Online Supplementary material

Title: Sputum microbiota in adults with CF associates with response to inhaled tobramycin

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Methods

Microbiome analysis

PCR and extraction blanks were used as quality controls. Sequencing reads were analyzed using an in-house designed pipeline[1]. Cutadapt was used to trim primers and low-quality reads[2]. PANDAseq was used to assemble paired-end reads[3]. Operational taxonomic units (OTUs) were picked using Abundant OTU+ for OTUs with ≥97% identity[4]. Taxonomy was assigned using the RDP classifier (version 2.2) against the Greengenes 2013 reference database[5,6]. OTUs were removed if they did not match to the Bacteria clade, were present as a single read in the entire dataset (singletons), or only present in one sample.

qPCR

The concentration of genomic DNA isolated from the sputum samples was measured and normalized to a concentration of 100 ng/µL for the qPCR *S. aureus* detection. This was the minimum concentration established to obtain a readable signal from samples, regardless of the relative abundance of *Staphylococcus sp*. Samples that did not meet that concentration were excluded for the qPCR analysis for detection of *S. aureus*, as the template for that reaction was set up at 100 ng/µL. For *S. aureus* detection, each 10 µl reaction contained 5 µl of TaqMan® Fast Advanced Master Mix (Applied Biosystems), 0.5 µl of TaqMan Gene Expression probe for *S. aureus* detection (Ba04646259_s1; Thermo Fisher Scientific), and 4 µl of the template DNA. Each run contained non-template control wells and a 9-fold dilution series of *S. aureus* genomic DNA strain ATCC 25923, which was used to estimate the absolute quantity (ng/µL) of *S. aureus*. For total bacterial load determination, each 10 µl reaction contained 5 µl of TaqMan® Fast Advanced Master Mix (Applied Biosystems), 0.5 µl of TaqMan Gene Expression probe for pan-bacterial detection of 16S rRNA (Ba04230899_s1; Thermo Fisher Scientific), and 2.5 µl of the template DNA (at a concentration of 10 ng/µL, which was the minimum concentration established to obtain a readable signal from samples). Each run contained non-template control wells and a 9-fold dilution series of *P. aeruginosa* genomic DNA PA01 strain. Standard curve samples were converted from DNA concentration to 16S rRNA copy number as previously described[7,8]. The number of 16S copies in a given 25 ng qPCR reaction was multiplied by the total number of nanograms in the entire sputum DNA prep and then divided by the volume

of sputum used for DNA extraction (i.e. ~0.3 ml) to give an absolute abundance of 16S copies/ml of sputum. For both qPCR assays, the qPCR program reaction consisted of an initial step at 50 ˚C for 2 min, then a step at 95 ˚C for 2 min followed by 40 cycles of 95 ˚C for 1 s, 60 ˚C for 20 s. All samples were assayed in duplicate and their results averaged. qPCR reactions were carried out in the QuantStudio 5 Real-time PCR systems (Applied Biosystems).

Supplemental Literature Cited

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4. Ye, Y. Identification and Quantification of Abundant Species from Pyrosequences of 16S rRNA by Consensus Alignment. *Proceedings. (IEEE. Int. Conf. Bioinformatics Biomed).* **2010**, 153–157 (2011).
5. Wang, Q., Garrity, G. M., Tiedje, J. M. & Cole, J. R. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* **73**, 5261–7 (2007).
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7. Zhao, J. *et al.* Decade-long bacterial community dynamics in cystic fibrosis airways. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 5809–14 (2012).
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Supplemental Tables

Table S1: Concurrent therapies at treatment initiation.

Characteristics of patient cohort	**Response†**		
	Responder (n=19) (46%	**Non-R‡ (n=22) (54%)**	**P-value**
Nutritional			
CF specific multi-vitamin	19(100%)	22(100%)	0.35
Vitamin D	15(79%)	17(77%)	0.90
Pancreatic enzymes	15(79%)	21(95%)	0.11
Nutritional supplements	3(16%)	6(27%)	0.38
Ultratums	6(32%)	10(45%)	0.36
Oral antibiotic therapies			
Azithromycin	8(42%)	9(41%)	0.94
Ciprofloxacin	2(11%)	3(14%)	0.76
Inhaled antibacterials			
Inhaled aztreonam (AZLI)	2(11%)	1(5%)	0.46
Respiratory			
Short acting beta-agonist	14(74%)	17(77%)	0.79
Long acting beta-agonist	11(58%)	15(68%)	0.50
Inhaled corticosteroid	11(58%)	15(68%)	0.50
Nasal steroid	1(5%)	4(18%)	0.21
Dornase alpha (DNase)	12(63%)	13(14%)	0.79
Hypertonic Saline	8(42%)	8(36%)	0.71
Gastrointestinal (GI)			
PPI (Pantoprazole/omeprazole)	8(42%)	8(36%)	0.71
Ranitidine	0	2(9%)	0.18
Bisphosphonate	6(32%)	1(5%)	0.022*
Domperidone	5(26%)	5(23%)	0.79
Ursodiol	6(32%)	4(18%)	0.32
Endocrine			
Birth control	2(11%)	6(27%)	0.18
Insulin	5(%)	6(%)	0.95
Mood			
SSRI	4(%)	7(%)	0.44

