CHEST Online Supplement

Persistent Airway Inflammation and Emphysema Progression on CT Scan in Ex-Smokers Observed for 4 Years

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e-Appendix 1.

MATERIAL AND METHODS

Longitudinal COPD-E study design

We have previously reported levels of biomarkers in sputum and BAL in a cross-sectional cohort of 16 subjects with a chest CT scan diagnosis of COPD-E who had quit smoking and 15 non-COPD control subjects as part of an NIH sponsored Biomarker study approved by the UCSD Human Subjects Protection Committee (# 040962)[S1]. This study is a longitudinal follow up study in 10/16 of the original cohort of COPD-E subjects who agreed to return for repeat assessments approximately four years after the baseline assessments to determine repeat levels of biomarkers in sputum, pulmonary function testing, cotinine levels, and assess evidence of chest CT scan progression of COPD-E.

None of the study subjects used oral corticosteroids during the six months prior to entering the study, and none had a history of upper or lower respiratory tract infection or the need for antibiotics in the six weeks prior to study. Study subjects were allowed to use short-acting bronchodilators, and were in a clinically stable condition.

COPD and control subjects

Baseline visits: The COPD-E study and control subjects were recruited as part of an NIH Biomarker study and their clinical characteristics, pulmonary function as well as sputum and BAL biomarkers are described in the manuscript and have also previously been described in detail [S1, S2].

Repeat visit 4 years later: We contacted all 16 COPD-E subjects approximately four years after their initial baseline assessments to have a return visit for sputum induction, blood cotinine level, pulmonary function, and chest CT scan in a protocol approved by the UCSD Human Subjects Protection Committee. Ten of the 16 COPD-E subjects consented for the repeat visit.

Chest CT scan

Each returning subject had a full-chest non-contrast helical CT scan during a single held inspiration, with 2.5 mm collimation, using the GE "standard" algorithm for reconstruction as previously described [S1] and reformatted to 10 mm slice thickness to enable comparability with the baseline scan. In co-operative study subjects, breath holding at full inspiration is the most reproducible lung volume, resulting in the lowest variation in lung density between scans [S3, S4]. All scans were done on a GE Light Speed scanner at 160 mA and 120 kVp, with water density calibrated daily. The % of voxels with density less than -910 HU exceeded 10% for subjects included in the COPD-E group[S1]. All CT scans were scored by an experienced chest CT scan radiologist (PJF)[S1, S4, S5]. All studies were done on a

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single scanner. The tube was changed 9 times from April 2001 to mid 2006. It was recalibrated each time, as well as having annual water calibrations. It was also calibrated against air daily. The CT number for a plastic phantom was stable at 125 Hounsfield units. The CTDI (vol) decreased from 1.87 to 1.77 mGy during this period.

Pulmonary function studies

All the study subjects had pulmonary function studies including spirometry (FEV_1 reported), lung volumes (by body plethysmography) as well as carbon monoxide single breath diffusion capacity (DLCO) and volume adjusted DLCO measurements according to ATS guidelines[S6]. Results of changes in lung function are all presented as % of control.

Cotinine levels

Serum cotinine levels were assayed in the UCSD clinical laboratory by gas chromatography as described[S7]. Serum cotinine levels < 10 ng/ml indicate no current use of nicotine containing products.

Sputum biomarkers

Sputum induction and processing was performed according to a standardized protocol [S8]. In brief, COPD-E or control subjects were exposed for 20 minutes to an aerosol of 3% hypertonic saline solution using a NOUVAG Ultrasonic nebulizer (Nouvag USa Inc, Lake Hughes, CA) and sputum was collected into 50-mL sterile ampoules. The volume of the induced sputum was determined, and an equal volume of dithiothreitol (0.1% in saline; Sigma Chemical Co, St Louis, Mo) was added. After homogenization, sputum samples were centrifuged at 2,000*g* for 5 minutes to separate the supernatants from the cell pellet. The supernatants were then aspirated and frozen at –80°C in separate aliquots for subsequent analysis.

Levels of selected sputum biomarkers including neutrophil biomarkers (MPO), neutrophil chemoattractants (IL-8, LTB_4), mononuclear cell chemoattractants (MCP-1), and metalloproteases (MMP-9) in samples were quantitated by ELISA. The detection limit of each assay is indicated in parentheses: MPO (1.6 ng/ml), IL-8 (31.2 pg/ml), LTB4 (6 pg/ml), MCP-1 (1.9 pg/ml), and MMP-9 (78.1 pg/ml). All the ELISA's were obtained from R&D Systems (Minneapolis, MN), other than the LTB4 ELISA which was obtained from Amersham (Piscataway, NJ).

