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Randomised trial of azithromycin to eradicate *Ureaplasma* in preterm infants

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ABSTRACT

Objective To test whether azithromycin eradicates *Ureaplasma* from the respiratory tract in preterm infants.

Design Prospective, phase IIb randomised, double-blind, placebo-controlled trial.

Setting Seven level III–IV US, academic, neonatal intensive care units (NICUs).

Patients Infants 24^0–28^6 weeks' gestation (stratified 24^0–26^6; 27^0–28^6 weeks) randomly assigned within 4 days following birth from July 2013 to August 2016.

Interventions Intravenous azithromycin 20 mg/kg or an equal volume of D5W (placebo) every 24 hours for 3 days.

Main outcome measures The primary efficacy outcome was *Ureaplasma*-free survival. Secondary outcomes were all-cause mortality, *Ureaplasma* clearance, physiological bronchopulmonary dysplasia (BPD) at 36 weeks' postmenstrual age, comorbidities of prematurity and duration of respiratory support.

Results One hundred and twenty-one randomised participants (azithromycin: n=60; placebo: n=61) were included in the intent-to-treat analysis (mean gestational age 26.2±1.4 weeks). Forty-four of 121 participants (36%) were *Ureaplasma* positive (azithromycin: n=19; placebo: n=25). *Ureaplasma*-free survival was 55/60 (92% (95% CI 82% to 97%)) for azithromycin compared with 37/61 (61% (95% CI 48% to 73%)) for placebo. Mortality was similar comparing the two treatment groups (5/60 (8%) vs 6/61 (10%)). Azithromycin effectively eradicated *Ureaplasma* in all azithromycin-assigned colonised infants, but 21/25 (84%) *Ureaplasma*-colonised participants receiving placebo were culture positive at one or more follow-up timepoints. Most of the neonatal mortality and morbidity was concentrated in 21 infants with lower respiratory tract *Ureaplasma* colonisation. In a subgroup analysis, physiological BPD-free survival was 5/10 (50%) (95% CI 19% to 81%) among azithromycin-assigned infants with lower respiratory tract *Ureaplasma* colonisation versus 2/11 (18%) (95% CI 2% to 52%) in placebo-treated infants.

Conclusion A 3-day azithromycin regimen effectively eradicated respiratory tract *Ureaplasma* colonisation in this study.

Trial registration number NCT01778634.

What is already known on this topic?

- Respiratory tract colonisation with the genital mycoplasmas *Ureaplasma parvum* and *U. urealyticum* is an independent risk factor for bronchopulmonary dysplasia in extremely low gestational age infants.
- The azalide antibiotic azithromycin has anti-inflammatory properties and exhibits high potency against *Ureaplasma* isolates in vitro.
- Open-label pharmacokinetic/pharmacodynamics studies of single and multiple dose aizithromycin indicate that 20 mg/kg x3 days was most effective in eradicating respiratory tract *Ureaplasma* in preterms.

What this study adds?

- In this randomised clinical trial that included 121 infants <29 weeks' gestation, *Ureaplasma*-free survival was significantly higher in the azithromycin compared with the placebo group.
- *Ureaplasma* was eradicated in all colonised infants assigned to azithromycin compared with 16% in colonised infants assigned to placebo.
- Since neonatal mortality and morbidity was concentrated in infants with lower respiratory tract *Ureasplasma* colonisation, this population should be targeted in future phase III randomised controlled trial.

INTRODUCTION

Ureaplasma respiratory tract colonisation is an independent risk factor for developing bronchopulmonary dysplasia (BPD) in preterm infants.[1–4] Evidence from clinical studies,[1 5] and experimental infection models,[6–10] established lung *Ureaplasma* as proinflammatory and profibrotic, contributing to BPD alone or when combined with inflammatory stimuli such as hyperoxia or mechanical ventilation.[8] Whether eradicating *Ureasplasma* from the developing lung will reduce the risk for BPD is unknown.

Since azithromycin exhibits high potency against clinical *Ureaplasma* isolates in vitro[11] and immunomodulatory properties,[12] it is an appropriate therapeutic candidate to eradicate *Ureaplasma* and reduce inflammation-mediated BPD in preterm infants.[13] We conducted open-label, pilot studies characterising the population pharmacokinetics, safety and microbiological efficacy of intravenous

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10 and 20 mg/kg single dose and 20 mg/kg x3d multidose azithromycin in 24–28 week gestation infants.[14–16] In the open-label, uncontrolled 20 mg/kg multidose study, azithromycin eradicated *Ureaplasma* and appeared safe, with no deaths or serious adverse events attributed to the drug. We performed the current pilot (phase IIb) randomised clinical trial to test: (1) the feasibility of recruitment and *Ureaplasma* detection; (2) whether the 3-day azithromycin regimen was safe and was more effective than placebo to eradicate *Ureaplasma* in colonised infants; and (3) whether azithromycin would be beneficial or harmful in the subgroups of *Ureaplasma*-positive and *Ureaplasma*-negative infants. Respiratory outcomes were explored as potential endpoints for a later phase III trial.

METHODS

Study design and oversight

Study design was a prospective, randomised, double-blind, placebo-controlled trial (clinicaltrials.gov NCT01778634). The U.S. Food and Drug Administration (IND78990) and the Institutional Review Board of each participating institution approved the study protocol. Written parental consent was obtained for all participants prior to randomisation. Recruitment was conducted in seven US academic, level III/IV neonatal intensive care units from July 2013 to August 2016. An independent data and safety monitoring committee reviewed unblinded data every 6 months to assess safety and study performance.

Participants

Eligible participants were extremely low gestation newborns (ELGAN) 24^0–28^6 weeks' gestation (November 2013–January 2016), <72-hour postnatal age who received positive pressure ventilation for at least 1 hour. Since rapid diagnostic testing for *Ureaplasma* was not feasible, presence of *Ureaplasma* colonisation was not an inclusion criterion and was unknown at the time of recruitment. To focus enrollment on infants with the highest *Ureaplasma* prevalence, the protocol was revised to limit eligibility to the lower gestation stratum (24^0–26^6 weeks) for the last 6 months of enrollment (February–August 2016). Exclusion criteria were: non-viability or planned life support withdrawal; lethal congenital anomalies; >twin gestation; delivery for maternal indications; ECG corrected QT interval ≥450 ms; significant hepatic impairment; other systemic macrolide exposure; clinically suspected *Ureaplasma* CNS infection or culture-confirmed sepsis; or participation in other clinical trials.

