Integrating airway microbiome and plasma proteomics data to identify multi-omic networks associated with cystic fibrosis pulmonary exacerbation treatment response

**Introduction**

Pulmonary exacerbations (PExs) are the leading cause of morbidity and decreased quality of life in cystic fibrosis (CF). Characterized by an acute decrease in lung function, PExs can occur repeatedly in people with CF. They are associated with a long-term decline in lung function and shortened survival (1-3). Intravenous (IV) antibiotic treatment is typically administered at the onset of a CF PEx in order to recover lost lung function. However, lung function is often not fully recovered after treatment (4, 5). There is therefore interest in characterizing the factors that contribute to incomplete PEx recovery. Identification of such determinants may improve our understanding of CF PEx disease mechanisms, provide evidence for the effectiveness and inadequacies of current CF therapies, as well as point to new therapeutic targets.

Blood and airway inflammatory biomarkers have been shown to decrease after treatment of a PEx, suggesting that airway infection in CF results in a robust host immune response (6-9). Identifying associations between specific airway bacteria or bacterial communities and host-response may be critical to understanding the pathogenicity of CF bacteria. Further, exploring microbiota—protein relationships in the context of PEx treatment response may provide novel insights into the mechanisms of PEx and the efficacy of PEx treatments.

In this study, we use an extension of canonical correlation analysis (CCA) called sparse multiple canonical correlation network (SmCCNet) to integrate plasma proteomics and airway microbiome data from the onset of PEx (i.e., pre-treatment CF PEx samples) (10). Specifically, these omics data are integrated into interpretable microbiota—protein networks that are associated with PEx treatment response. We use change in PEx score (ΔPExS) between hospital admission (i.e., PEx onset) and hospital discharge (i.e., late in PEx, post-treatment) as a clinical measure of PEx treatment response. Our aim was to identify potential biomarkers at PEx onset that are indicative of PEx recovery.

**Materials & Methods**

**Study Design & Population**

This study includes 34 PEx events from a cohort of 30 subjects aged 10 to 22 years old with a confirmed diagnosis of CF. Participants could be reenrolled in the study if PEx events were separated by at least six months. Accordingly, the study population includes four subjects with two PEx events and 26 subjects with one PEx event. Participants were recruited prospectively and enrolled at the time of hospital admission for IV antibiotic therapy of a clinically diagnosed PEx. A PEx was defined by a PEx score > 5 and at least three out of 11 PEx criteria being met, as defined by the CF foundation (CFF) Consensus Conference guidelines. Cohort characteristics, stratified by PEx time point, are described in Table 1. Subjects were treated with IV antibiotics targeting their specific CF pathogens as determined through microbial culture.

The study consists of two time points: hospital admission (i.e., PEx onset, day 0-2) and hospital discharge (i.e., after the IV treatment had been administered, day 4-21). Study procedures at each visit included a physical, a spirometry test following standard American Thoracic Society guidelines [ref], a standardized PEx score [ref], a validated quality-of-life measure, and specimen collection (blood plasma and sputum).

**Clinical Phenotype Definition**

The phenotype of interest in this study is the observed difference in PEx score (PExS) between hospital admission (*t1*) and hospital discharge (*t2*) (ΔPExS). ΔPExS is defined in Equation 1. A PExS is a standardized measure of PEx severity. The PEx scoring system was originally developed to establish a systematic definition of PEx in the clinic that does not rely on physician discretion (11). PEx scores are measured using patient symptoms (two week change in exercise tolerance, cough, sputum production, chest congestion, school or work attendance, and appetite) and physical examination findings (increased adventitial sounds on auscultation of the chest and change in FEV1 over the preceding month) (11). Increased measures of PExS correspond to increased PEx severity. We interpret ΔPExS in this study as a clinical measure of PEx treatment response. Negative ΔPExS values represent an improved PExS between hospital admission and discharge, with larger negative values indicating greater improvement (or PEx treatment response). The distribution of ΔPExS in our cohort is shown in Supplemental Figure 1.



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**Microbiome Data**

*Sample Collection*

Spontaneously expectorated sputum was collected into a sterile container for microbiologic analysis. Participants unable to spontaneously expectorate underwent sputum induction using a standardized, published protocol with 3% hypertonic saline [ref]. Standard CF culture including quantitative bacterial culture and fungal culture was performed on sputum (expectorated and induced) samples following CFF guidelines [ref]. Respiratory viral panel was performed on admission sputum specimen (Luminex Molecular Diagnostics X-Tag® RVP). Residual sputum samples were frozen at −70°C for microbiome analyses.

