	6 (6.0)	6 (5.8)

[a] Percentage calculation is based on number of patients in the ITT population who had data available for the assessment of the parameter.

[b] Response = at least a -5 mV improvement in total chloride transport from baseline

[c] Hyperpolarization = total chloride transport value at least as electrically negative as -5 mV

Abbreviations: ITT = intent-to-treat, NA = not applicable

In addition to the total chloride transport described in the paper, other nasal parameters assessed by NPD were basal potential difference, sodium transport, intrinsic chloride transport, stimulated chloride transport, total potential difference, and adenosine triphosphate (ATP)-mediated chloride transport. (Supplementary Table 10).

Supplementary Table 10: Nasal Potential Difference Assessment Parameters (ITT Population)

Parameter, Mean Change from Baseline to Week 48, mV (SD) [a]	Treatment Arm		
	Ataluren (N=116)	**Placebo (N=116)**	p-value[b]
Basal potential difference[c]	5.66 (15.43)	3.03 (15.37)	0.0774
Sodium transport[d]	-5.20 (12.46)	-3.52 (12.40)	0.1696
Intrinsic chloride transport[e]	0.90 (4.10)	0.64 (4.91)	0.7538
Stimulated chloride transport[f]	-0.59 (3.87)	-0.50 (3.67)	0.9703
Total chloride transport[g]	0.31 (5.06)	0.14 (5.81)	0.8587
Total potential difference[h]	-4.95 (12.84)	-3.39 (13.06)	0.1729
ATP-mediated chloride transport[i]	2.35 (11.53)	2.05 (10.28)	0.9081

[a] Mean Δ from baseline calculation is based on number of patients who had data available for the assessment of the parameter.

[b] P-values were obtained from an MMRM analysis. Covariates were baseline value, treatment, visit, treatment-by-visit interaction, baseline value-by-visit interaction, and the stratification factors of baseline inhaled antibiotics (yes vs no), baseline age (<18 vs ≥18 years), and baseline % predicted FEV_1 (40 to <65% vs ≥65 to 90%).

[c] Measurement at the end of Ringer's solution

[d] Measurement at end of amiloride solution minus measurement at end of Ringer's solution.

[e] Measurement at end of chloride-free gluconate minus measurement at end of amiloride solution.

[f] Measurement at end of isoproterenol solution minus measurement at end of chloride-free gluconate solution.

[g] Measurement at the end of isoproterenol solution minus measurement at the end of amiloride solution.

[h] Measurement at the end of isoproterenol solution minus measurement at the end of Ringer's solution.

[i] Measurement at the end of ATP solution minus measurement at the end of isoproteronol solution.

Abbreviations: ATP = adenosine triphosphate, ITT = intent-to-treat, mV = millivolt

General Well Being

Body weight and BMI were stable over 48 weeks, resulting in a small and not statistically significant difference between treatment groups (body weight Δ=0·04 kg; BMI Δ=-0·108).

Health-Related Quality of Life

The Cystic Fibrosis Questionnaire-Revised (CFQ-R) respiratory domain is a health-related quality-of-life (HRQL) questionnaire that is scored on a 100-point scale, with higher numbers indicating a lower effect of symptoms on the patient's quality of life. CFQ-R assessed separately for children age 6-13 years, whose questionnaires consisted of the same questions, and for adolescents and adults (≥14 years), whose questionnaire consisted of a different set of questions than the 6-13 year olds questions. The difference between ataluren and placebo in mean change of this endpoint was small not statistically significant. In children, the mean change in the CFQ-R respiratory domain score from baseline to Week 48 was -0·7 in the ataluren arm, and -3·6 in the placebo arm (Δ= 2·9, p=0·8152). In adolescents and adults, the mean change in the score from baseline to Week 48 was -2·8 for ataluren vs -3·3 for placebo (Δ= 0·5, p=0·3723).

Cough

The VivoMetrics LifeShirt® is a measurement device that has been shown to be an objective and a safe method for measuring cough frequency in CF patients.[31] The device includes motion-sensing transducers, electrodes, a microphone, and a 3-axis accelerometer, all of which are housed within a wearable, lightweight vest. A Phase 2 ataluren study (Study 005e) piloted this novel assessment of quantitative cough frequency,[31] which has not been attempted in other CF clinical trials published to date. In Study 005e, a baseline mean [range] awake cough rate of 43 [20-82] coughs/hour was observed (N=18). Based on the limited experience with this outcome measure, cough assessment was included in Study 009. Because coughs were seen in much greater frequency while patients are awake, the pre-specified primary analysis of this tertiary endpoint in Study 009 was the change in awake cough rate. Also collected via the LifeShirt® were changes in total, awake, and asleep cough parameters, ie, rate, duration, intensity, and area under the cough-intensity vs time curve (AUC_{cough}).