Randomisation and intervention

Participants were stratified by gestational age (24^0–26^6 vs 27^0–28^6 weeks) and assigned in 1:1 ratio to azithromycin or placebo using separate randomisation schedules for each clinical site and stratum with twins assigned to the same treatment. The web-based randomisation system (Axio Research, Seattle, Washington, USA) used a permuted block design with varying block sizes of 2, 4 and 6. Baseline respiratory specimens were obtained; infants were randomised; and the first study drug dose administered within 24 hours of signed consent. Participants received azithromycin (American Pharmaceuticals Partners, Schaumburg, Illinois, USA) 20 mg/kg at a concentration of 2 mg/mL in 5% dextrose water or equal volume of 5% dextrose water (10 mL/kg) as a placebo intravenously via a peripheral or central line over 60 min every 24 hours for three doses. The primary care team at each site determined the fluid management of enrolled patients. Participants, care providers and study staff were blinded to treatment assignment.

Ureaplasma culture, antibiotic susceptibility testing and real-time PCR

Two tracheal aspirates (TAs) at least 2 hours apart and one nasopharyngeal sample from intubated infants, or two nasopharyngeal samples at least 2 hours apart from non-intubated infants were obtained for *Ureaplasma* culture and PCR before the first dose. Subsequent samples were obtained at 2 and 4–5 days after the last dose and 21 days postnatal age. Each specimen was frozen for later shipment to the University of Alabama at Birmingham Diagnostic Mycoplasma Laboratory for culture and azithromycin susceptibility testing. Species-specific real-time PCR[15] was performed on all respiratory samples and each *Ureaplasma* isolate. Culture positivity was defined as a positive 10B broth culture from either TA or nasopharyngeal specimens confirmed by typical morphology. A culture was considered negative after no growth was detected for 7 days. Patients who were culture or PCR positive at any time point were classified as positive. *Ureaplasma* eradication was defined as three negative cultures post-treatment.

Outcomes

To accomplish an analysis according to the principle of intention to treat and avoid treating death as a good or neutral outcome, we defined our primary outcome as *Ureaplasma*-free survival (ie, survival to NICU discharge with three negative cultures post-treatment). Secondary outcomes were mortality, *Ureaplasma* clearance, physiological BPD at 36 weeks' postmenstrual age (PMA) determined by a room air challenge (RAC), comorbidities of prematurity and duration of respiratory support. Participants were assessed at 36±1 weeks' PMA and physiological BPD classified as present if they were receiving positive pressure respiratory support, nasal cannula flow ≥4 liters per minute (LPM or effective fractional inspired oxygen >0.3[17–19] or failed a RAC.[20] To compare with BPD rates in other neonatal trials, participants were also classified according to the BPD severity[21] and the modified Shennan classification[22] that assigned infants on supplemental oxygen at 36^0 week as BPD present regardless of respiratory support and infants discharged home on room air <36 weeks' PMA as BPD absent.

Sample size and statistical analysis

Using a two-sided α level of 0.05 and assuming an 80% overall survival rate in both groups, 20% twins and a 25% placebo clearance rate,[23] the study would have power of 0.8 to detect an absolute 40% difference in the primary outcome of *Ureaplasma*-free survival with enrolment of 30 *Ureaplasma*-positive infants in each group. With an expected 45% respiratory prevalence[23] and 5% drop-out rate, we planned to enrol 140 participants. The principal investigator (RV) ended recruitment without any information on the unblinded treatment comparisons when 121 neonates had been randomised because of interruption to funding.

For the efficacy analysis, we compared *Ureaplasma* eradication and other outcomes among all randomised participants according to the principle of intention to treat and in the subgroups of *Ureaplasma*-positive and *Ureaplasma*-negative participants to estimate the extent to which azithromycin had efficacy beyond clearance. To account for possible correlation between outcomes in twins, we used generalised estimating equations[24] and multiple outputation.[25] When observed counts were small, we used exact methods without accounting for twinning to calculate p values and CIs. Additional details of

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Table 1 Baseline characteristics of the study participants for the total cohort and stratified by *Ureaplasma* status

