Increasing relative abundance of *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* via 16S sequencing predicts chronic rejection after lung transplant

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Background

- Chronic rejection is the leading cause of long-term morbidity and mortality after lung transplantation[1]
- Chronic rejection, also called chronic lung allograft dysfunction (CLAD), has been linked with colonization of the lung allograft by "Pseudomonads" in culture-dependent studies[2,3]
  - "Pseudomonads" is a historical name generally meant to include species of the genera *Pseudomonas, Burkholderia*, *Stenotrophomonas,* and *Alcaligenes*
- Culture-independent methods to describe the lung microbiome have been associated with clinically important outcomes, including the development of CLAD[4]
- The ***purpose of this study*** was to investigate the relationship of airway colonization with "Pseudomonads" and the subsequent development of CLAD using culture-independent methods

Methods

Patient Cohort
- Lung transplant recipients undergoing routine 12-month post-transplant surveillance bronchoscopy at the University of Michigan; patients with pulmonary symptoms or lung function decline at time of bronchoscopy were excluded from analysis

Clinical Outcomes & Variables
- Primary outcome: CLAD or death within 500 days of bronchoscopy
- CLAD defined according to ISHLT criteria as a persistent decline in FEV_1 by at least 20% of the post-transplant baseline value[1]

Lung Microbiome Characterization
- Cell-free BAL fluid surplus to clinical requirements were prospectively collected and stored at -80° C prior to processing
- Bacterial DNA burden was measured with a QX200 Droplet Digital PCR System (BioRad) and quantified as total number of 16S gene copies per mL of BAL fluid
- The V4 region of the 16S rRNA gene was sequenced using the MiSeq platform (Illumina)
- Sequence data was processed and analyzed using the software *mothur*,[5] and a phylotyped file was generated using operational taxonomic units (OTU)
  - OTU representative of *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* were identified by comparing the homologous representative nucleotide sequences generated for each OTU to the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST)

Statistical Analysis
- Correlation between continuous microbiome variables was evaluated via Spearman rank correlation method
- PERMANOVA was used to compare beta-diversity between samples with and without evidence of Pseudomonads
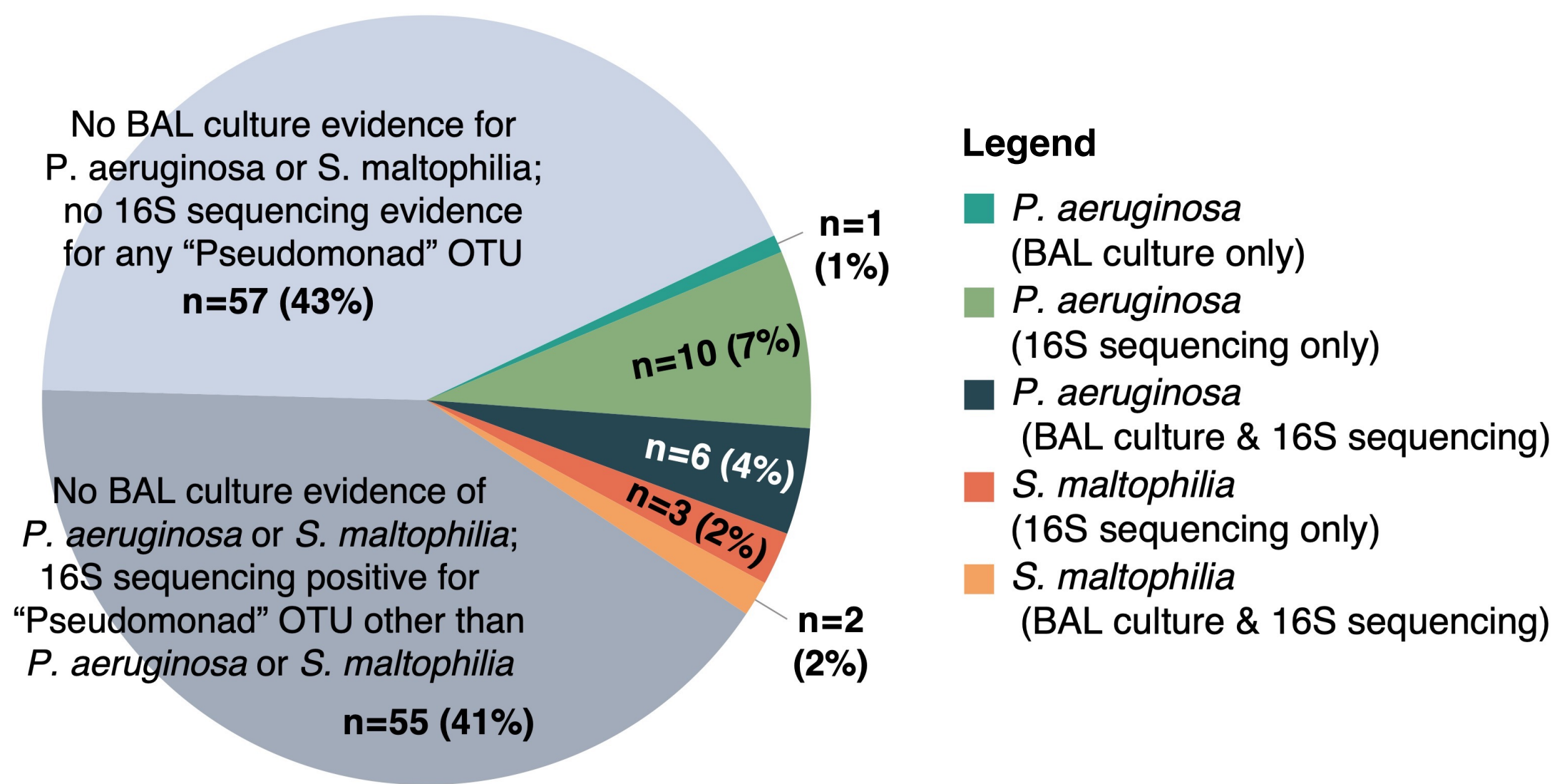- Survival was analyzed using Cox proportional hazards multivariate regression