Chi-squared tests were used to determine differences between dichotomous variables. Wilcoxon rank sum tests were conducted for analysis of continuous.
† Response was *a priori* defined as achieving stable lung function as measured by FEV_1 percent predicted in the year following TIP/S initiation (no net decline).
‡ non-R = non-responder

Table S2A Assessment of microbiome for baseline samples and its association with response to inhaled tobramycin.

		Beta-diversity (Bray-Curtis)		Alpha-diversity		
Net Improvement in percent predicted Force Expiratory Value in the 18 months preceding TIP/S initiation†	**Number of responders (of a possible total n= 41) (%)**	**PERMANOVA (p-value)**	**R^2**	**Median (IQR) Shannon Diversity Responders**	**Median (IQR) Shannon Diversity non-Responders**	**Wilcox (p-value)**
-2.5	24(59%)	0.22	0.03	1.63(1.29-2.42)	1.45(1.07-2.13)	0.32
-2	22(54%)	0.18	0.04	1.73(1.25-2.45)	1.45(1.13-1.98)	0.28
-1.5	21(51%)	0.15	0.04	1.70(1.23-2.40)	1.47(1.16-2.16)	0.53
-1	21(51%)	0.16	0.04	1.70(1.23-2.40)	1.47(1.16-2.16)	0.53
-0.5	21(51%)	0.15	0.04	1.7(1.23-2.40)	1.48(1.16-2.16)	0.53
0 (A priori definition)	**19(46%)**	**0.035**	**0.06**	**1.76(1.28-2.44)**	**1.38(1.18-2.95)**	**0.28**
0.5	15(37%)	0.011	0.08	1.76(1.43-2.42)	1.33(1.10-2.05)	0.16
1	13(32%)	0.002	0.11	1.76(1.53-2.47)	1.33(1.01-2.11)	0.08
1.5	11(27%)	0.007	0.10	2.26(1.62-2.48)	1.33(1.10-2.04)	0.05
2	11(27%)	0.006	0.10	2.26(1.62-2.48)	1.35(1.10-2.04)	0.05
2.5	10(24%)	0.013	0.09	2.26(1.62-2.48)	1.33(1.10-2.04)	0.08
3	10(24%)	0.007	0.09	2.08(1.57-2.49)	1.49(1.12-2.12)	0.08

† values were selected predicted FEV_1 of responders 2.5% (IQR 0.5-6.5%).

Table S2B Genus level assessment of the sputum microbiome for baseline samples and its association with response to inhaled tobramycin.

		Taxonomy											
		Staphylococcus			***Pseudomonas***			***Mogibacterium***			***Scardovia***		
Net Improvement in percent predicted Force Expiratory Value in the 18 months preceding TIP/S initiation†	**Number of responders (of a possible total n= 41) (%)**	**Base Mean of Reads Responder**	**Log2 Fold Change in non-Responders**	**adjusted p-value‡**	**Base Mean of Reads Responder**	**Log2 Fold Change in non-Responders**	**adjusted p-value‡**	**Base Mean of Reads Responder**	**Log2 Fold Change in non-Responders**	**adjusted p-value‡**	**Base Mean of Reads Responder**	**Log2 Fold Change in non-Responders**	**adjusted p-value‡**
-2.5	24(59%)	877.51	-4.61	2.42E-04	23690.84	2.75	0.014	15.13	-3.73	0.03	2.24	0.15	0.99
-2	22(54%)	877.51	-4.89	2.11E-05	23690.84	2.63	0.027	3.47	-0.76	0.97	2.24	-0.14	0.97
-1.5	21(51%)	877.51	-5.03	6.00E-06	23690.84	2.53	0.047	3.04	-0.56	0.98	2.24	-0.34	0.98
-1	21(51%)	877.51	-5.03	6.00E-06	23690.84	2.53	0.047	3.04	-0.56	0.98	2.24	-0.34	0.98
-0.5	21(51%)	877.51	-5.03	6.00E-06	23690.84	2.53	0.047	3.04	-0.56	0.98	2.24	-0.34	0.98
0 (A priori definition)	**19(46%)**	**877.51**	**-5.28**	**5.59E-07**	**23690.84**	**2.49**	**0.061**	**3.47**	**-0.18**	**0.95**	**2.24**	**-0.69**	**0.95**
0.5	15(37%)	3280.67	-5.73	5.62E-06	23231.07	3.07	4.50E-04	15.13	-3.54	0.04	14.26	-5.19	0.05
1	13(32%)	6094.78	-6.95	6.79E-09	23509.40	3.48	8.00E-04	15.13	-3.87	0.01	14.26	-5.56	0.03
1.5	11(27%)	947.12	-2.32	0.93	23835.37	3.87	4.00E-04	3.47	-1.19	0.93	2.24	-1.37	0.93
2	11(27%)	947.12	-2.32	0.93	23835.37	3.87	4.00E-04	3.47	-1.19	0.93	2.24	-1.37	0.93
2.5	10(24%)	947.12	-2.07	0.94	23835.37	3.89	8.97E-04	3.47	-1.32	0.94	2.24	-1.59	0.94
3	10(24%)	947.12	-2.07	0.94	23835.37	3.89	8.97E-04	3.47	-1.32	0.94	2.24	-1.59	0.94