*16S rRNA Sequencing*

Bacterial profiles were determined by broad-range amplification and sequence analysis of 16S rDNA following previously described methods [ref]. Amplicons were generated using primers targeting approximately 300 base pairs of the V1/V2 variable region of the 16S rRNA gene. Illumina paired-end sequencing was performed on the MiSeq platform using a 500 cycle v2 reagent kit.

*Analysis of Illumina Paired-End Reads*

Quality control procedures were performed on paired-end sequences as described in the online supplement. Assembled sequences were aligned and classified with SINA (1.2.11) [ref] using the Silva 111 database [ref] as reference configured to yield the Silva taxonomy. Sorted paired-end sequence data were deposited in the NCBI Short Read Archive under accession number xxx. The software package Explicet (v2.10.5) [ref] was used for analysis of Good’s coverage and the calculation of richness, evenness, and Shannon diversity, a composite measure of evenness and richness, at the rarefaction point of xxx sequences.

*Data Preprocessing*

Microbiome count data were filtered to include only prevalent taxa. Detection and prevalence thresholds were set to 0.1% and 10%, respectively (i.e., taxa were filtered out if they did not exceed 0.1% relative abundance (RA) in at least 10% of samples). Count data were transformed using the centered log-ratio (CLR) transformation. A pseudocount (RAmin/2) was applied to exact zero RA entries before CLR transformation was performed. Microbiome features were standardized prior to downstream statistical analysis by removing the mean and scaling to unit variance.

**Proteomics Data**

*Sample Collection*

Blood was collected and analyzed for complete blood count (CBC), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and calprotectin (S100A8/9; ALPCO-Buhlman, LLD 0.4 mcg/mL). An aliquot of 3.5 mL was processed following a standard operating procedure, frozen and batch shipped on dry ice to SomaLogic in Boulder, Colorado for proteomics analysis.

*SomaLogic Proteomics Assay*

Proteomics data were measured using the SomaScan multiplex proteomics assay, an aptamer-based quantitative proteomic biomarker discovery platform. Approximately 4,000 aptamers, or single-stranded deoxyribonucleic acid (ssDNA) molecules that bind specific protein targets, were measured in this assay. Aptamers targeted approximately 3,600 unique proteins.

*Data Preprocessing*

SomaScan measurements, reported in relative fluorescent units (RFUs), were normalized using internal hybridization controls added to the assay prior to hybridization. Normalization was performed to adjust for inter-sample, inter-plate, and inter-run variation. Normalized RFU values were log-2 transformed to handle skewness and improve normality. Proteomics features were standardized prior to downstream statistical analysis by removing the mean and scaling to unit variance.

**Statistical Analysis**

*Statistical Software*

All statistical analysis work, including multi-omic integration, network identification with, gene ontology (GO) enrichment analysis, and sensitivity analysis, was performed using R version 4.1.1.

*SmCCNet*

Airway microbiome (Χ1) and plasma proteomics (Χ2) data from PEx onset were integrated using SmCCNet (version 0.99.0). SmCCNet is an extension of canonical correlation analysis (CCA) that incorporates a quantitative phenotype to construct interpretable multi-omic networks. Given our omic data types and phenotype of interest, we use SmCCNet to construct microbiota—protein networks at PEx onset that are correlated with PEx treatment response (ΔPExS, or Υ).

CCA is an unsupervised learning approach that aims to identify relationships between two multivariable data sets by finding the linear combination of variables that maximizes correlation between the sets. In the most general form, CCA seeks to maximize Cor(Χ1*w*1, Χ2*w*2) where *w*1 and *w*2 are canonical weights corresponding to Χ1 and Χ2, respectively. The canonical weight objective function is defined in Equation 2.