The mean change in awake cough hourly rate from baseline to Week 48 was -0.60 coughs per hour for ataluren vs 0·88 coughs/hour for placebo. The difference was not statistically significant (p=0·9695). Of note, the baseline mean [range] awake cough rates were substantially lower in this study than in the Phase 2 pilot study: 28 [2, 107] coughs/hour for ataluren and 25 [0·4, 101] coughs/hour for placebo. In the evaluable ITT population (N=229), a total of the 99 (43%) patients (47 ataluren and 52 placebo) had a baseline awake cough hourly rate <20 coughs/hour, which was the minimum baseline value observed in the Phase 2 study. Thus, a floor effect may have limited the ability to detect improvements in awake cough hourly rate in this study.

The other parameters assessed by LifeShirt resulted in differences between ataluren and placebo that were small and not of clinical significance.

Inflammation

Sputum and serum inflammatory markers were also assessed as endpoints. The mean change from baseline to Week 48 for sputum interleukin-8 (IL-8) and neutrophil elastase were small in both arms, and the differences between ataluren and placebo at Week 48 were not statistically significant (p=0·7966 and p=0·5955, respectively).

Concentrations of inflammatory markers, including IL-8 and c-reactive protein (CRP), in serum were assessed. Over 48 weeks, the mean changes were small and the differences between ataluren and placebo were not statistically different at the end of treatment (p=0·3115 and p=0·6000, respectively). Blood neutrophil levels in whole blood were assessed and the differences between treatment arms at Week 48 were small and not statistically significant (p=0·5360).

Also assessed were hepatobiliary inflammatory markers via concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), alkaline phosphatase, and total bilirubin in serum. Over 48 weeks, differences in mean changes in ALT, AST, and GTT between ataluren and placebo were small and not statistically significant. Although statistically significant (p=0·0160), the difference in total bilirubin between ataluren and placebo was small (-0·06 mg/dL).

Computerized Tomography

Computerized tomography (CT) of the lungs have been utilized as a monitoring tool for CF patients in multiple clinical trials,[35] and has shown potential for assessment of the pulmonary pathology of CF.[35-38] Scoring systems have been devised to convert scan imagery to more useful quantitative numerical data for evaluating disease severity.[36,37] In Study 009, as a tertiary endpoint, changes in lung CT scores were assessed.

A CT scan of the chest was obtained at baseline and at end of treatment (~Week 48) for all study participants. Total lung score was established by the sum of five characteristics from the Brody scoring system[36]; resulting in a total possible score range of 0-40·5. The characteristics scored were bronchiectasis (score range 0 – 12), mucus plugging (score range 0 – 6), peribronchial thickening (score range 0 – 9), parenchyma (score range 0-9), and hyperinflation (score range 0 – 4·5). The average of 2 blinded reviewers was used to determine total lung score and specific subscores, a lower score indicating improvement of the disease.

Baseline (BL) and End of Treatment (EOT) CT scans were obtained for 203 patients in the ITT population who completed 48 weeks of blinded treatment. The difference in total lung score

between the ataluren and placebo groups at Week 48 (-0·28) was not significant (Supplementary Table 11). The mean change from baseline in total lung score at Week 48 was small for the ataluren group (0·28±1·34; n=99) and the placebo group (0·56±1·56; n=104) and was not significant. The changes in total lung scores in patient subgroups of inhaled aminoglycoside use and inhaled antibiotics use also did not reveal significant differences between ataluren and placebo.

An increase in score from baseline to end of treatment would be consistent with the anticipated disease progression but was not seen in the placebo group. Importantly, this lack of change in the placebo group is contrasted to the mean relative change in % predicted FEV_1 of -5·5% seen during the same time period. The lack of change in total lung score in the placebo arm, despite a mean relative change in % predicted FEV_1 of -5·5% seen during the same time period, suggests that morphologic change on CT scan does not parallel functional change in FEV_1 over 48weeks. The lack of disease progression as observed on CT scan may limit its use in study designs dependent on progressive disease in the placebo arm over 48 weeks.