Results

Identification of *P. aeruginosa*, *S. maltophilia* and other "Pseudomonads" in BAL

- 7 (5.2%) patients had evidence of *P. aeruginosa* and 2 (1.5%) had evidence of *S. maltophilia* on BAL bacterial culture
  - No other cultures were positive for bacterial spp. from genera considered to be "Pseudomonads"
- 16 (11.9%) patients had evidence of *P. aeruginosa*, 5 (3.7%) had evidence of *S. maltophilia*, and 55 (41.0%) had OTU classified as being from genera considered to be a "Pseudomonad" on 16S sequencing

No BAL culture evidence for P. aeruginosa or S. maltophilia; no 16S sequencing evidence for any "Pseudomonad" OTU n=57 (43%)

No BAL culture evidence of P. aeruginosa or S. maltophilia; 16S sequencing positive for "Pseudomonad" OTU other than P. aeruginosa or S. maltophilia n=55 (41%)

n=1 (1%)
n=10 (7%)
n=6 (4%)
n=3 (2%)
n=2 (2%)

Legend
- *P. aeruginosa* (BAL culture only)
- *P. aeruginosa* (16S sequencing only)
- *P. aeruginosa* (BAL culture & 16S sequencing)
- *S. maltophilia* (16S sequencing only)
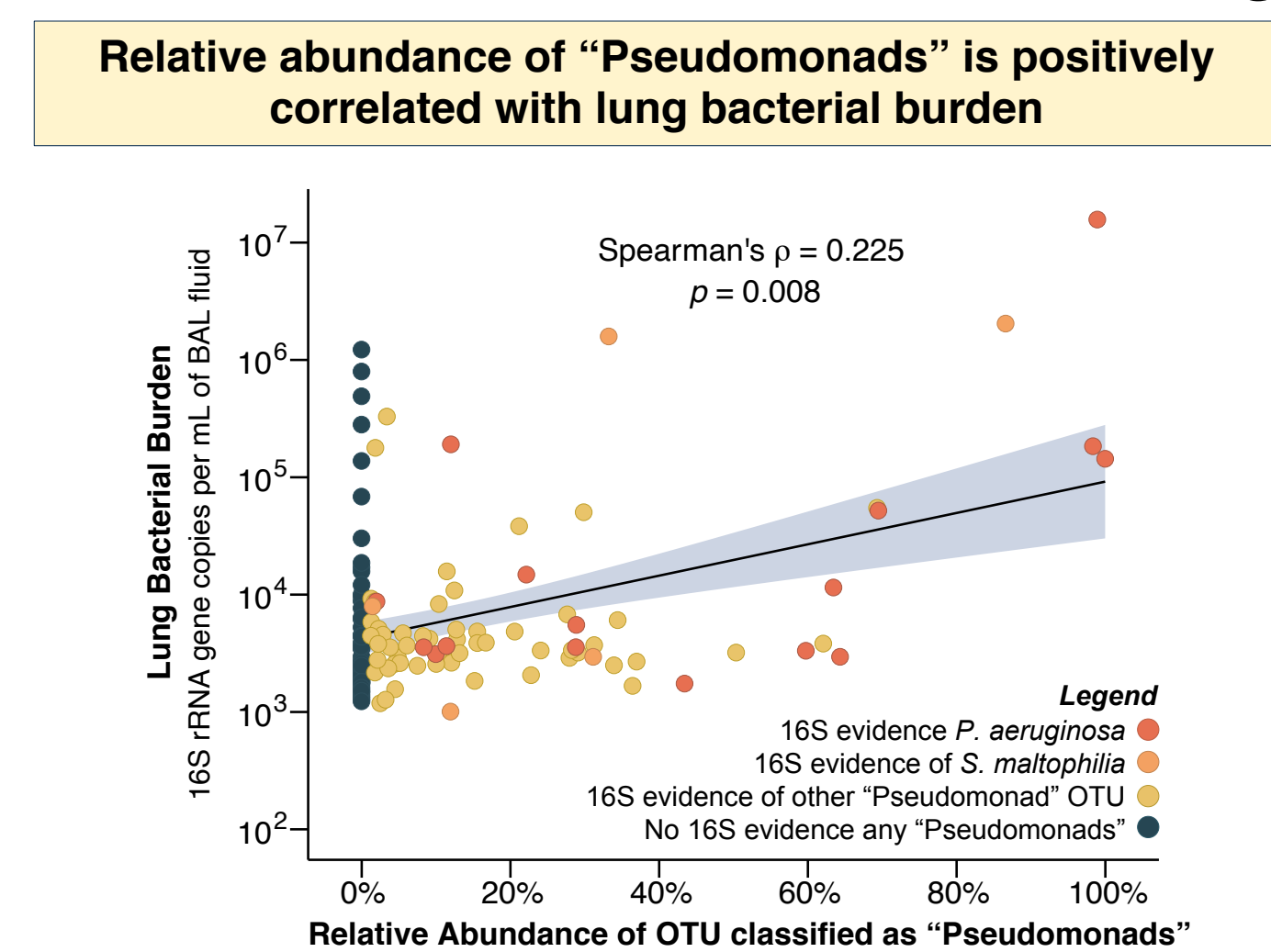- *S. maltophilia* (BAL culture & 16S sequencing)

Increasing relative abundance of *P. aeruginosa*, *S. maltophilia* and other "Pseudomonads" is associated with subsequent CLAD development