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SmCCNet uses an adaptation of CCA, called sparse multiple CCA (SmCCA), to incorporate a third data type (i.e., the phenotype of interest) into the integration task. SmCCA accomplishes multimodal integration by accounting for phenotype—omic correlation within the canonical weight objective function. Sparsity is imposed on the canonical weight vectors by further adding penalization terms to Equation 2 (functions *P*1(•) and *P*2(•)). The least absolute shrinkage and selection operation (LASSO) is used within the SmCCA implementation used by SmCCNet. The extended canonical weight objective function that allows for sparse, multivariate integration is defined in Equation 3. In this objective function, *c*1 and *c*2 are the pre-specified penalty constants and *a*, *b*, and *c* are scaling constants that can be used to prioritize specific between-set correlations (i.e., microbiota—protein, microbiome—ΔPExS, or protein—ΔPExS correlation). Shi et al suggest that weighted SmCCA (i.e., the case in which *a*, *b*, and *c* are not all equal) might be preferred when the correlation between phenotype and omic data is weaker than the correlation between the omic data sets.

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SmCCNet uses SmCCA and a feature subsampling approach to obtain a set of canonical weight vectors, each represented by *u* = (*w*1, *w*2). The canonical weight vectors are used to construct a similarity matrix *A* following *A* = *u* ⊗ *u* and subsequently a dissimilarity (or distance) matrix following *D* = 1 − *A*. *D* is used as input for hierarchical clustering and the resultant hierarchical tree is cut to form distinct networks that are associated with the phenotype of interest. 500 feature subsampling iterations and default parameters were used for SmCCNet analysis unless stated otherwise.

*SmCCNet Hyperparameter Tuning*

As mentioned, SmCCNet induces sparsity into the CCA framework by incorporating two LASSO-based penalty parameters (i.e., one penalty parameter for each of Χ1 and Χ2; *c*1 and *c*2, respectively). Penalty parameters were selected using five-fold cross-validation (CV) and a randomized grid search approach. The selected penalty pair corresponds to the penalty pair that minimizes the prediction error between the CV training and test sets. Counterintuitively, increasing the value of a penalty parameter weakens the strength of regularization applied to a given omic set. We searched a range of larger values for *c*1 (ranging from 0.40 to 0.60) and a range of larger values for *c*2 (ranging from 0.10 to 0.40) since the dimensionality of Χ1 (60 taxa) was much smaller than the dimensionality of Χ1 (4,001 aptamers). The aim was to impose a greater penalization on Χ2 in order to generate networkswith an improved balance of proteins and taxa. We further used imbalanced feature subsampling proportions (i.e., 0.90 and 0.70 for Χ1 and Χ2, respectively) to additionally account for the feature imbalance between omics sets. Ten feature subsampling iterations were used during CV due to computational limitations.

SmCCA objective function scaling coefficients *b* and *c* were additionally tuned using a manual hyperparameter tuning process similar to the one used by Mastej et al [ref]. We explored this *weighted* version of SmCCNet since the correlation between omic sets was found to be stronger than the correlation between the phenotype and each of the omic sets (Supplemental Figure 2). Separate five-fold CVs were performed to select penalty parameters for each of the tested weighting schemes. We considered the number of networks, the strength of network–phenotype correlations, network sizes (i.e., number of nodes, or features), and microbiota-protein balance when determining the optimal weighting scheme (Supplemental Table 1). The aim was to generate robust and reasonably sized multi-omic networks that are strongly correlated with PEx treatment response (ρ > 0.30).

*Network Summarization*

The association between each network and ΔPExS was assessed by performing principal component analysis (PCA) and calculating the correlation between ΔPExS and the first principal component (PC1) of the network-specific matrix of taxa and proteins (i.e., a merged matrix of subnetwork-specific taxa and proteins in Χ1 and Χ2, respectively). Absolute correlation values are reported since the use of PC1 obscures the interpretability of negative and positive correlations.

*Network Pruning*

We aimed to limit networks to 200 or less nodes, which represents just less than 5% of the total feature space. The reason for setting this maximum network size was two-fold. First, signals from gene enrichment analysis are typically too weak to detect if gene lists are too large relative to the full set of background genes[ref]. We aim to extract more meaningful results from gene enrichment analyses by trimming our networks into high-quality and highly connected lists of genes and taxa. Second, constraining network sizes to 200 nodes or less aids network interpretability, especially with regard to network visualizations.

We speculated that not every node in a large network (i.e., a network with more than 200 nodes) contributes heavily to network—phenotype relatedness. We therefore carried out the following network pruning procedure which aims to both yield reasonable network sizes and maximize network—phenotype correlations. First, nodes are ranked by importance using the PageRank algorithm [ref]. Generally, the PageRank algorithm measures node importance by assessing the number and importance of connected nodes. Second, we determine network—phenotype correlation when including a variable number of the top ranked nodes. Tested node counts ranged from 100 to the original node count generated by SmCCNet. Third, and finally, we select *k* (i.e., the optimal number of nodes) by determining the *k* top-ranked nodes that maximize network-phenotype correlation. This procedure was only performed on large networks, or SmCCNet networks with more than 200 nodes. An example visualization of the procedure is provided in Supplemental Figure 3.