Supplementary Table 11: Change in Total Lung CT Score and Subscores (ITT Population)

Score, ±SD	Ataluren			Placebo			Δ	P-value[1]
	BL	EOT	EOT− BL	BL	EOT	EOT− BL		
Total Lung (primary)	9.53±3.75	9.85±3.69	0.28±1.34	9.62±3.42	10.19±3.43	0.56±1.56	-0.28	0.833
Bronchiectasis	3.00±1.65	3.13±1.67	0.12±0.62	3.02±1.56	3.24±1.55	0.23±0.73	-0.11	0.359
Air Trapping	2.21±0.70	2.28±0.67	0.05±0.52	2.17±0.72	2.29±0.69	0.10±0.53	-0.04	0.713
Mucus Plugging	1.83±0.83	1.89±0.82	0.05±0.46	1.96±0.83	2.03±0.79	0.06±0.44	-0.02	0.568
Parenchyma	0.72±0.43	0.73±0.38	0.02±0.28	0.72±0.41	0.75±0.39	0.02±0.33	0.00	0.971
Peribronchial Thickening	1.77±0.90	1.82±0.91	0.04±0.39	1.75±0.83	1.89±0.81	0.15±0.49	-0.11	0.125

P-value for the Total Lung score is based on nonparametric Rank ANCOVA; other p-values are based on an ANCOVA analysis.

Abbreviations: BL = baseline, EOT = end of treatment, ITT = intent to treat, SD = standard deviation

Effect of Antibiotics on Readthrough Activity of Ataluren In Vitro

Since aminoglycoside antibiotics have been reported to bind to ribosomal RNA, this study was designed to evaluate the effect of antibiotics on the readthrough activity of ataluren in a nonsense-mutation-containing in vitro reporter system.

Methods and results

Four antibiotics were selected for analysis that included two aminoglycosides (gentamicin and tobramycin) and two nonaminoglycoside antibiotics (aztreonam and colistin), reflecting the antibiotics used in the Phase 3 clinical trial. Tobramycin and gentamicin both interact with prokaryotic ribosomal RNA[38,39] and are reported to demonstrate nonsense suppression (i.e. ribosomal readthrough) activity in an in vitro eukaryotic system.[39,40]

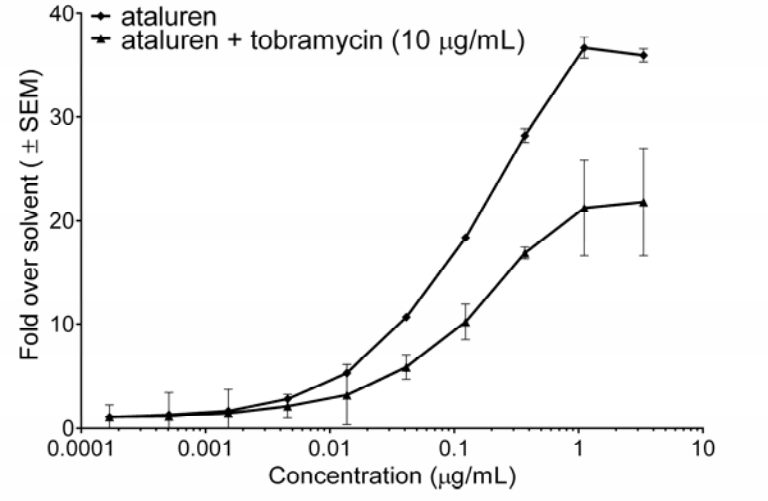
A luciferase reporter construct containing an intron, a premature termination codon and a FLAGepitope tag was stably transfected into human embryonic kidney cells (HEK293 LUC-190 FLAG). Activity was determined using BrightGlo (Promega,Madison WI).[41]

The antibacterial activity of each antibiotic was tested on a panel of sensitive bacterial strains and shown to be active (data not shown). The cytotoxicity of each compound was determined after treatment for 20 hours; no cytotoxicity was observed (Supplementary Figure 2 panel C).

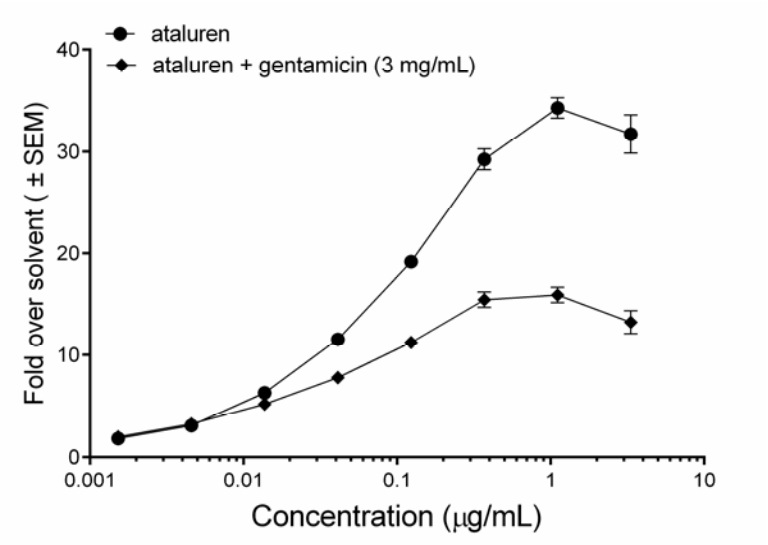
To evaluate the effect of ataluren in combination with antibiotics, the antibiotic concentration that resulted in maximal readthrough activity or that was not cytotoxic was utilized. For tobramycin, a concentration of 10 μg/mL was used based on the work of Keeling 2002 (Figure 3B).[42] For gentamicin, a concentration of 3 mg/mL was used. For both aztreonam and colistin, a concentration of 100 μg/mL was used. Cells harboring the luciferase reporter (HEK293 LUC-190 FLAG) were grown in Dulbecco's Modified Eagle Media (DMEM) containing 10% fetal