	No. of participants (%)					
	Total cohort		*Ureaplasma* positive		*Ureaplasma* negative	
	(n=121)		(n=44)		(n=77)	
Characteristic	AZM (n=60)	Placebo (n=61)	AZM (n=19)	Placebo (n=25)	AZM (n=41)	Placebo (n=36)
Male, n (%)	26 (43)	32 (52)	11 (58)	10 (40)	15 (37)	22 (61)
Race, n (%)						
White	36 (60)	15 (25)	13 (68)	5 (20)	23 (56)	10 (28)
African-American	21 (35)	43 (70)	6 (32)	19 (76)	15 (37)	24 (67)
Asian	0 (0)	1 (2)	0	1 (4)	0	0
Multiple/biracial	3 (5)	2 (3)	0	0 (0)	3 (7)	2 (6)
Hispanic ethnicity, n (%)	2 (3)	0 (0)	0 (0%)	0 (0)	2 (5)	0 (0)
Birth weight, mean (SD), g	895 (215)	903 (245)	897 (195)	851 (282)	895 (226)	939 (213)
Gestational age, mean (SD), weeks	26.2 (1.4)	26.2 (1.4)	25.8 (1.1)	25.8 (1.4)	26.4 (1.5)	26.5 (1.4)
Gestational age strata, n (%)						
24^0–26^6 weeks	40 (67)	43 (70)	16 (84)	20 (80)	24 (59)	23 (64)
27^0–28^6 weeks	20 (33)	18 (30)	3 (16)	5 (20)	17 (41)	13 (36)
SGA, n (%)	2 (3)	1 (2)	0 (0)	1 (4)	2 (5)	0 (0)
Preterm labour, n (%)	47 (78)	49 (80)	17 (89)	18 (72)	30 (73)	31 (86)
PPROM, n (%)	23 (38)	29 (48)	9 (47)	17 (68)	14 (34)	12 (33)
Duration rupture of membranes, n (%)						
<1 hour	36 (60)	29 (48)	9 (47)	7 (28)	27 (66)	22 (61)
≥1 hour	22 (37)	29 (48)	9 (47)	16 (64)	13 (32)	13 (36)
Unknown	2 (3)	3 (5)	1 (5)	2 (8)	1 (2)	1 (3)
Maternal Pe-eclampsia, n (%)	0 (0)	2 (3)	0 (0)	1 (4)	0 (0)	1 (3)
Antenatal steroids, n (%)	51 (85)	48 (79)	16 (84)	19 (76)	35 (85)	29 (81)
Maternal macrolide, n (%)						
Erythromycin	10 (17)	11 (18)	4 (21)	8 (32)	6 (15)	3 (8)
Azithromycin	9 (15)	9 (15)	2 (11)	1 (4)	7 (17)	8 (22)
Both	1 (2)	0	0 (0)	0	1 (2)	0 (0)
Neither	40 (67)	41 (67)	13 (68)	16 (64)	27 (66)	25 (69)
Route of delivery, n (%)						
SVD	27 (45)	27 (44)	9 (47)	13 (52)	18 (44)	14 (39)
C/S	33 (55)	34 (56)	10 (53)	12 (48)	23 (56)	22 (61)
Apgar 1 min, median (IQR)	5 (3,7)	4 (2,6)	4 (2,8)	4 (2,5)	5 (3,7)	5 (2.5 to 6.5)
Apgar 5 min, median (IQR)	7 (6,8)	7 (6,8)	6.5 (5,8)	6 (6,8)	7 (6,8)	7 (5.5 to 8)
Respiratory support at enrolment, n (%)						
None	2 (3)	1 (2)	1 (5)	0 (0)	1 (2)	1 (3)
Non-invasive*	28 (47)	34 (56)	10 (53)	15 (60)	18 (44)	19 (53)
Invasive†	30 (50)	26 (43)	8 (42)	10 (40)	22 (54)	16 (44)
Duration IMV at enrolment, median (IQR), hours	24.9 (10.3,52.3)	29 (15.0,46.8)	20.5 (0.3,53.0)	30.9 (21.1,49.3)	26.5 (12,49.2)	22.2 (10.5,46.4)
Effective FiO_2 at enrolment, median (IQR)	0.24 (0.21,0.28)	0.25 (0.21,0.33)	0.26 (0.21,0.30)	0.27 (0.21,0.30)	0.22 (0.21,0.27)	0.25 (0.21,0.36)
Postnatal age at time of first dose, mean (SD), hours	58.5 (23.1)	56.2 (19.4)	58.3 (24.1)	50.4 (18.7)	58.5 (22.9)	60.3 (19.0)
Ureaplasma spp. respiratory colonisation, n (%)	19 (32)	25 (41)	19 (100)	25 (100)	N/A	N/A
U. parvum	14 (23)	19 (31)	14 (74)	19 (76)		
U. urealyticum	3 (5)	4 (7)	3 (16)	4 (16)		
Both species	1 (2)	2 (3)	1 (5)	2 (8)		
Untyped	1 (2)	0 (0)	1 (5)	0 (0)		

*Non-invasive ventilation included oxyhood, low flow nasal cannula, high flow nasal cannula, nasal continuous positive pressure and nasal intermittent positive pressure ventilation.
†Invasive ventilation included synchronised intermittent mechanical ventilation, high frequency oscillatory ventilation and high frequency jet ventilation.
AZM, azithromycin; C/S, caesarean section; FiO_2, fractional inspired oxygen; IMV, intermittent mandatory ventilation; PPROM, preterm premature rupture of membranes; SGA, small for gestational age; SVD, spontaneous vaginal delivery.

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Table 2 Primary and secondary outcomes of total cohort and stratified by *Ureaplasma* respiratory colonisation status

	No. of participants (%)								
	Total cohort (n=121)			*Ureaplasma* positive (n=44)			*Ureaplasma* negative (n=77)		
Outcome	AZM (n=60)	Placebo (n=61)	P value*	AZM (n=19)	Placebo (n=25)	P value*	AZM (n=41)	Placebo (n=36)	P value*
Ureaplasma-free survival, n (%)	55 (92)	37 (61)	<0.001	16 (84)	3 (12)	<0.001	39 (95)	34 (94)	>0.99
Survival, n (%)	55 (92)	55 (90)	0.78	16 (84)	21 (84)	>0.99	39 (95)	34 (94)	>0.99
Ureaplasma clearance post-treatment, n (%)	19/19 (100)	4/25 (16)	<0.001	19/19 (100)	4/25 (16)	<0.001	N/A	N/A	
Discharged to home, n (%)	39 (65)	30 (49)	0.10	13 (68)	8 (32)	0.03	26 (63)	22 (61)	0.86
Survival free of physiological BPD, n (%)†	31/59 (53)	36/59 (61)	0.42	9 (47)	13/24 (54)	0.54	22 (55)	23 (66)	0.33
Physiological BPD, n (%)†‡	25/56 (45)	18/54 (33)	0.28	8/17 (47)	8/21 (38)	0.49	17/39 (44)	10/33 (30)	0.25
Modified Shennan BPD, n (%)‡	28/57 (49)	23/56 (41)	0.45	8/17 (47)	11/22 (50)	0.99	20/40 (50)	12/34 (35)	0.21
Moderate-severe BPD, n (%)‡	31/57 (54)	23/56 (39)	0.20	9/17 (53)	10/22 (45%)	0.51	22/40 (55)	13/34 (38)	0.15
Postnatal steroids exposure, n (%)	15 (25)	14 (23)	0.86	7 (37)	6 (24)	0.33	8 (20)	8 (22)	0.74
Passed hearing screen, n (%)§	50/54 (93)	52/54 (96)	0.68	13/16 (81)	19/21 (90)	0.63	37/38 (97)	33/33 (100)	>0.99
Duration IMV, median (IQR), days¶	12 (3–31)	4 (1–44)	0.36	15 (5–66)	3 (1–44)	0.25	11 (2–20)	4 (1–47)	0.51
Duration supplemental oxygen, median (IQR), days¶	73 (39–114.5)	68 (33–118)	0.94	87 (30–140)	75 (55–135)	0.98	70 (40–91)	60 (26–94)	0.81
Duration hospitalisation, median (IQR), days¶	87 (62.5–138.5)	87 (67–111)	0.91	109 (54–147)	87 (59–111)	0.62	83 (66–136)	87 (72–112)	0.53

*P values for binary outcomes are based on a score test from generalised estimating equations to account for correlations between twins, or Fisher's exact test when one of the cell sizes has an expectation of less than 5. P values for quantitative outcomes are based on non-parametric tests using multiple outputation to account for correlations between twins.
†Three participants could not be classified with respect to physiological BPD and are excluded from these percentages.
‡Excludes eight participants (three azithromycin and five placebo) who died prior to BPD assessment.
§Based on only those who survived until discharge but excludes two survivors who did not have a hearing screen.
¶In computing the median and IQR, those who died are included as having the worst outcomes.
AZM, azithromycin; BPD, bronchopulmonary dysplasia; IMV, intermittent mandatory ventilation.

the statistical analysis plan are described in the online supplementary file 1.