RESULTS

Validation of sputum biomarker assays

We have performed experiments in which sputum was spiked with a known quantity of each cytokine or mediator (MPO, LTB4, IL-8, MCP-1, or MMP-9) and processed as for our regular sputum processing and assay. The results of the amount of spiked cytokine or mediator recovered, as well as the inter-assay and intra-assay variability are presented in Table A below.

Correlation between measurements of airway inflammation and changes on CT scan

The change in CT scan index (visit 1 to visit 2) was plotted against the change in sputum biomarker (visit 1 to visit 2) for MPO, LTB4, IL-8, MCP-1, and MMP-9. The median change in CT scan score per year in the COPD-E subjects over the 4 year period was 4.39 (range -0.43 to 7.88). There was a significant correlation between the change in sputum biomarker levels and change in CT scan score for MPO ($r=0.8$; $p<0.02$) and MCP-1 ($r=0.6$; $p<0.03$), but not for LTB4, IL-8, or MMP-9.

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We also investigated the following sputum mediator and CT correlations:

a) Baseline Sputum Mediator and Change in CT from baseline to year 4
   No significant correlation with MPO, LTB4, IL-8, MCP-1, or MMP-9.
b) Baseline Sputum Mediator and Baseline CT
   No significant correlation.
c) Baseline Sputum Mediator and Year 4 CT
   No significant correlation.
d) Year 4 Sputum Mediator and Baseline CT
   No significant correlation.
e) Year 4 Sputum Mediator and Year 4 CT
   No significant correlation.

Correlation between measurements of airway inflammation and changes in FEV1

The change in FEV1 (visit 1 to visit 2) was plotted against the change in sputum biomarker (visit 1 to visit 2) for MPO, LTB4, IL-8, MCP-1, and MMP-9. There was no significant correlation between the change in FEV1 and any of these sputum biomarkers.

We also performed the following correlations for each of the 5 individual sputum biomarkers (MPO, LTB4, IL-8, MCP-1, MMP-9) with baseline or year 4 FEV1.

a) Baseline Sputum Mediator and Change in FEV1 from baseline to year 4
   MMP-9 (r= 0.71; p=0.02)
   No significant correlation with MPO, LTB4, IL-8, or MCP-1.
b) Baseline Sputum Mediator and Baseline FEV1
   No significant correlation.
c) Baseline Sputum Mediator and Year 4 FEV1
   No significant correlation.
d) Year 4 Sputum Mediator and Baseline FEV1
   No significant correlation.
e) Year 4 Sputum Mediator and Year 4 FEV1
   No significant correlation.

Correlation between measurements of airway inflammation and changes in DLCO

The annual change in volume adjusted DLCO (i.e. KCO) over 4 years was plotted against the change in sputum biomarker (visit 1 to visit 2) for MPO, LTB4, IL-8, MCP-1, and MMP-9. There was a significant correlation between the change in sputum biomarker levels and change in volume adjusted DLCO for MCP-1 (r= -0.98; p=0.0001), but not for MPO, LTB4, IL-8, or MMP-9.

We also performed the following correlations for each of the 5 individual sputum biomarkers (MPO, LTB4, IL-8, MCP-1, MMP-9) with baseline or year 4 volume adjusted DLCO.

a) Baseline Sputum Mediator and Change in volume adjusted DLCO from baseline to year 4
   MPO (r= 0.86; p=0.02)
   MCP-1 (r= 0.69; p=0.05)
   No significant correlation with LTB4, IL-8, or MMP-9.
b) Baseline Sputum Mediator and Baseline volume adjusted DLCO
   No significant correlation.
c) Baseline Sputum Mediator and Year 4 volume adjusted DLCO

No significant correlation.

d) Year 4 Sputum Mediator and Baseline volume adjusted DLCO
No significant correlation.

e) Year 4 Sputum Mediator and Year 4 volume adjusted DLCO
MCP-1 (r= 0.90; p=0.01)
No significant correlation with MPO, LTB4, IL-8, or MMP-9

The non-volume adjusted DLCO only showed a significant correlation between year 4 DLCO and the change in sputum MCP-1 from visit 1 to visit 2 (r=0.90, p=0.01). There was no correlation of DLCO with any of the other above biomarkers.

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e-Table 1. Cytokine spiking assays.

Assay	Spiked cytokine recovery (%)	Inter-assay variability (%)	Intra-assay variability (%)
MPO	96.8	11.2	6.8
LTB4	97.6	12.7	6.5
IL-8	95.3	11.0	7.7
MCP-1	98.3	14.7	9.6
MMP-9	96.3	5.3	6.2