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Supplementary appendix

Supplement to:

Efficacy and safety of azithromycin maintenance therapy in primary ciliary dyskinesia (BESTCILIA): a multicentre, double-blind, randomized, placebo-controlled phase 3 trial.

Methods

Study design and participants

Diagnostic criteria for PCD

A confirmed diagnosis of PCD was based on characteristic clinical symptoms and high speed video microscopic recordings of abnormal ciliary beat pattern and/or frequency, combined with either an abnormally low nasal nitric oxide production <77 nl/min,[1] abnormal ciliary ultra-structure demonstrated by transmission electron microscopy or high resolution immunofluorescence (excluding isolated inner dynein arm defects), and/or a genetic mutation recognized to cause PCD. Table A1 shows the diagnostic criteria fulfilled by the participants.

Table A1: Diagnostic criteria fulfilled by the participants

	Azithromycin (n=49)	Placebo (n=41)	Total (n=90)
Characteristic clinical symptoms	49 (100%)	41 (100%)	90 (100%)
Abnormal ciliary beat pattern/frequency by HSVM	49 (100%)	41 (100%)	90 (100%)
Abnormally low nasal NO	45 (91·8%)	36 (87·8%)	81 (90·0%)
Abnormal ciliary ultra-structure by TEM or IF	35 (71·4%)	31 (75·6%)	66 (73·3%)
Genetic mutation*	7 (14·3%)	7 (17·1%)	14 (15·6%)
CCDC39	1 (2·0%)	1 (2·4%)	2 (2·2%)
CCDC40	1 (2·0%)	1 (2·4%)	2 (2·2%)
CCDC114	0	2 (4·9%)	2 (2·2%)
DNAI1	1 (2·0%)	0	1 (1·1%)
HYDIN	1 (2·0%)	1 (2·4%)	2 (2·2%)
LRRC6	1 (2·0%)	1 (2·4%)	2 (2·2%)
RSPH9	1 (2·0%)	0	1 (1·1%)
Other	1 (2·0%)	1 (2·4%)	2 (2·2%)

Data are presented as no. (%).
*The data was collected at the screening visits, which took place between 2014 and 2016, and therefore less participants had known genetic mutations compared to nowadays.
HSVM=high speed video microscopy. NO=nitric oxide. TEM=transmission electron microscopy. IF=immunofluorescence.

Prohibited medications

According to the exclusion criteria treatment with certain medicinal products known to possibly interact with azithromycin or prolong QT interval were prohibited during study participation: Ciclosporin, coumarin-like oral anticoagulants (e.g. warfarin), digoxin, ergotamine derivatives (e.g. methylergometrine), nelfinavir, rifabutin, and active substances known to prolong QT interval such as amiodarone and other class IA and class III antiarrhythmics, cisapride, terfenadin, moxifloxacin, levofloxacin, antipsychotic agents such as pimozide, and antidepressants such as citalopram.

Procedures

QOL-PCD

The QOL-PCD questionnaire was completed before any other assessments to reduce bias. The QOL-PCD instrument has developmentally appropriate versions for children 7-12 years, adolescents 13-17 years, and adults from 18 years, and a parent-proxy version for parent caregivers of children 7-12 years old.[2,3] The original QOL-PCD questionnaire, developed in English language and recently validated,[2-5] was translated into the primary languages of the participating countries (Danish, Dutch, and German/Swiss German) according to international guidelines prior to the start of the trial. In this study, the QOL-PCD was available as both 'paper-pencil' questionnaire and electronic questionnaire with a speaking questionnaire for the children, as preferred by the study sites and the participants. Participants and parent caregivers reported on their health or their child's health over the past week using a 4-point Likert scale. Responses were standardized from 0-100 with higher scores representing better health-related quality of life.[2-5] Data from the child, adolescent, and adult versions of the QOL-PCD were pooled for the statistical analysis. The analysis was repeated including the parent-proxy responses instead of the child responses.

Nitrogen multiple breath washout

Ventilation distribution inhomogeneity was assessed by nitrogen multiple breath washout (N_2 MBW) according to European Respiratory Society/American Thoracic Society (ERS/ATS) consensus statement for inert gas washout measurement[6] utilising the commercially available instrument EXHALYZER®D and the associated software SPIROWARE® version $3.1.6_{extended}$ (ECO MEDICS AG, Duernten, Switzerland). The N_2 MBW measurements were performed prior to spirometry. The lung clearance index (LCI) is an index of overall ventilation distribution inhomogeneity in the lungs, whereas the indices $S_{cond}*V_T$ and $S_{acin}*V_T$ are thought to reflect ventilation inhomogeneity in the small conducting and acinar airways, respectively.[7] The average of the N_2 MBW indices from intentionally three and at least two technically acceptable measurements from each study visit were reported as results of the test.