In post hoc analyses, we explored the impact of lower respiratory tract *Ureaplasma* colonisation on the primary and major secondary outcomes.

All analyses were performed using SAS V.9.4.

RESULTS

Study participants

Infants were recruited from seven sites over 37 months (July 2013–August 2016). A total of 982 patients were screened, of whom 434 (44%) were eligible (online supplementary figure 1S). Of 121 randomised, 60 were assigned to azithromycin and 61 were assigned to placebo; 119 (98%) received at least one dose of assigned treatment, one in each treatment group did not receive any doses of assigned treatment and treatment was discontinued in four azithromycin participants (one parent request and three clinical team request). All participants who received <3 doses were *Ureaplasma* negative. One placebo-assigned infant who was nasopharyngeal *Ureaplasma* positive received a single dose of azithromycin due to pharmacy error.

The baseline characteristics of randomised patients and stratified by *Ureaplasma* status are summarised in table 1. An imbalance in race distribution occurred with 40% non-white in the azithromycin versus 75% in the placebo group. Other baseline characteristics were similar comparing treatment arms for the entire study cohort and when stratified by *Ureaplasma* colonisation status.

Forty-four of 121 participants (36%) were *Ureaplasma* positive at one or more time points with 19 (32%) randomised to azithromycin and 25 (41%) to placebo (table 1 and online supplementary figure 2S). *Ureaplasma* prevalence was higher in the 24^0–26^6 weeks' gestation stratum compared with 27^0–28^6 weeks (36/83 (43%) vs 8/38 (21%), p=0.02). *Ureaplasma parvum* was the most common species detected in both treatment arms (*U. parvum*, n=33 (75%); *U. urealyticum* n=7 (16%); both species, n=3 (7%); untyped n=1 (2%)). The

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Table 3 Baseline characteristics and outcomes of participants on non-invasive respiratory support, invasive ventilation with TA *Ureaplasma*-negative specimens and invasive ventilation with TA *Ureaplasma*-positive specimens

Outcome	No. (%) of participants*			
	Never intubated (no TA specimen) (n=47)	TA *Ureaplasma* negative (n=52)	TA *Ureaplasma* positive (n=21)	P value†
Baseline characteristics				
Male, n (%)	24 (51)	25 (48)	9 (43)	0.88
Non-white race, n (%)	34 (72)	24 (46)	12 (57)	0.12
Birth weight, mean (SD), g	994 (244)	854 (207)	805 (188)	0.004
Gestational age, mean (SD), weeks	26.9 (1.2)	26.0 (1.4)	25.4 (1.0)	<0.001
Gestational age strata, n (%)				
24^0–26^6 weeks	24 (51)	39 (75)	19 (90)	0.004
27^0–28^6 weeks	23 (48)	13 (25)	2 (10)	
Preterm labour, n (%)	37 (79)	40 (77)	18 (86)	0.62
PPROM	23 (49)	16 (31)	13 (62)	0.03
Antenatal steroids	39 (83)	42 (81)	17 (81)	0.95
Maternal macrolide exposure	20 (43)	13 (25)	6 (29)	0.26
C/S delivery	28 (60)	29 (56)	9 (43)	0.40
Admission WCC × 10^3, mean (SD)	14.5 (9.4)	11.3 (7.2)	21.4 (17.8)	0.05
Ureaplasma spp. respiratory colonisation, n (%)	18 (38)	5 (10)	21 (100)	0.01
Primary and secondary outcomes				
Ureaplasma-free survival, n (%)	37 (78)	46 (88)	8 (38)	0.002
Survival, n (%)	47 (100)	47 (90)	15 (71)	<0.001
Ureaplasma clearance post-treatment, n (%)	8/18 (44)	4/5 (8%)	11/21 (52)	0.44
Survival free of physiological BPD, n (%)‡	37/45 (82)	22/51 (43)	7 (33)	<0.001
Physiological BPD, n (%)‡§	8/45 (18)	26/48 (54)	9/16 (56)	0.001
Modified Shennan BPD, n (%)§	14 (29)	28/49 (57)	9/16 (56)	0.02
Moderate-severe BPD, n (%)§	14 (29)	30/49 (61)	9/16 (56)	0.009
Discharge home, n (%)	33 (70)	28 (54)	7 (33)	0.02
Postnatal steroids exposure, n (%)	3 (6)	16 (31)	10 (48)	0.001
Passed hearing screen, n (%)¶	44/46 (96)	45/46 (98)	12/15 (80)	0.27
Total duration IMV, median (IQR)¶**	1 (1–2)	19.5 (9.5–55)	44 (24 to –)	<0.001
Total duration supplemental oxygen, median (IQR)‡**	38 (15–64)	85 (59–125)	135 (77 to –)	<0.001
Duration hospitalisation, median (IQR)¶**	71 (56–87)	99 (81–142)	110 (76 to –)	<0.001

*One surviving participant with moderate-severe BPD who was discharged home was intubated but had no TA specimens and is not included in this analysis.
†P values for binary outcomes are based on a score test from generalised estimating equations to account for correlations between twins, or Fisher's exact test when one of the cell sizes has an expectation of less than 5. P values for quantitative outcomes are based on non-parametric tests using multiple outputation to account for correlations between twins.
‡Excludes three participants who could not be classified with respect to physiological BPD.
§Excludes eight participants who died prior to BPD assessment.
¶Based on only those who survived until discharge but excludes two survivors who did not have a hearing screen.
**In computing the median and IQR, those who died are included as having the worst outcomes. For the TA *Ureaplasma*-positive participants, more than 25% died, so it was not possible to specify the actual 75th percentile.
BPD, bronchopulmonary dysplasia; C/S, caesarean section; IMV, intermittent mandatory ventilation; PPROM, preterm premature rupture of membranes; TA, tracheal aspirate; WCC, white cell count.