bovine serum (FBS) at 37°C and 5% CO2. The combination of each antibiotic with ataluren (169 pg/mL to 3·3 μg/mL; 55 nM to 11 μM) was added to the cells and incubated for 20 hours. The dose response curves for ataluren with and without the two aminoglycosides are shown in Supplementary Figures 2A and 2B. The decrease in activity was not due to cytotoxicity (Supplementary Figure 2C).

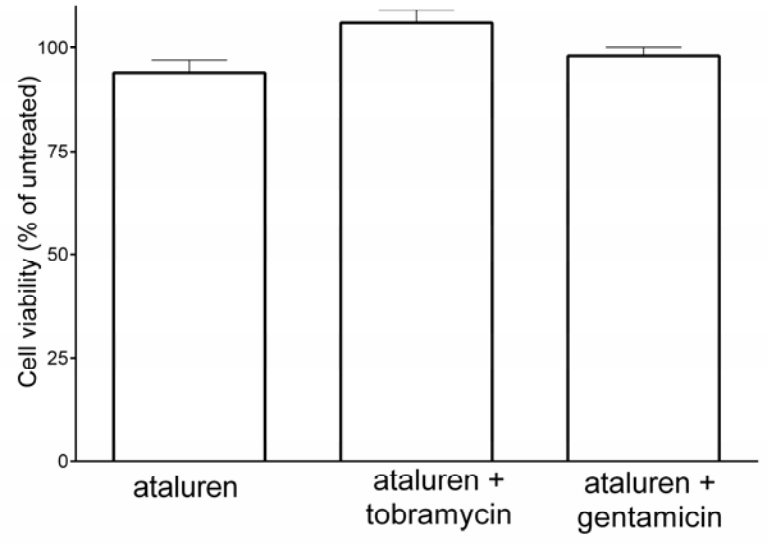
Supplementary Figure 2: Aminoglycoside antibiotics reduce ataluren activity in the cell-based reporter assay.



A) HEK293 LUC-190 FLAG cells were incubated with ataluren alone (169 pg/mL to 3.3 μg/mL) and in combination with tobramycin (10 μg/mL).



B) HEK293 LUC-190 FLAG cells were incubated with ataluren alone (1.5 ng/mL to 3.3 µg/mL) and in combination with gentamicin (3 mg/mL).



C) Cell viability of HEK293 LUC-190 FLAG cells incubated with ataluren alone (3.3 µg/mL) and in combination with tobramycin (10 µg /mL) or gentamicin (3 mg/mL) is not affected. Abbreviation: SEM = standard error of the mean.

To determine whether ataluren antagonizes the antimicrobial activity of tobramycin, ataluren was tested in combination with tobramycin. Ataluren and tobramycin were dissolved in DMSO at a concentration 40-fold higher than the highest concentration tested. The DMSO was then serially diluted 2-fold in DMSO to generate diluted stock solutions that were added to the 96-well plates, in duplicate, at a 1:40 dilution. Prior to testing, *P. aeruginosa* bacteria were isolated on agar plates to ensure that a monoclonal population was used. Logarithmically growing cells were diluted to approximately 5 x 10^5 colony forming units (CFU)/mL and subjected to test compounds solubilized and serially diluted in DMSO, with a final DMSO concentration of 2·5%. After 18 hours of incubation at 37^{o}C, the optical density at 600 nm (OD_{600}) was determined by reading the 96-well microtiter plates on a plate reader. For a given concentration, a minimum inhibitory concentrations (MIC) determination was noted if: [OD600 Control – OD600 Test Concentration]/[OD600 Control – OD600 Media] x 100 ≥90%, which correlated with no observable turbidity.