Spirometry

Dynamic lung function measured by spirometry was performed according to ERS/ATS standards.[8] Short- and long-acting bronchodilators and anticholinergics were withheld 6 and 12 hours prior to spirometry, respectively.

Body plethysmography

Measurement of static lung volumes and airway resistance by body plethysmography was performed using a standardised whole-body plethysmograph and according to ERS/ATS recommendations.[9] Reference values for calculation of percent predicted were from Koopman et al.[10] for participants aged 7-18 years and from Verbanck et al.[11] for participants ≥19 years.

Audiometry and tympanometry

Pure tone audiometry and tympanometry was performed at local hospital otorhinolaryngology departments. Hearing threshold was determined as the pure tone average (at 0·5, 1, 2, 4, and 8 kHz) by air conduction and as discrimination loss, measured by audiometry. Bone conduction thresholds were measured only when hearing thresholds by air conduction were above 10 dB - to distinguish conductive hearing loss from sensorineural hearing loss. The average of the hearing thresholds on the right and left ears was used for the statistical analysis.

Sputum microbiology

Sputum was, preferably, collected by expectoration. In participants, who could not cough up sputum due to lack of expectoration or young age, sputum was collected according to local routine procedures (laryngeal suction, cough swab, or throat swab). Sputum was cultured for pathogenic airway bacteria (cystic fibrosis panel) and tested for antibiotic susceptibility at the local microbiological departments.

Inflammatory markers

Blood samples were analysed locally for white blood cells including differential cell counts, and C-reactive protein. Blood samples for measurement of cytokines were centrifuged locally to separate the serum from the blood cells. Serum- and sputum samples were stored at minus 70 to 80 degrees Celsius until centralised measurement of cytokines was performed at the Department of Clinical Microbiology, Copenhagen University Hospital Rigshospitalet, Denmark, after the end of the study. A heat fixated smear of sputum was prepared locally and stored at room temperature. Gram staining and microscopy of the smears were performed centrally by two microbiologists to determine if the sputum samples contained material originating from the lower respiratory tract. Only sputum rated as representing the lower respiratory tract was analysed for cytokines. Prior to cytokine analysis 0.1 gram of sputum was added to 800 µL phosphate-buffered saline (PBS), vortexed vigorously and centrifuged at 3000 g for 15 minutes at 4°C. The subsequent supernatant was removed and stored at -80 °C until later use. Expression of IL-1β, G-CSF, IL-8, IL-10, TNF-α, Gro-α and MCP-1 in sputum supernatant and serum cytokines (IL-1β, G-CSF, IL-8) were determined by multiplex analysis (Human Magnetic Luminex Assay, R&D systems) according to manufacturer's instructions and analyzed by a LUMINEX® 200™ platform (Luminex Corporation, Austin, TX). Participants with cytokine values below lower limit of quantification (LLOQ) at both baseline and the 6-month visit were excluded from the statistical analysis of the particular cytokines.

Table A2: Schedule of assessments					
	Screening	Randomisation	Treatment period		
			Follow-up 2 months	Follow-up 4 months	Follow-up 6 months
Electrocardiogram	X				
Quality of life questionnaire (QOL-PCD)		X	X	X	X
Symptoms of exacerbation – by interview and weekly diary card	X	X	X	X	X
Adverse events			X	X	X
Concomitant medications	X	X	X	X	X
Physical examination incl. vital signs	X	X	X	X	X
N_2 multiple breath washout		X	X	X	X
Spirometry	X	X	X	X	X
Body plethysmography		X	X	X	X
Sputum culture & susceptibility testing	X	X	X	X	X
Sputum analysis for NTM	X				X
Urine pregnancy test	X	X	X	X	X
Audiometry & tympanometry		X			X
Blood tests (haematology, C-reactive protein, kidney- & liver function)		X			X
Sampling of serum & sputum for analysis of cytokines		X			X
Adherence (count of returned study drug)			X	X	X

QOL-PCD=PCD-specific health-related quality of life questionnaire. N_2=Nitrogen. NTM=non-tuberculous mycobacteria.