† values were selected predicted FEV1 of responders 2.5% (IQR 0.5-6.5%)

‡ DESeq2 (test="Wald" fitType=parametric and Benjamin Hochberg multiple test correction)

Supplemental Figures

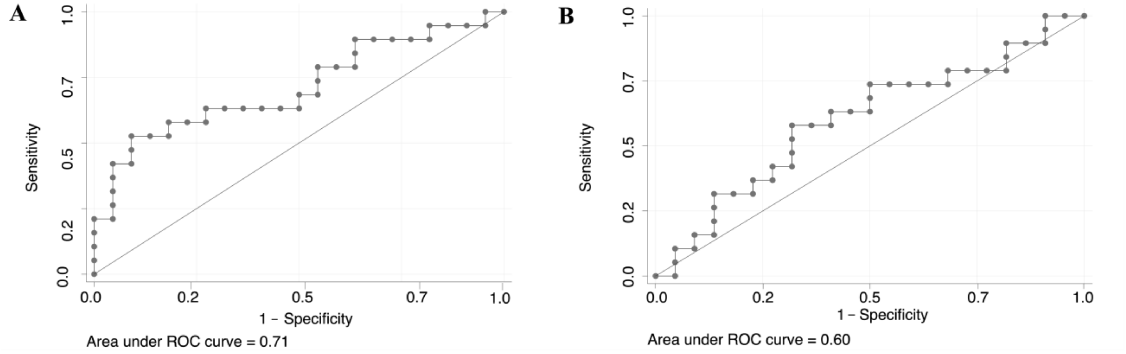

Figure S1: Receiver operator characteristic curve analysis of **A)** *Staphylococcus* relative abundance and **B)** Shannon Diversity Index as a predictors of patient response at baseline. ROC curves and area under the curve analysis demonstrates that the relative abundance of *Staphylococcus* at baseline is superior to SDI in predicting patient response.

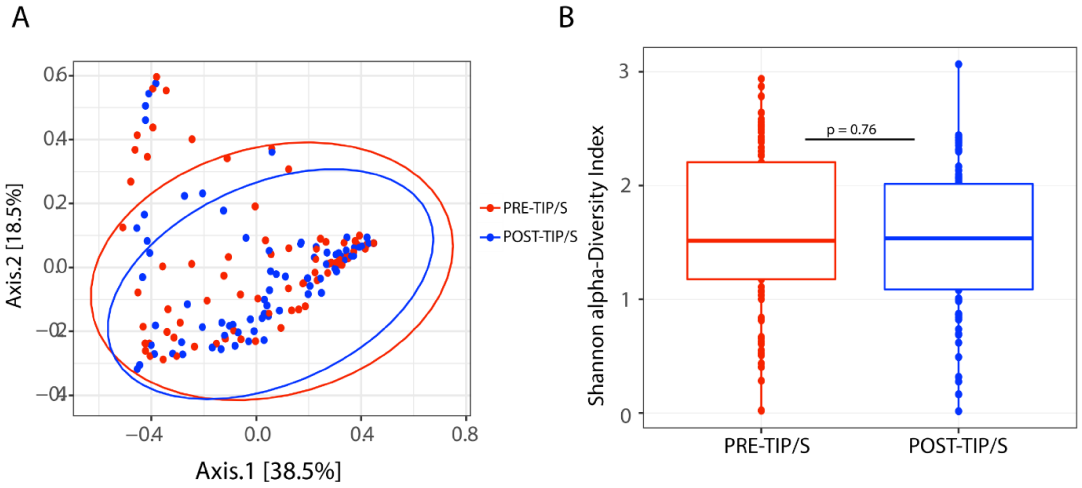

Figure S2: Shifts in the microbiome of samples collected prior (n=77) and post (n=74) initiation of TIP/S. **A)** Bray-Curtis based PCoA plot showing community wide no significant differences between samples collected prior (red) and post (blue) initiation of TIP/S (PERMANOVA,

R^2=0.0033), p=0.84). **B)** No significant differences were observed in Shannon alpha-diversity index prior to and post initiation of TIP/S.