Minimum inhibitory concentration $(MIC)_{50}$ and MIC_{90} for *Ureaplasma* isolates were 2 µg/mL and 4 µg/mL, respectively. No tested isolate was resistant to azithromycin (MIC ≥16 µg/mL).

Efficacy analysis

The *Ureaplasma*-free survival was higher in the azithromycin group (92% (95% CI 82% to 97%)) compared with the placebo group (61% (95% CI 48% to 73%)) (p<0.001) (table 2) and was sustained in analyses stratified by race (online supplementary table 1). For *Ureaplasma*-positive infants, *Ureaplasma*-free survival was higher in the azithromycin group (16/19 (84%), (95% CI 60% to 97%)) than in the placebo group (3/25 (12%), (95% CI 3% to 31%)) (p<0.001) (table 2). The proportion of infants who survived until discharge was similar in each treatment group (92% vs 90%, table 2). All follow-up cultures were negative in the azithromycin group, but 21/25 (84%) of colonised placebo subjects were culture positive at one or more follow-up time point (online supplementary figure S2). Seven azithromycin-assigned participants were PCR positive, but culture-negative post-treatment.

Secondary outcomes

Two-thirds (12/19) of participants who met criteria for RAC, failed and were classified as physiological BPD. Three infants did not have a RAC completed so they could not be classified. There were no significant differences between treatment groups for the entire cohort or stratified by *Ureaplasma* colonisation status (table 2) or race (online supplementary table 1S)

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Table 4 Primary and secondary outcomes among tracheal aspirate *Ureaplasma*-positive participants by treatment assignment

Outcome	No. of participants (%)		
	Azithromycin (n=10)	Placebo (n=11)	P value*
Ureaplasma-free survival, n (%)	8 (80)	0 (0)	<0.001
Survival, n (%)	8 (80)	7 (64)	0.64
Ureaplasma clearance post-treatment, n (%)	10 (100)	1 (9)	<0.001
Survival free of physiological BPD, n (%)†	5 (50)	2 (18)	0.18
Physiological BPD, n (%)†	3/8 (38)	6/8 (75)	0.31
Modified Shennan BPD, n (%)†	3/8 (38)	6/8 (75)	0.31
Moderate/severe BPD, n (%)†	3/8 (38)	6/8 (75)	0.31
Discharge home, n (%)	5 (50)	2 (18)	0.18
Postnatal steroids, n (%)	4 (40)	6 (55)	0.67
Passed hearing screen, n (%)‡	6/8 (75)	6/7 (86)	>0.99
Total duration IMV, median (IQR)§	24.5 (8–72)	53 (31 to –)	0.11
Total duration supplemental oxygen, median (IQR)§	95.5 (39–174)	142 (114 to –)	0.13
Duration of hospitalisation, median (IQR)§	80.5 (27–173)	134 (91 to –)	0.08

*P values for categorical outcomes are based on Fisher's exact tests. P values for quantitative analysis are based on two-sample Wilcoxon tests.
†Excludes five participants (two azithromycin, three placebo) who died prior to 36 weeks PMA.
‡Excludes six (two azithromycin, four placebo) participants who died before hearing screen was obtained.
§In computing the median and IQR, those who died are included as having the worst outcomes. For the tracheal aspirate *Ureaplasma*-positive participants, more than 25% died, so it was not possible to specify the actual 75th percentile.
BPD, bronchopulmonary dysplasia; IMV, intermittent mandatory ventilation; PMA, postmenstrual age.

in overall survival, physiological BPD-free survival, frequency BPD by any classification or other secondary outcomes.

Post hoc analyses

Patients with lower respiratory tract *Ureaplasma* colonisation (n=21) were of lower gestation and birth weight than TA-negative intubated participants (n=52) and non-intubated infants (n=47) (table 3). In 5/52 (10%) TA-negative and 18/47 (38%) non-intubated neonates, one or more nasopharyngeal samples were *Ureaplasma* positive. Outcomes including *Ureaplasma*-free survival, overall survival, physiological BPD-free survival, durations of hospitalisation, mechanical ventilation and supplemental oxygen and postnatal steroid exposure were less favourable in patients with lower respiratory tract *Ureaplasma* colonisation than intubated infants without lower tract involvement or non-intubated patients (table 3). In patients with lower respiratory tract *Ureaplasma* colonisation, physiological BPD-free survival was 50% (5/10), (95% CI 19% to 81%) in azithromycin-treated versus 18% (2/11), (95% CI 2% to 52%) in placebo-treated infants (p=0.18) (table 4).

Safety

Common morbidities of prematurity occurring after randomisation and prior to hospital discharge were similar between treatment groups (table 5) and when stratified by race (online supplementary table 2S). Posthaemorrhagic hydrocephalus (PHH) was more common in the azithromycin-assigned compared with the placebo group (6 vs 0). Prior to dosing, IVH status was unknown in 4/6 of these infants; 1/6 had grade 2 IVH; and 1/6 received no azithromycin. Among those assigned to azithromycin, 11/56 (20%) had retinopathy of prematurity (ROP) >stage 2 compared with 4/56 (7%) assigned to placebo. ROP was more common in white infants in both treatment groups than non-white infants, which appears to explain most of this difference (online supplementary table 2S). There were no reported cases of infantile hypertrophic pyloric stenosis (IHPS) or QT-interval prolongation.

DISCUSSION

This pilot clinical trial demonstrates that: (1) respiratory tract *Ureaplasma* colonisation persists in untreated infants during the first three postnatal weeks; (2) 20 mg/kg x3d intravenous azithromycin effectively eradicates *Ureaplasma* from the respiratory tract in colonised ELGAN infants; and (3) ELGANs with lower respiratory tract *Ureaplasma* colonisation are a high risk group to target in future randomised trials. There is no evidence of an impact of azithromycin among *Ureaplasma*-negative infants.

