

Blood mRNA biomarkers for detection of treatment response in acute pulmonary exacerbations of cystic fibrosis

Methods supplement

Study population The study was approved by the National Jewish Institutional Review Board. The inclusion criteria required a clinical diagnosis of an acute pulmonary exacerbation, which does not have a consensus diagnostic criteria. Thus, in the current study, diagnosis was based on clinical diagnostic criteria from CFF Clinical Practice guidelines, requiring at least 3 of 11 new findings or changes in status compared to a baseline visit (1) as assessed by a faculty member of the Denver Adult CF Program. In addition, inclusion required a calculated Rosenfeld pulmonary exacerbation score of greater than or equal to 2.6, which has been validated in prospective multicenter study to establish a standardized pulmonary exacerbation definition (2). Recruited subjects had blood drawn at therapy initiation and completion (± 2 days). The sample collection time window was commensurate with accepted ranges in previous CF exacerbation biomarker studies (3). Based on preliminary power calculations, a sample of 60 subjects was necessary to have 80% power to detect clinically significant changes in gene expression. Sixty two subjects were recruited secondary to 2 subjects dropping out throughout the trial, as the goal was for recruitment to proceed until 60 was reached.

Laboratory and lung function testing . During sample collection, sputum samples were obtained in the morning on the day of enrollment and subsequently on the morning of completion of the study. Blood and sputum were

collected simultaneously. Initial FEV₁ was measured at the time that the examining physician determined that patients met criteria for acute pulmonary exacerbation and qualified for study enrollment. The post-treatment FEV₁ was obtained at the time of completion of antibiotic therapy. Bronchodilators were not administered immediately prior to performing spirometry. Serum CRP levels were analyzed via immunoturbidimetry on a UniCel DxC 800 Analyzer (Beckman-Coulter). Mean bacterial density was calculated based on the total sum of all pathogens cultured from subjects. Quantitative microbiology culture methods were specific for CF patients and conformed to the CFF Guidelines Statement for Microbiology and Molecular Typing (4).

Measurement of leukocyte RNA from whole blood and PBMCs: RNA was isolated from whole blood samples with the PAXgene™ Blood RNA Kit. Tubes were centrifuged for 10 min at 5,000×g, the supernatant was discarded and 500 μL of RNase-free water added to the pellet. The tubes were vortexed thoroughly to re-suspend the pellet, centrifuged for 10 min at 5000×g and the entire supernatant discarded. The pellets were resuspended in 350 μL of buffer BR1 by vortexing and further purification of RNA was done following the manufacturer's protocol with on-column DNase digestion. RNA purification was fully automated on a QIAcube® instrument. RNA concentrations were determined using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). First strand cDNA was made from 1 μg total RNA, using a BioRad C1000 PCR machine. High quality RNA extraction was defined based on MIQE guidelines (5). All quantification was done using a single method on the same

instrument, a NanoDrop spectrophotometer (Thermo Scientific). All samples underwent a genomic DNA elimination step. The A260/A280 ratio was measured for all samples at neutral pH as an indication of RNA purity. Nucleic acid quality was further tested during the RT-PCR process, at which time a dissociation curve of nucleic acids was included to determine the melting temperatures of nucleic acid sequences within each sample. This step evaluates for nonspecific product formation and is important in ruling out nonspecific amplification. As described previously, quantitative RT-PCR quantified transcript abundance for pre and post antibiotic samples, for ten genes using Sybrgreen® indicator on an Applied Biosystems 7300 Real Time PCR System: CD36, CD64, CD163, TLR2, PLXND1, HCA112, HPSE, ADAM9, CSPG2, and IL-32 α (6). Each measurement was made in triplicate and expressed relative to the detection of the housekeeping gene, hypoxanthine guanine phosphoribosyl transferase (HPRT).

PBMCs were isolated via density gradient centrifugation followed by RNA isolation, cDNA synthesis, and quantitative RT-PCR as described previously. PBMC counts at each isolation were compared by paired t tests (6)

RNA samples which failed threshold quality requirements were not included in the PCR analysis and therefore in the statistical analyses below.

Statistics. Paired t-tests were used to compare pre- and post-treatment changes; two-sample t-tests (unequal variance) were used for comparisons between groups of subjects (i.e., 'unpaired' t-tests). Variables with right-skewed distributions were log transformed for t-tests and Pearson correlations. Log transformed variables were approximately normally distributed. The 10 gene

panel was originally identified by microarray analysis of CF PBMCs before and after antibiotic treatment (6). Predicted probabilities from each fitted logistic regression model were dichotomized using various cut-points for the probability. For each cut-point, performance statistics were calculated, and the cut-point achieving the highest accuracy was of special interest. ROC curves were constructed based on sensitivity and specificity values obtained for a range of cut-points between 0 and 1. The logistic regression model has the form:

$$\log[P(Y = 1) / P(Y = 0)] = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \dots + \beta_p x_p$$

where $Y=1$ denotes exacerbation state and $Y=0$ denotes resolution; x variables denote the predictors (e.g., FEV₁ plus 6 gene expression variables, for which $p=7$). Exponentiating β terms yields odds ratios which are listed in Table 3.

References:

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