Outcomes

Table A3: Assessment of outcomes			
	Time of assessment		
	All visits	Baseline & 6-month visit	At time of event
No. of respiratory system exacerbations	X		X
Outcomes from spirometry: - FEV_1 % predicted - FVC % predicted - FEF_{25-75} % predicted	X		
Outcomes from body plethysmography: - RV % predicted - RV/TLC % predicted - Raw % predicted	X		
Outcomes from N_2 MBW: - LCI - $S_{cond}*V_T$ - $S_{acin}*V_T$	X		
Outcomes from QOL-PCD: - Respiratory Symptoms - Sinus Symptoms - Ear & Hearing Symptoms	X		
Outcomes from audiometry: - Pure tone average - Discrimination loss		X	
Tympanograms		X	
Inflammatory markers		X	
No. of pathogenic airway bacterial species	X		
Macrolide resistance	X		
Adverse events & serious adverse events	X		X

FEV_1=Forced expiratory volume in one second. FVC=Forced vital capacity. FEF_{25-75}=Forced expiratory flow at 25-75% of forced vital capacity. RV=Residual volume. RV/TLC=residual volume divided by total lung capacity. Raw=Airway resistance. N_2 MBW=nitrogen multiple breath washout. LCI=lung clearance index. $S_{cond}*V_T$=regional ventilation inhomogeneity of the conducting airways corrected for tidal volume. $S_{acin}*V_T$=regional ventilation inhomogeneity of the intra-acinar airways corrected for tidal volume. QOL-PCD=PCD-specific health-related quality of life questionnaire.

Details on statistical analysis

According to the study protocol, the primary outcome would be analysed using Poisson regression. However, the Negative Binomial model better accommodated overdispersion and gave the best model fit, and hence was used for the analysis of the primary outcome instead of Poisson regression.

Results

Randomization at each site

Table A4: No. of patients randomized at each study site

Study site	No. of patients randomized
Rigshospitalet, Copenhagen, Denmark	36
VU Medical Center, Amsterdam, Netherlands	16
University Hospital, Muenster, Germany	19
Royal Brompton Hospital, London, United Kingdom	10
Inselspital, Bern, Switzerland	5
University Hospital Southampton, United Kingdom	4
Total	**90**

Post hoc analyses

The mean (SD) duration of intervention were 159·0 days (48·5) in the azithromycin group and 159·8 days (49·4) in the placebo group. Counts of returned study drug showed that adherence was 93·7% in the azithromycin group and 92·6% in the placebo group (missing data on four participants from each group).