*Network Visualization*

Networks were visualized using CytoScape (version 3.9.1). The following additional pruning steps were applied to networks prior to visualization in order to generate interpretable visualizations reflective of the underlying biology. First, networks often contain a set of insubstantial node-to-node connections (or edges) that might incorrectly suggest erroneous associations. Our intention was to remove these edges to improve biological interpretability. We used between-node pairwise correlations for systematic removal of weak edges, removing edges between nodes that were weakly correlated (ρ < 0.30). Second, interpretation of large network visualizations is difficult due to the number of nodes, edges, and network attributes. We aimed to reduce large networks into their core components to create coherent and comprehensible visualizations. To accomplish this, we determined the maximum weight connected to every node. Between-node weights, which are calculated in the previously described *A* matrix, are reflective of the mutual importance of two nodes in maximizing the correlation between all data sets (Χ1, Χ2, and Υ). We arbitrarily selected all nodes associated with a maximum weight of greater than 0.20 for visualization. This pruning step was only performed for large networks (e.g., Network 1 in Figure 1).

*Gene Ontology Enrichment Analysis*

GO enrichment analyses were performed on select networks using network-specific protein/gene sets and Metascape [ref]. The aim was to identify over-represented GO pathways (specifically biological processes, or BP) associated with networks of interest. The full set of proteins/genes targeted by the SomaScan proteomics assay was used as the background gene list. P-value and minimum enrichment (i.e., -log10(p)) cutoffs of 0.001 and 3 were used to guard against false discoveries (i.e., type I errors). GO enrichment analyses were only performed for networks containing more than 25 genes. Networks containing less than 25 genes were assessed using a more manual process.

*Network Sensitivity Analysis*

Work in progress.

**Results**

*Selected SmCCNet Configuration & Networks*

A weighting scheme of (1, 2, 2) (i.e., *a* = 1, *b* = *c* = 2) with a CV selected penalty parameter pair of (0.25, 0.40) was found to generate the most desirable networks when considering the following network characteristics: network–phenotype correlation, network size (i.e., number of n­odes), and protein–microbiota balance (Supplemental Table 1). Eight networks, summarized in Figure 1, resulted from this SmCCNet configuration, including six networks with a strong correlation to ΔPExS (ρ > 0.30). Comprehensive analyses are provided for Networks 1 and 3 in the subsequent sections, including network visualizations, GO enrichment analyses, and network sensitivity analyses. Network 1 is a large and robust network comprised of bacteria that are not historically associated with severe clinical outcomes in CF. Correspondingly, many of these are not traditionally tracked in CF airways.

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**Table 1.** Study cohort demographics and clinical measures at hospital admission. ΔPExS, the phenotype of interest in this study, is reported as well. ΔPExS represents change in PEx Score from hospital admission to hospital discharge. Continuous demographic and clinical information are reported as median [range].

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**Figure 1.** SmCCNet network summarizations for the selected SmCCNet configuration. The scatterplot (left) shows correlations between individual network nodes and ΔPExS. Protein nodes are represented as blue circles and taxon nodes are represented as pink triangles. The heatmap (right) reports the absolute correlation, |ρ|, observed between PC1 of each network and ΔPExS. Bold typeface is used to specify the networks that are analyzed in-depth.

*The Traditional CF Pathogen Network*

The *Traditional CF Pathogen* *Network* (Network 3 in Figure 1) is a small network comprised of 12 nodes (Figure 2, Table 2). Nodes include two taxa commonly found in CF airways, *Pseudomonas aeruginosa* (*P. aeruginosa*)and *Prevotella*, and 10 proteins. Individual correlations between nodes and ΔPExS range from -0.301 to 0.394. The full network has a 0.327 correlation with ΔPExS (*p* = 0.059). Of the 12 nodes, peptidoglycan recognition protein 1 (PGRP-S) is most correlated with ΔPExS (ρ = 0.394; *p* = 0.021). PGRP-S is involved in the humoral immune response to bacterium [ref]. Interleukin-6 receptor subunit alpha (IL-6 sRa), a protein that serves as part of the receptor for interleukin-6 (IL-6) and is also involved in immune response, serves as the most heavily connected node in the network with 10 connected edges [ref]. The largest edges in the network exist between *P. aeruginosa*, IL-6 sRa, and HEM4, indicating that these nodes share mutually large canonical weights relative to other nodes in the network.