The *Ureasplasma* eradication rate (100%) with the 3 days 20 mg/kg/day azithromycin regimen that was based on our open-label pharmacokinetics/pharmacodynamics studies[14–16]

Table 5 Morbidities of prematurity by treatment group

Morbidity	Azithromycin (n=60)	Placebo (n=61)	
	N (%) acquired prior to discharge	N (%) acquired prior to discharge	P value*
Pneumothorax	**7/55 (13)**	**4/57 (7)**	**0.49**
PDA	25/55 (45)	21/56 (38)	0.33
Feeding intolerance	20/51 (39)	34/58 (59)	0.04
Gastro-oesophageal reflux	14/60 (23)	11/61 (18)	0.54
Intestinal perforation	2/60 (3)	4/61 (7)	0.68
NEC ≥stage 2	4/60 (7)	5/61 (8)	>0.99
Culture-confirmed sepsis	8/60 (13)	14/61 (23)	0.18
IVH†			0.33
None	31/53 (58)	40/54 (74)	
Grade 1	10/53 (19)	7/54 (13)	
Grade 2	5/53 (9)	5/54 (9)	
Grade 3	5/53 (9)	1/54 (2)	
Grade 4	2/53 (4)	1/54 (2)	
Shunted PHH	6/60 (10)‡	0/61 (0)	0.01
PVL	4/60 (7)	5/61 (8)	>0.99
ROP (highest stage)§			0.28
None	18/56 (32)	25/56 (45)	
Stage 1	17/56 (30)	17/56 (30)	
Stage 2	10/56 (18)	10/56 (18)	
Stage 3	11/56 (20)	3/56 (5)	
Stage 4	0/56 (0)	1/56 (2)	

*P values for binary outcomes are based on a score test from generalised estimating equations to account for correlations between twins, or Fisher's exact test when one of the cell sizes has an expectation of less than 5.
†The IVH proportions exclude 12 participants who had IVH prior to their first dose and who did not progress. It also excludes two who never received the treatment to which they were randomised.
‡For azithromycin-assigned participants with shunted PHH, IVH status at baseline was unknown in four participants; grade 2 in one participant; and one infant was never dosed.
§Four assigned to azithromycin and five assigned to placebo were never assessed for ROP and are not included.
IVH, intraventricular haemorrhage; NEC, necrotising enterocolitis; PDA, patent ductus arteriosus; PHH, posthaemorrhagic hydrocephalus; PVL, periventricular leukomalacia; ROP, retinopathy of prematurity.

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was higher than but not inconsistent with eradication rates in previous trials of erythromycin (82%–86%)[26 27] and clarithromycin (68.5%).[28] However, some infants in the azithromycin group remained PCR-positive after treatment. This may represent residual DNA from dead organisms since no isolate was resistant. Effective clearance likely is dependent on factors such as pathogen virulence[1] and variability in host immune response due to polymorphisms in host defence genes that may alter susceptibility to *Ureaplasma* and the inflammatory response.[29]

No current BPD definition is a strong predictor of long-term pulmonary outcomes.[30] The recent increase in use of non-invasive respiratory support with room air has challenged classifications of BPD based on supplemental oxygen use. We selected three common BPD definitions as exploratory outcomes in the current trial. Completion of the study 2-year follow-up will allow us to compare the modified Shennan,[22] BPD severity and physiological definitions' predictive ability for later respiratory outcomes to better design a definitive phase III clinical trial.

Published reports on racial differences in preterm outcomes differ on which races experience more adverse perinatal outcomes[31–33] or whether differences exist.[34] In a recent prospective cohort of infants<29 weeks' gestation, Wai *et al*[35] observed a lower incidence of BPD in black than white infants, but the frequency of respiratory morbidity during the first year of life was higher in black than white infants. In the Trial of Late Surfactant for Prevention of BPD (TOLSURF) clinical trial, black infants administered inhaled nitric oxide were less likely to develop BPD but experienced greater frequency of wheezing illness in the first 18–24 months of life.[35 36] Since there was an imbalance by race in treatment groups in the current trial, we examined outcomes stratified by race. Our primary outcome finding of greater *Ureaplasma*-free survival with azithromycin was sustained in analyses stratified for race.

Azithromycin side effects are infrequent in adults and children.[37] A recent study demonstrated an association of IHPS with oral azithromycin exposure in the first 14d of life in term[38] and preterms 33–36 weeks' gestation but not ≤32 weeks' gestation.[39] In addition, azithromycin is proarrhythmogenic with prior reports of occurrences of QT-interval prolongation and torsades de pointes in adults.[40] Although there were no reported incidences of IHPS or QT interval prolongation in the infants in the current trial, adverse events must be monitored closely in any subsequent trial of azithromycin in the ELGAN population.

Study limitations

Since *Ureaplasma* spp. lack cell walls, they are susceptible to drying and heat contributing to false negatives, so that some affected infants may have been missed. We made efforts to avoid this misclassification by providing central laboratory culture medium, collection procedures optimised for organism recovery, multiple sampling sites at timepoints before and after study treatment and inclusion of PCR methods to better detect *Ureaplasma*. The race imbalance in randomisation did not influence the primary outcome, *Ureaplasma*-free survival, but influenced some secondary clinical outcomes. Future trials should consider stratifying on race. Brain imaging prior to randomisation was not required for this trial but, due to the observed differences in PHH, may be warranted in any future trial to better delineate the timing of IVH in relation to treatment.

Study implications summary

The results of this trial demonstrate the efficacy of azithromycin to eradicate *Ureaplasma* in ELGAN infants but do not support treatment of all ELGAN infants with azithromycin. Perinatal mortality and prolonged respiratory support are concentrated in ELGANs who have *Ureaplasma* in the lower respiratory tract. A phase III clinical trial in ELGAN infants with lower respiratory tract *Ureaplasma* would determine whether or not a 3-day course of azithromycin is of clinical benefit.

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DATA SUPPLEMENT

Randomized Trial of Azithromycin to Eradicate *Ureaplasma* in Preterm Infants

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3. Supplementary Figure S1. Consolidated Standards of Reporting Trials Diagram of the Azithromycin in Preterms Trial
4. Supplementary Figure S2. *Ureaplasma* spp. clearance from the respiratory tract in neonates randomized to azithromycin and placebo
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1. SUPPLEMENTARY TABLE 1S

Primary and Secondary Outcomes, Stratified by Race.