Secondary outcomes

Table A5: Difference in secondary outcomes

	Change from baseline to follow-up (95% CI)		Between-group difference (95% CI)	p value
	Azithromycin (n=49)	Placebo (n=41)		
Dynamic lung function				
FEV1 % predicted				
2 months follow-up	1·22 (-0·68 to 3·13) [45]	-0·52 (-2·61 to 1·58) [37]	1·74 (-1·05 to 4·54)	0·22
4 months follow-up	-0·17 (-2·17 to 1·82) [40]	0·73 (-1·41 to 2·87) [35]	-0·90 (-3·79 to 1·99)	0·54
6 months follow-up	0·05 (-1·94 to 2·05) [40]	-3·04 (-5·20 to -0·87) [34]	3·09 (0·18 to 6·00)	0·038
FVC % predicted				
2 months follow-up	1·48 (-0·47 to 3·43) [45]	-1·06 (-3·20 to 1·09) [37]	2·53 (-0·31 to 5·38)	0·08
4 months follow-up	0·07 (-1·97 to 2·11) [40]	1·88 (-0·31 to 4·06) [35]	-1·81 (-4·75 to 1·14)	0·23
6 months follow-up	-0·13 (-2·18 to 1·91) [40]	-1·48 (-3·69 to 0·73) [34]	1·35 (-1·61 to 4·31)	0·37
FEF_{25-75} % predicted*				
2 months follow-up	2% (-5% to 9%) [45]	-2% (-9% to 5%) [37]	4% (-6% to 14%)	0·46
4 months follow-up	-3% (-10% to 4%) [40]	-3% (-10% to 4%) [35]	0% (-10% to 10%)	0·96
6 months follow-up	0% (-7% to 7%) [40]	-9% (-16% to 2%) [34]	10% (-1% to 22%)	0·06
Lung volumes				
RV % predicted				
2 months follow-up	-3·17 (-12·31 to 5·98) [43]	-2·82 (-13·06 to 7·42) [34]	-0·35 (-13·57 to 12·87)	0·96
4 months follow-up	4·70 (-4·94 to 14·34) [38]	-2·51 (-13·00 to 7·98) [32]	7·21 (-6·55 to 20·98)	0·30
6 months follow-up	-4·98 (-15·24 to 5·29) [32]	-3·12 (-13·94 to 7·71) [29]	-1·86 (-16·32 to 12·60)	0·80
RV/TLC % predicted				
2 months follow-up	-2·87 (-9·67 to 3·93) [43]	-1·15 (-8·77 to 6·47) [34]	-1·72 (-11·58 to 8·15)	0·73
4 months follow-up	1·65 (-5·52 to 8·82) [38]	-1·66 (-9·47 to 6·15) [32]	3·31 (-6·96 to 13·57)	0·53
6 months follow-up	-5·69 (-13·32 to 1·94) [32]	-0·55 (-8·61 to 7·50) [29]	-5·14 (-15·92 to 5·64)	0·35
Airway resistance				
Raw % predicted†				
2 months follow-up	0·05 (-0·19 to 0·28) [44]	-0·04 (-0·30 to 0·22) [32]	0·09 (-0·26 to 0·43)	0·62
4 months follow-up	-0·13 (-0·37 to 0·12) [38]	-0·05 (-0·31 to 0·22) [32]	-0·08 (-0·44 to 0·28)	0·66
6 months follow-up	-0·02 (-0·27 to 0·23) [32]	-0·33 (-0·59 to -0·06 [29]	0·31 (-0·05 to 0·67)	0·09
Ventilation inhomogeneity				
LCI				
2 months follow-up	5e-04 (-0·47 to 0·47) [40]	-0·11 (-0·62 to 0·39) [36]	0·11 (-0·56 to 0·79)	0·74
4 months follow-up	-0·06 (-0·55 to 0·42) [36]	-0·46 (-0·96 to 0·05) [34]	0·39 (-0·30 to 1·08)	0·26
6 months follow-up	0·06 (-0·43 to 0·55) [36]	-0·40 (-0·91 to 0·11) [33]	0·46 (-0·23 to 1·15)	0·19
S_{cond}*V_T				
2 months follow-up	-0·002 (-0·010 to 0·006) [40]	0·002 (-0·006 to 0·010) [36]	-0·004 (-0·015 to 0·006)	0·43
4 months follow-up	0·003 (-0·006 to 0·011) [36]	1e-04 (-0·008 to 0·009) [33]	0·002 (-0·009 to 0·013)	0·67
6 months follow-up	0·001 (-0·007 to 0·010) [36]	0·003 (-0·006 to 0·011) [32]	-0·001 (-0·013 to 0·010)	0·80
S_{acin}*V_T†				
2 months follow-up	-0·026 (-0·051 to -0·001) [40]	-0·004 (-0·031 to 0·023) [36]	-0·022 (-0·058 to 0·014)	0·22
4 months follow-up	-0·010 (-0·036 to 0·016) [36]	-0·003 (-0·030 to 0·025) [33]	-0·007 (-0·044 to 0·030)	0·70
6 months follow-up	-0·028 (-0·054 to -0·002) [36]	0·009 (-0·019 to 0·036) [32]	-0·036 (-0·074 to 7e-04)	0·054
QOL-PCD scale scores‡				
Respiratory Symptoms				
2 months follow-up	4·76 (0·05 to 9·47) [39]	0·05 (-5·01 to 5·12) [33]	4·71 (-1·77 to 11·19)	0·15
4 months follow-up	4·87 (0·17 to 9·57) [39]	5·59 (0·63 to 10·56) [35]	-0·72 (-7·12 to 5·68)	0·82
6 months follow-up	2·13 (-2·56 to 6·83) [39]	3·80 (-1·26 to 8·86) [33]	-1·66 (-8·15 to 4·82)	0·61
Sinus Symptoms				
2 months follow-up	4·74 (-0·40 to 9·89) [39]	1·53 (-4·02 to 7·07) [33]	3·22 (-4·02 to 10·45)	0·38
4 months follow-up	4·42 (-0·71 to 9·54) [39]	4·92 (-0·52 to 10·36) [35]	-0·50 (-7·65 to 6·65)	0·89
6 months follow-up	3·62 (-1·51 to 8·75) [39]	-0·22 (-5·76 to 5·32) [33]	3·84 (-3·39 to 11·07)	0·30
Ear & Hearing Symptoms				
2 months follow-up	1·77 (-3·53 to 7·08) [39]	3·42 (-2·30 to 9·15) [33]	-1·65 (-9·18 to 5·88)	0·67
4 months follow-up	7·11 (1·82 to 12·39) [39]	2·79 (-2·83 to 8·41) [35]	4·32 (-3·13 to 11·77)	0·25
6 months follow-up	2·80 (-2·49 to 8·08) [39]	1·60 (-4·12 to 7·31) [33]	1·20 (-6·33 to 8·72)	0·75
Hearing				
Pure tone average (dB)				
6 months follow-up	-0·11 (-0·20 to -0·02) [39]	-0·04 (-0·14 to 0·06) [32]	0·07 (-0·06 to 0·20)	0·30
Discrimination Loss (%)				
6 months follow-up	-1·17 (-2·29 to -0·05) [30]	-0·86 (-2·03 to 0·32) [27]	-0·31 (-1·85 to 1·23)	0·69
Tympanograms				
6 months follow-up	[35]	[30]		0·18