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**Figure 2.** The *Traditional CF Pathogen Network* identified by SmCCNet. Protein nodes are represented as circles and taxon nodes are represented as triangles. A divergent color palette is used for node color. Dark shades of brown and green indicate strong negative and positive correlations between the node and ΔPExS, respectively. Lighter shades of brown and green indicate weak negative and positive correlations between the node and ΔPExS. Node size corresponds to the absolute correlation observed between a node and ΔPExS. Edge thickness corresponds to between-node canonical weight-based connectivity (i.e., pairwise weights found in matrix *A*). Positively associated nodes are connected by purple edges and negatively associated nodes are connected by grey edges.

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**Table 2.** Node summarization for the *Traditional CF Pathogen Network* identified by SmCCNet, including node names and individual node–ΔPExS correlations. UniProt identifiers are included for protein nodes.

*The Robust Network*

The *Robust Network* (Network 1 in Figure 1) is a large network comprised of the most influential nodes and edges as determined by SmCCA (Figure 3, Table 3). Features in this network are associated with large canonical weights relative to other networks, suggesting their importance in maximizing the canonical correlation between Χ1, Χ2, and ΔPExS (Equation 3). The full network considered for GO enrichment analysis contains 175 nodes, including 13 taxa and 162 unique proteins. Correlations between nodes and ΔPExS range from -0.599 to 0.584. A correlation of 0.443 is observed between PC1 of the full network and ΔPExS. The central node of the network is *Atopobium*, which is strongly correlated to ΔPExS (ρ = 0.507; *p* = 0.002). The edge that connects *Atopobium* and *Actinomyces* is associated with the largest node-to-node weight observed in the integrated data set (i.e., the maximum weight calculated in matrix *A*). Proteins most associated with the phenotype ΔPExS (ρ > 0.50) include Chemokine-like protein TAFA-5, IL-23, PACN1, AIF, and TAK1-TAB1.

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**Figure 3.** Pruned representation of the *Robust* *Network* identified by SmCCNet. Protein nodes are represented as circles and taxon nodes are represented as triangles. A divergent color palette is used for node color. Dark shades of brown and green indicate strong negative and positive correlations between the node and ΔPExS, respectively. Lighter shades of brown and green indicate weak negative and positive correlations between the node and ΔPExS. Node size corresponds to the absolute correlation observed between a node and ΔPExS. Edge thickness corresponds to between-node canonical weight-based connectivity. Positively associated nodes are connected by red edges and negatively associated nodes are connected by blue edges. Weakly connected nodes are indicated by thin and near-white edges.

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**Table 3.** Node summarization for the visualized (i.e., edge-pruned) *Robust Network* identified by SmCCNet, including node names and individual node–ΔPExS correlations. UniProt identifiers are included for protein nodes. Pruned nodes, including proteins used for GO enrichment analysis, are shown in Supplemental Table 2.

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**Figure 4.** GO biological processes enriched in the *Robust Network* identified by SmCCNet. Similar biological processes (i.e., biological processes that share genes) are clustered into GO enrichment clusters. Figure 4A shows the amount of enrichment associated with each of the identified biological processes. GO enrichment clusters are signified by color. Figure 4B shows the correlation between genes associated with each GO enrichment cluster and the prominent taxa of the network. Strong positive and negative between-node correlations are indicated by purple and orange, respectively. A binary tile map is included at the top of Figure 4B to indicate which genes are included in which GO biological process. Yellow tiles indicate that a given gene is associated within a given biological process, while black tiles indicate that a given gene is not associated within a given biological process.

*Sensitivity Analysis*

Work in progress.

**Discussion**

**Limitations**

* Assuming independent observations even though we have repeats
* Cohort limitations (sample size, generalizability)
* The aptamer approach – difficult to rationalize how to deal with aptamers that hit multiple genes

**Supplementary Materials**

**Supplemental Figures**

Supplemental Figure 1.

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Supplemental Figure 2.

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Supplemental Figure 3.

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**Supplemental Tables**

Supplemental Table 1.

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**Citations**

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