- Increasing relative abundance of *P. aeruginosa* or *S. maltophilia*, when analyzed together, was associated with increased risk of CLAD or death in a univariate model
- Likewise, increasing total relative abundance of all OTU representing genera that could be classified as "Pseudomonads" was associated with an increased risk of CLAD or death in a univariate model
- *P. aeruginosa* or *S. maltophilia* was not associated with CLAD or death when analyzed as present vs. absent using either culture-dependent or culture-independent methods
- In multivariate models which included other relevant microbiome characteristics, such as lung bacterial burden[4], the associations above were attenuated
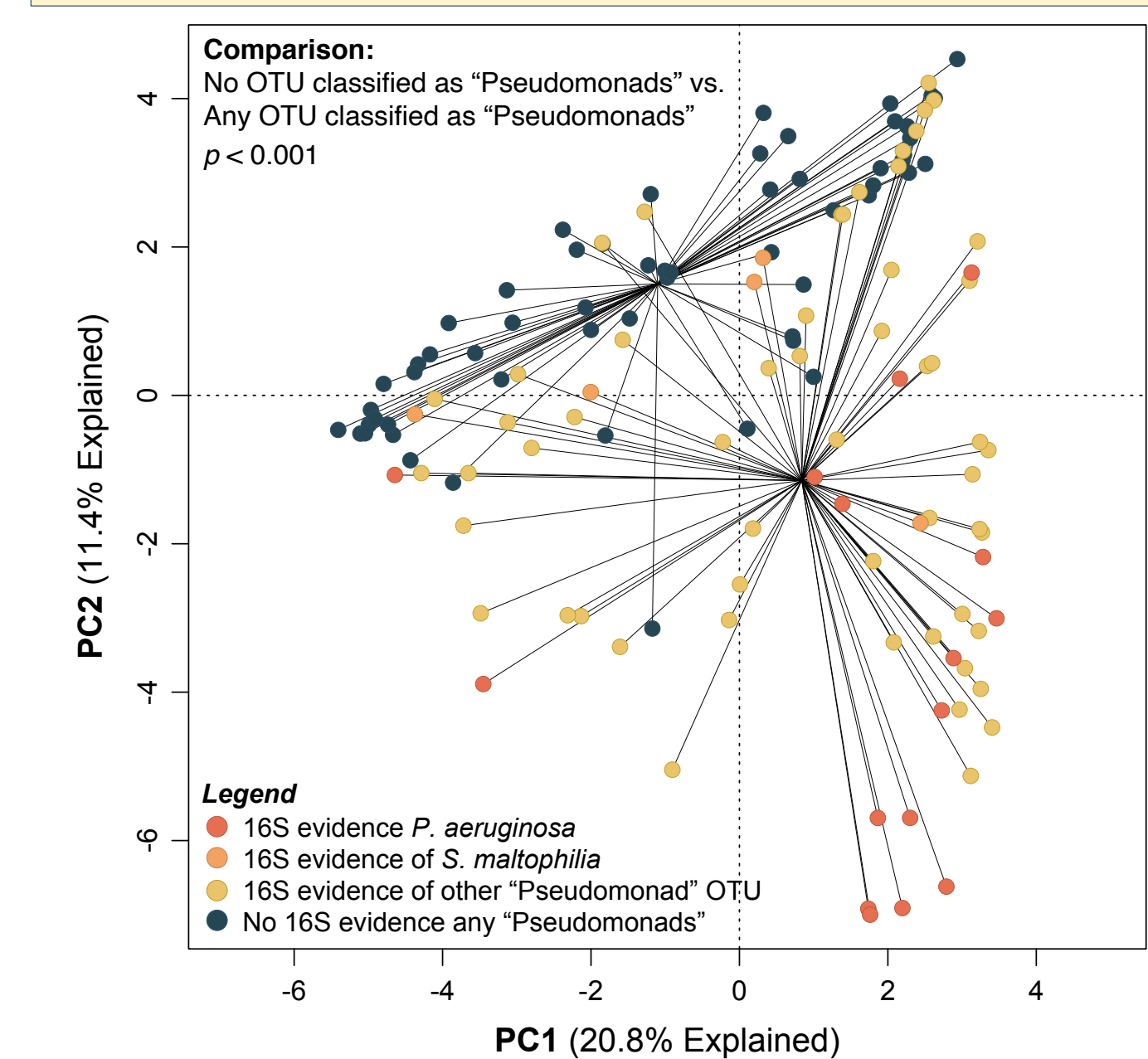
Culture-Dependent	HR (95% CI)	*p*-value
Presence vs. Absence		
P. aeruginosa	1.52 (0.36 - 6.41)	0.566
S. maltophilia	3.76 (0.51 - 27.72)	0.193
P. aeruginosa or *S. maltophilia*	1.95 (0.59 - 6.46)	0.272
Colonization[2]		
P. aeruginosa	0.92 (0.32 - 2.64)	0.877
S. maltophilia	2.06 (0.49 - 8.65)	0.325
P. aeruginosa or *S. maltophilia*	1.31 (0.53 - 3.22)	0.557
Culture-Independent[3]	**HR (95% CI)**	***p*-value**
Presence vs. Absence		
P. aeruginosa	1.28 (0.44 - 3.67)	0.651
S. maltophilia	0.80 (0.11 - 5.86)	0.823
P. aeruginosa or *S. maltophilia*	1.22 (0.47 - 3.20)	0.687
Any "Pseudomonad" OTU	1.27 (0.60 - 2.68)	0.536
Increasing relative abundance, by 10% increase		
P. aeruginosa	1.13 (0.96 - 1.34)	0.137
S. maltophilia	1.45 (1.09 - 1.93)	**0.010**
P. aeruginosa or *S. maltophilia*	1.18 (1.02 - 1.35)	**0.024**
Any "Pseudomonad" OTU	1.17 (1.02 - 1.34)	**0.021**