The first column shows the mean change from baseline to the respective follow-up visits (95% CI) in each treatment group, the second column the mean between-group difference (95% CI) at the respective follow-up visits, and the p-value for the between-group difference. The changes and differences are estimated from a linear mixed model with an interaction between treatment group and follow-up visit based on all four study visits. The hearing outcomes were only measured at baseline and the 6 months follow-up visit. The number of participants measured at the specified time points is stated in square brackets in the table cells.

*FEF_{25-75} % predicted was log2 transformed for the analysis and has been back-transformed. The data are presented as relative changes.
†Raw % predicted and $S_{acin}*V_T$ were square root transformed for the analyses and are presented on square root scale.
‡The QOL-PCD scores are based on data from the QOL-PCD questionnaires completed by the children themselves, adolescents and adult participants. Data from the parent proxy version of the QOL-PCD questionnaire is not included in this table.
FEV_1=forced expiratory volume in one second. FVC=forced vital capacity. FEF_{25-75}=forced expiratory flow at 25–75% of forced vital capacity. RV=residual volume. RV/TLC=residual volume divided by total lung capacity. Raw=airway resistance. LCI=lung clearance index. $S_{cond}*V_T$=regional ventilation inhomogeneity of the conducting airways corrected for tidal volume. $S_{acin}*V_T$=regional ventilation inhomogeneity of the intra-acinar airways corrected for tidal volume. QOL-PCD=PCD-specific health-related quality of life questionnaire.

Table A6: Change in inflammatory markers from baseline to 6-month visit

	Change from baseline		
	Azithromycin (n=40)	**Placebo (n=34)**	**p value**
Peripheral blood cells			
White blood cells ($\times10^9$/mL)	-0·5	0·1	0·36
Neutrophils ($\times10^9$/mL)	-0·3 [38]	0·2	0·40
Eosinophils ($\times10^9$/mL)	0·0 [38]	0·0	0·75
Lymphocytes ($\times10^9$/mL)	-0·1 [38]	0·0	0·41
Monocytes ($\times10^9$/mL)	0·0 [38]	0·0	0·70
Basophils ($\times10^9$/mL)	0·0 [38]	0·0	0·79
C-reactive protein (mg/L)*	0·0	0·0	0·13
Sputum cytokines			
IL-1β (pg/mL)	48·4 [23]	-112·2 [25]	0·82
G-CSF (pg/mL)	80·5 [22]	58·4 [23]	0·92
IL-8 (pg/mL)	271·8 [24]	-60·5 [24]	0·52
IL-10 (pg/mL)	1·4 [17]	-0·7 [17]	0·89
TNF-α (pg/mL)	12·1 [23]	-15·5 [23]	0·45
Gro-α (pg/mL)	1906·1 [24]	60·5 [24]	0·99
MCP-1 (pg/mL)	24·3 [23]	18·4 [25]	0·76
Serum cytokines			
IL-1β (pg/mL)	N/A#	N/A#	
G-CSF (pg/mL)	0·0 [38]	0·0 [32]	0·85
IL-8 (pg/mL)	-0·9 [38]	0·3 [32]	0·28

Data are presented as median change from baseline to 6-month visit. Between-group difference was analysed using Wilcoxon signed rank test. The number of participants available for the specific variables is stated in square brackets in the table cells if different from the total number of participants in the treatment group.
*At some study sites, C-reactive protein <2·5 was not measured more accurately. In these cases, C-reactive protein was set to zero.
Unsuccessful/failed analysis.
IL-1β=Interleukin-1 beta. G-CSF=Granulocyte-colony stimulating factor. IL-8=Interleukin-8. IL-10=Interleukin-10. TNF-α=Tumor necrosis factor alpha. Gro-α=Growth-regulated oncogene alpha. MCP-1=Monocyte chemoattractant protein-1.

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