

ORIGINAL ARTICLE

## Decreased Histone Deacetylase Activity in Chronic Obstructive Pulmonary Disease

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### ABSTRACT

#### BACKGROUND

Chronic obstructive pulmonary disease (COPD) is characterized by chronic airway inflammation that is greater in patients with advanced disease. We asked whether there is a link between the severity of disease and the reduction in histone deacetylase (HDAC) activity in the peripheral lung tissue of patients with COPD of varying severity. HDAC is a key molecule in the repression of production of proinflammatory cytokines in alveolar macrophages.

#### METHODS

HDAC activity and histone acetyltransferase (HAT) activity were determined in nuclear extracts of specimens of surgically resected lung tissue from nonsmokers without COPD, patients with COPD of varying severity, and patients with pneumonia or cystic fibrosis. Alveolar macrophages from nonsmokers, smokers, and patients with COPD and bronchial-biopsy specimens from nonsmokers, healthy smokers, patients with COPD, and those with mild asthma were also examined. Total RNA extracted from lung tissue and macrophages was used for quantitative reverse-transcriptase–polymerase-chain-reaction assay of HDAC1 through HDAC8 and interleukin-8. Expression of HDAC2 protein was quantified with the use of Western blotting. Histone-4 acetylation at the interleukin-8 promoter was evaluated with the use of a chromatin immunoprecipitation assay.

#### RESULTS

Specimens of lung tissue obtained from patients with increasing clinical stages of COPD had graded reductions in HDAC activity and increases in interleukin-8 messenger RNA (mRNA) and histone-4 acetylation at the interleukin-8 promoter. The mRNA expression of HDAC2, HDAC5, and HDAC8 and expression of the HDAC2 protein were also lower in patients with increasing severity of disease. HDAC activity was decreased in patients with COPD, as compared with normal subjects, in both the macrophages and biopsy specimens, with no changes in HAT activity, whereas HAT activity was increased in biopsy specimens obtained from patients with asthma. Neither HAT activity nor HDAC activity was changed in lung tissue from patients with cystic fibrosis or pneumonia.

#### CONCLUSIONS

Patients with COPD have a progressive reduction in total HDAC activity that reflects the severity of the disease.

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**T**HE GLOBAL BURDEN OF CHRONIC OBSTRUCTIVE pulmonary disease (COPD) — a common and debilitating chronic inflammatory disease that is characterized by the progressive development of airflow limitation and is poorly reversible — is increasing.<sup>1</sup> Cigarette smoking is strongly linked with the ongoing inflammation in the airways and lung parenchyma, and the severity of airflow limitation is correlated with the degree of pulmonary inflammation.<sup>2,3</sup>

The inflammatory processes in COPD are complex.<sup>4</sup> Neutrophil chemotactic mediators, such as interleukin-8 and leukotriene B<sub>4</sub>, and proinflammatory cytokines, such as tumor necrosis factor  $\alpha$ , are increased in the sputum of patients with COPD, as compared with that of normal subjects.<sup>5</sup> Histone acetyltransferase (HAT) and histone deacetylase (HDAC) are families of nuclear enzymes that modify the expression of inflammatory genes by regulating chromatin structure.<sup>6</sup> Acetylation of the core histones by transcriptional coactivator proteins, which possess intrinsic HAT activity, leads to changes in the chromatin structure that subsequently allow the transcription factors and RNA polymerase II to bind to DNA and enhance gene transcription. Conversely, deacetylation of the core histones is generally associated with the repression of transcription.

We have previously shown that HDAC is a key molecule in the repression of production of proinflammatory cytokines in alveolar macrophages<sup>7</sup>; thus, a decrease in the HDAC could be associated with enhanced inflammation in COPD.<sup>8</sup> The present study was designed to test the hypothesis that the magnitude of the inflammatory response in the peripheral lung that has been described in COPD is associated with a decrease in HDAC activity.

## METHODS

### PATIENTS

We followed the guidelines for grading disease severity in COPD of the Global Initiative for Obstructive Lung Disease<sup>9</sup>: stage 0 denotes normal measurements on spirometry with chronic symptoms (i.e., cough and sputum); stage 1 denotes mild disease, with a ratio of forced expiratory volume in one second (FEV<sub>1</sub>) to forced vital capacity (FVC) of less than 70 percent and an FEV<sub>1</sub> of more than 80 percent of the predicted value with or without symptoms (i.e., cough and sputum); stage 2 denotes moderate disease, with an FEV<sub>1</sub>:FVC ratio of less than 70 percent and an FEV<sub>1</sub> of 80 to 50 percent of the predicted value with or without symptoms (i.e.,

cough, sputum, and dyspnea); stage 3 denotes severe disease, with an FEV<sub>1</sub> of 50 to 30 percent of the predicted value; and stage 4 denotes very severe disease, with an FEV<sub>1</sub> of less than 30 percent of the predicted value.

We obtained specimens of lung tissue and data on patients' lung function from a tissue bank that was linked to an established patient registry.<sup>10</sup> Specimens of peripheral lung tissue were obtained from 11 patients who were nonsmokers without symptoms who had normal lung function and from 29 patients who were smokers: 9 with stage 0 COPD, 10 with stage 1 COPD, and 10 with stage 2 COPD. Specimens of peripheral lung tissue were obtained from an additional six patients with stage 4 COPD who were undergoing lung-volume-reduction surgery. The patient registry and the tissue bank also provided specimens of peripheral lung tissue from five patients who had a tumor that had produced obstructive pneumonia, as well as from five explanted lungs of patients with cystic fibrosis. Baseline characteristics of the patients are summarized in Table 1.