Abbreviations: HR = Hazard ratio; 95% CI = 95% confidence interval, OTU = operational taxonomic unit
[1]Each comparison is performed independently, no correction for multiple comparisons
[2]Defined as 2 consecutive positive cultures, taken between 4 weeks and 6 months apart, with no clinical signs of infection or other clinical instability (NB: includes patients who had resolved their previous colonization at time of 12-month surveillance bronchoscopy)
[3]Only measured in 12-month post-transplant surveillance bronchoscopy

Association of "Pseudomonads" presence with other features of the lung microbiome

Relative abundance of "Pseudomonads" is positively correlated with lung bacterial burden

Spearman's ρ = 0.225
p = 0.008

Legend
- 16S evidence *P. aeruginosa*
- 16S evidence of *S. maltophilia*
- 16S evidence of other "Pseudomonad" OTU
- No 16S evidence any "Pseudomonads"

Presence of "Pseudomonads" is a key community composition difference among lung transplant patients

Comparison:
No OTU classified as "Pseudomonads" vs.
Any OTU classified as "Pseudomonads"
p < 0.001

PC2 (11.4% Explained)
PC1 (20.8% Explained)

Legend
- 16S evidence *P. aeruginosa*
- 16S evidence of *S. maltophilia*
- 16S evidence of other "Pseudomonad" OTU
- No 16S evidence any "Pseudomonads"

- Relative abundance of OTU classified as "Pseudomonads" is positively correlated with total lung bacterial burden (Spearman's rho = 0.225, p=0.008)
- Relative abundance of OTU classified as "Pseudomonads" is negatively correlated with Shannon Diversity Index (Spearman's rho = -0.225, p=0.009, data not shown)
- Unsurprisingly, having any OTU classified as a genus considered to be a "Pseudomonad" distinguished unique overall bacterial communities
  - Yet, as seen in the Principal Components Analysis plot, there remains some overlap between patients with and without evidence of Pseudomonads

Conclusions

- Among asymptomatic lung transplant recipients undergoing 12-month post-transplant surveillance bronchoscopy, having evidence of *P. aeruginosa* or *S. maltophilia* was rare via both culture-dependent and culture-independent methods
- Increasing relative abundance of *P. aeruginosa* and *S. maltophilia* (as well as any OTU representative of genera broadly classified as "Pseudomonads") predicts shorter CLAD-free survival in univariate analyses
- Whether these bacteria directly contribute to the pathogenesis of CLAD or are indicative of a dysbiotic lung microbiome requires further study

References:
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5. Schloss PD, Westcott SL, Ryabin T, et al. Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. *Applied and Environmental Microbiology* 2009; 75(23): 7537.