As shown in Supplementary Table 12, the MICs for ataluren (PTC124, sodium salt form) were >250 μg/mL, indicating a lack of antibacterial activity at all testing concentrations up to and including 250 μg/mL. The MIC for tobramycin was ~0·4 μg/mL when tested alone, and was not changed in the presence of ataluren concentrations ranging from 0·24 to 125 μg/mL.

Supplementary Table 12: MICs for Tobramycin Against *P. aeruginosa* When Tested inthe Presence and Absence of Ataluren

Compound	MIC (μg/mL)	Ataluren concentration (μg/mL)
tobramycin	0.39	0
tobramycin	0.39	0.24
tobramycin	0.39	0.49
tobramycin	0.78	0.98
tobramycin	0.39	1.95
tobramycin	0.78	3.91
tobramycin	0.39	7.81
tobramycin	0.78	15.63
tobramycin	0.39	31.25
tobramycin	0.39	62.5
tobramycin	0.39	125
ataluren	>250	250

Conclusion

The data demonstrate that the ability of ataluren to enable readthrough is reduced in the presence of antibiotics that are known to interact with ribosomal RNA (gentamicin and tobramycin), but not in the presence of antibiotics that act through alternative mechanisms (aztreonam and colistin). The reduced activity is not due to effects on cell viability. Conversely, ataluren did not affect the antibacterial activity of tobramycin as tested against *Pseudomonas aeruginosa*.

Adverse Events

Treatment-emergent adverse events occurring at a by-patient frequency ≥10% are shown in Supplementary Table 13. The most common types of disorders were respiratory, thoracic and mediastinal disorders; infections and infestations; gastrointestinal disorders. Most treatment-emergent adverse events were mild (Grade 1) or moderate (Grade 2) in severity

Supplementary Table 13: Treatment-Emergent Adverse Events with a Patient Frequency of >10% by System Organ Class (As-Treated Population)

MedDRA System Organ Class/Preferred Term[a], n (%)	Treatment Arm		All Patients
	Ataluren	Placebo	
	N=120	N=118	N=238
Gastrointestinal disorders	**58 (48.3%)**	**51 (43.2%)**	**109 (45.8%)**
Diarrhoea	13 (10.8%)	21 (17.8%)	34 (14.3%)
Abdominal pain	18 (15.0%)	15 (12.7%)	33 (13.9%)
Vomiting	14 (11.7%)	10 (8.5%)	24 (10.1%)
Nausea	11 (9.2%)	12 (10.2%)	23 (9.7%)
General disorders and administration site conditions	**33 (27.5%)**	**34 (28.8%)**	**67 (28.2%)**
Pyrexia	18 (15.0%)	21 (17.8%)	39 (16.4%)
Infections and infestations	**76 (63.3%)**	**77 (65.3%)**	**153 (64.3%)**
Viral upper respiratory tract infection	21 (17.5%)	29 (24.6%)	50 (21.0%)
Sinusitis	15 (12.5%)	14 (11.9%)	29 (12.2%)
Rhinitis	12 (10.0%)	12 (10.2%)	24 (10.1%)
Upper respiratory tract infection	5 (4.2%)	12 (10.2%)	17 (7.1%)
Nervous system disorders	**27 (22.5%)**	**23 (19.5%)**	**50 (21.0%)**
Headache	20 (16.7%)	14 (11.9%)	34 (14.3%)
Renal and urinary disorders	21 (17.5%)	5 (4.2%)	26 (10.9%)
Acute kidney injury[b]	**18 (15.0%)**	**1 (0.01%)**	**19 (0.08%)**
Respiratory, thoracic and mediastinal disorders	**105 (87.5%)**	**111 (94.1%)**	**216 (90.8%)**
Pulmonary exacerbation	92 (76.7%)	94 (79.7%)	186 (78.2%)
Cough	26 (21.7%)	35 (29.7%)	61 (25.6%)
Haemoptysis	11 (9.2%)	18 (15.3%)	29 (12.2%)

MedDRA System Organ Class/Preferred Term[a], n (%)	Treatment Arm		All Patients
	Ataluren	Placebo	
	N=120	N=118	N=238
Productive cough	12 (10.0%)	11 (9.3%)	23 (9.7%)
Oropharyngeal pain	4 (3.3%)	14 (11.9%)	18 (7.6%)

[a] Adverse events with a frequency of >10% in either treatment arm are displayed alphabetically by MedDRA System Organ Class and from highest to lowest incidence across both treatment arms within each System Organ Class. Patients who had the same adverse event more than once are counted only once for that adverse event. Adverse events with a frequency of ≤10% across both treatment arms are not shown.

[b] Includes reported terms including renal failure, acute renal failure, renal impairment, and hypercreatininaemia.

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