	***White* (N=51)**			***Non-White* (70)**		
Outcome	Azithromycin (N=36)	Placebo (N=15)	P-value[e]	Azithromycin (N=24)	Placebo (N=46)	P-value[e]
Ureaplasma-free survival	32 (89%)	9 (60%)	0.04	23 (96%)	28 (61%)	<.001
Survival	32 (89%)	13 (87%)	>.99	23 (96%)	42 (91%)	.65
Ureaplasma clearance post-treatment	13/13 (100%)	0/5 (0%)	<0.001	6/6 (100%)	4/20 (20%)	<0.001
Discharge to home	24 (67%)	7 (47%)	.16	15 (63%)	23 (50%)	.47
Survival free of Physiologic BPD[a]	14/35 (40%)	8 (53%)	.44	17 (71%)	28/44 (64%)	.49
Physiologic BPD[a,b]	19/33 (58%)	5/13 (38%)	.27	6/23 (26%)	13/41 (32%)	.57
Modified Shennan BPD[b]	20/34 (59%)	7/13 (54%)	.84	8/23 (35%)	16/43 (37%)	.78
Moderate-Severe BPD[b]	23/34 (68%)	7/13 (54%)	.46	8/23 (35%)	16/43 (37%)	.78
Postnatal Steroids	13 (36%)	3 (20%)	.26	2 (8%)	11 (24%)	.08
Passed Hearing Screen[c]	27/31 (87%)	13/13 (100%)	.30	23/23 (100%)	39/41 (95%)	.53
Total Duration IMV, median (IQR), d[d]	19.0 (7.5, 42.0)	10 (2, 59)	.46	8 (2, 12.5)	2.5 (1, 44)	.95
Total Duration Supplemental Oxygen, Median (IQR), d[d]	87.5 (52.0, 129.5)	69 (58, 118)	.51	56.5 (31, 83.5)	62.5 (27, 125)	.20
Duration hospitalization, median (IQR), d[d]	105 (77.5, 150.0)	98 (73, 120)	.31	72.5 (54.5, 85.0)	87.0 (66.0, 111.0)	.02

Abbreviations: IMV, intermittent mandatory ventilation; IQR, interquartile range

[a]Three participants could not be classified with respect to physiologic BPD and are excluded from these percentages.

[b]Excludes 8 participants (3 azithromycin and 5 placebo) who did not live long enough to be assessed for BPD

[c] Based on those who survived until discharge but excludes 2 survivors who did not have a hearing screen.

[d]In computing the median and interquartile range (IQR), those who died are included as having the worst outcomes

[e]P-values for binary outcomes are based on a score test from Generalized Estimating Equations to account for correlations between twins, or Fisher's Exact Test when one of the cell sizes has an expectation of less than 5. P-values for quantitative outcomes are based on non-parametric tests using multiple outputation to account for correlations between twins

2. SUPPLEMENTARY TABLE 2S

Morbidities of Prematurity Stratified by Race.

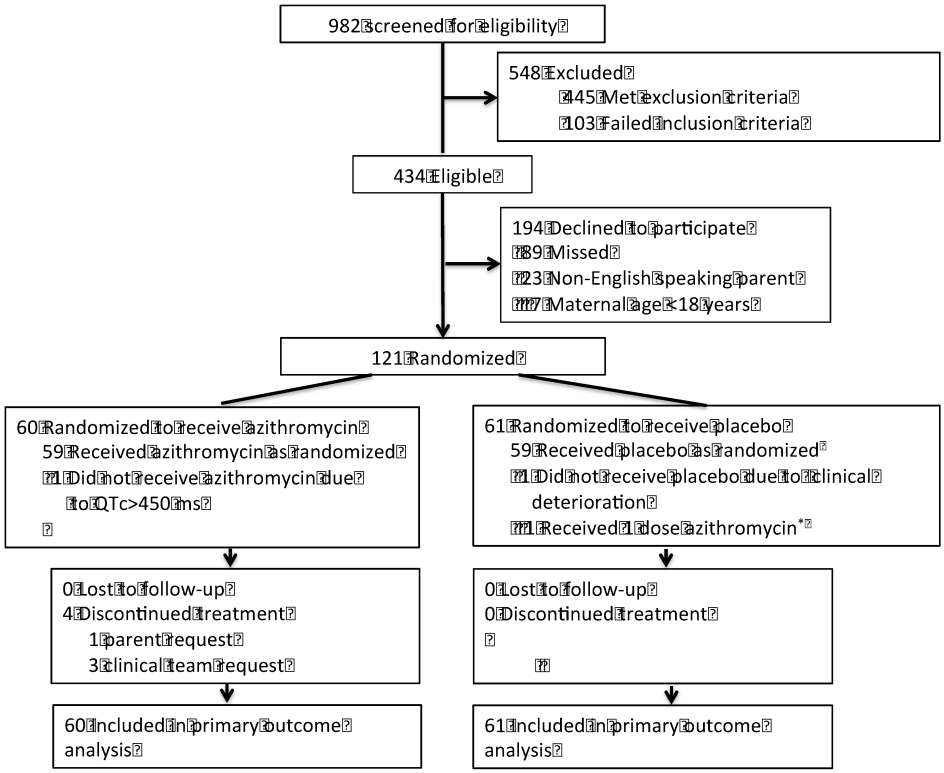
	White (N=51)			Non-White (N=70)		
Morbidity	Azithromycin (N=36)	Placebo (N=15)	P-value[a]	Azithromycin (N=24)	Placebo (N=46)	P-value[a]
Pneumothorax	6/32 (19%)	0/13 (0%)	.16	1/23 (4%)	4/44 (9%)	.65
PDA	18/35 (51%)	5/12 (42%)	.34	7/20 (35%)	16/44 (36%)	.93
Feeding Intolerance	16/32 (50%)	8 (53%)	.37	4/19 (21%)	26/43 (60%)	.008
Gastro-esophageal Reflux	8/36 (22%)	1 (7%)	.25	6 (25%)	10 (22%)	.76
Intestinal Perforation	1 (3%)	2 (13%)	.20	1 (4%)	2 (4%)	>.99
NEC ≥Stage 2	3 (8%)	1 (7%)	>.99	1 (4%)	4 (9%)	.65
Culture-confirmed Sepsis	6 (17%)	2 (13%)	>.99	2 (8%)	12 (26%)	.12
IVH>Grade 2	4/33 (12%)	1/12 (8%)	>.99	3/20 (15%)	1/42 (2%)	.095
PHH requiring shunt	4 (11%)	0 (0%)	.31	2 (8%)	0 (0%)	.11
PVL	3 (8%)	2 (13%)	.62	1 (4%)	3 (7%)	>.99
ROP> Stage 2	10/33 (30%)	3/13 (23%)	0.73	1/23 (4%)	1/43 (2%)	>.99

Abbreviations: PDA, patent ductus arteriosus; NEC, necrotizing enterocolitis; IVH, intraventricular hemorrhage; PHH, post-hemorrhagic hydrocephalus; PVL, periventricular leukomalacia; ROP, retinopathy of prematurity

[a]P-values are based on a score test from Generalized Estimating Equations to account for correlations between twins, or Fisher's Exact Test when one of the cell sizes has an expectation of less than 5.

3. SUPPLEMENTARY FIGURE S1

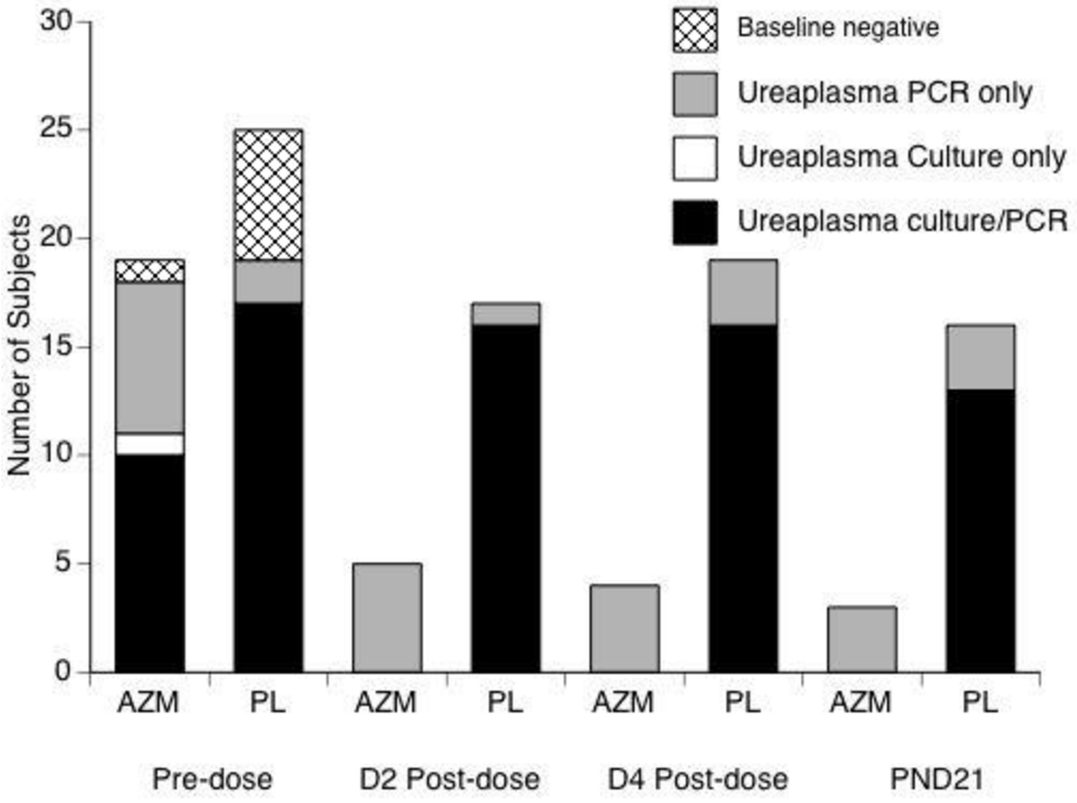
Consolidated Standards of Reporting Trials Diagram of the Azithromycin in Preterms Trial



Consolidated Standards of Reporting Trials Diagram of the Azithromycin in Preterms Trial. One participant in each treatment arm did not receive assigned treatment. *One participant assigned to the placebo group, received one dose of azithromycin due to investigational pharmacy error, but received placebo for other 2 doses. All participants were included in intent-to-treat analysis.

4. SUPPLEMENTARY FIGURE S2

The number of participants positive by culture or PCR at each time point by treatment assignment among those who were *Ureaplasma*-positive by culture or PCR at any time during the study.



Ureaplasma spp. clearance from the respiratory tract in neonates assigned to azithromycin (AZM) and placebo (PL). The number of participants in the azithromycin (AZM) and placebo (PL) groups with *Ureaplasma* detected by both culture and PCR (black), culture alone (white), and PCR alone (gray) at baseline pre-dose, 2 and 4 d post-third dose, and postnatal day 21 (PND21). The number of positive subjects who were negative at baseline, but positive at 1 or later timepoint are indicated by hatched bar. All follow-up cultures were negative in the azithromycin-treated group, but 21/25 (84%) of placebo-treated subjects were culture-positive at one or more follow-up time point.

5. SUPPLEMENTARY METHODS

Statistical Methods

The statistical analyses are influenced by twins who cannot be assumed to be independent, and the small number of some outcome events. To address these challenges, we used generalized estimating equations [1], multiple outputation[2], bootstrap, nonparametric tests, and exact tests as described below.

Analysis of Binary Outcomes

Treatment groups were compared with respect to the proportion of infants who survived *Ureaplasma*-free, and other secondary binary outcomes such as survival, physiologic BPD, etc. To take into consideration correlation of outcomes in twins, the statistical significance of observed differences was assessed based on generalized estimating equations, using an identity link, and assuming an exchangeable correlation between outcomes from twins. Since in simulations, these models did not perform well when the number of outcomes was very small, when any expected cell size was 5 or less in the implicit 2x2 table, we used Fisher's Exact Test by inverting two separate one-sided tests as implemented in SAS 9.4[3].

Analysis of Quantitative Outcomes

Treatment groups were compared with respect to the median value of several quantitative outcomes including duration of mechanical ventilation, duration of supplemental oxygen, and duration of hospital stay. One challenge in these analyses is how to include children who died during hospitalization. To include these children in the analysis appropriately, we used a rank-based (Wilcoxon test) analysis and gave these children the worst ranks. To address the challenge of the correlation between twins, we used multiple outputation [2]. This technique involves repeated analyses after randomly removing one child in each twin set, and combining the results.

REFERENCES

1. Zeger SL, Liang KY, Albert PS. Models for longitudinal data: a generalized estimating equation approach. *Biometrics*. 1988;44:1049-60.
2. Follmann D, Proschan M, Leifer E. Multiple outputation: inference for complex clustered data by averaging analyses from independent data. *Biometrics*. 2003;59:420-9.
3. Santner TJ, Snell MK. Small sample confidence intervals for p1-p2 and p1/p2 in 2x2 contingency tables. *J American Statistical Association*. 1980;75:386-94.