Alveolar macrophages were obtained by bronchoalveolar lavage from six healthy nonsmokers, six healthy current smokers, and seven patients with stage 2 or 3 COPD (Table 2). Bronchial-biopsy specimens were collected from 14 normal subjects who were nonsmokers, 10 patients with mild asthma and 13 age-matched subjects who were smokers, and 7 patients with COPD (stage 2 or 3) and 10 age-matched smokers (Table 2). Normal healthy subjects for bronchoscopy studies were volunteers recruited through advertisement. Although these samples were not specifically collected for this study, our study was part of a project that examined the molecular mechanism of inflammation in COPD, and it was approved by the local research ethics committees. All subjects had provided written informed consent for the deposition of their tissues in the tissue bank from which we obtained the specimens and for their use in research studies of this type.

### CELL PREPARATION AND ASSAYS

Bronchoalveolar-lavage fluid was collected as previously described.<sup>11</sup> Cells from the fluid were centrifuged (at 500×g for 10 minutes) and washed twice with Hanks' salt solution. Cell viability was assessed with the use of the trypan-blue exclusion method. Macrophages from the bronchoalveolar-lavage fluid were separated by means of adhesion to plastic and identified as previously described.<sup>11</sup> In these experiments, the mean ( $\pm$ SE) viability of

the macrophages was  $66.3 \pm 4.7$  percent (range, 30 to 95) and the mean purity was  $93.4 \pm 1.3$  percent (range, 83 to 98).

#### Preparation of Cell Extracts

Tissue specimens (three pieces approximately 0.5 cm by 0.5 cm by 0.5 cm in size) and biopsy specimens (two pieces approximately 1 mm by 1 mm by 3 mm in size) were ground under liquid nitrogen with the use of a pestle and mortar. Hypotonic buffer (10 mM HEPES–sodium hydroxide, pH 7.9, 1.5 mM magnesium chloride, 10 mM potassium chloride, 10 mM 2-mercaptoethanol, and a commercially available mixture of protease inhibitors [Complete Protease-Inhibitor Cocktail Tablets, Roche Diagnostics]) were added to the sample to remove contaminating red cells and secretions and to loosen the cell membranes. The sample was then left on ice for 15 minutes. NP-40 solution (Sigma), a nonionic detergent, was added to reach a 0.5 percent final concentration, and the samples were vortexed to isolate nuclei by lysing the cell membranes. The samples were microcentrifuged (at 3000 rpm for 1 minute), to remove the larger debris, and the supernatants were then microcen-

trifuged (at 14,000 rpm for 30 seconds), to obtain the nuclear-rich fraction. The nuclei of alveolar macrophages were prepared by means of suspension with mild lysis buffer on ice for 10 minutes.<sup>12</sup> Nuclear proteins were extracted as previously described.<sup>13</sup> The protein concentration of each sample was measured (Bradford Bio-Rad Protein Assay kit, Bio-Rad), with bovine serum albumin used as a standard.

#### HDAC Activity and HAT Activity

HDAC activity and HAT activity were measured with the use of a nonisotopic assay that used a fluorescent derivative of epsilon-acetyl lysine (HDAC Fluorescent Activity Assay Kit, Biomol) and an enzyme immunosorbent assay (HAT Activity Assay Kit, Upstate Biotechnology) to detect antiacetylated lysine antibody on synthesized histone-4 partial peptide. The results are expressed as micromolar values of the provided standard per microgram of protein.

#### Quantitative Reverse-Transcriptase–Polymerase-Chain-Reaction Assay

Total RNA was extracted from approximately  $1 \times 10^6$  cells or from two specimens of lung tissue  $0.3 \text{ cm}^3$

**Table 1. Characteristics of the Study Subjects.\***

| Characteristic                                    | Nonsmokers<br>(N=11) | Patients with COPD |                   |                   |                  | Pneumonia<br>(N=5) | Cystic<br>Fibrosis<br>(N=5) |
|---|----------------------|--------------------|-------------------|-------------------|------------------|--------------------|-----------------------------|
|   |                      | Stage 0<br>(N=9)   | Stage 1<br>(N=10) | Stage 2<br>(N=10) | Stage 4<br>(N=6) |                    |                             |
| Age (yr)  | 52.2±4.9             | 66.0±3.3           | 63.9±3.1          | 63.0±3.6          | 57.3±2.8         | 62.4±5.1           | 30.0±4.0                    |
| Sex (M/F)   | 3/8                  | 7/2                | 6/4               | 7/3               | 3/3              | 3/2                | 2/3                         |
| FEV <sub>1</sub> (% of predicted normal value)    | 96.6±4.3             | 93.9±4.3           | 87.2±1.9          | 6.5±2.0           | 24.4±3.0         | 74.8±5.2           | ND                          |
| FEV <sub>1</sub> :FVC (%)                         | 82.4±0.9             | 75.2±1.8           | 64.6±1.4          | 62.0±1.6          | 41.7±3.7         | 67.8±4.7           | ND                          |
| K <sub>CO</sub> (% of predicted normal value)     | 79.3±3.2             | 71.0±9.5           | 69.9±9.4          | 83.9±5.7          | 51.8±9.2         | ND                 | ND                          |
| Smoking (pack-years)                              | 0                    | 55.5±13.2          | 49.7±11.2         | 58.0±14.7         | 42.0±9.0         | 9.5±3.4            | 0                           |
| Former smoker (no.)                               | 0                    | 2                  | 3                 | 3                 | 6                | 2                  | 0                           |
| Medication (no.)                                  |                      |                    |                   |                   |                  | ND                 | ND                          |
| Oral corticosteroid                               | 0                    | 1                  | 0                 | 0                 | 1                |                    |                             |
| Inhaled corticosteroid                            | 0                    | 0                  | 1                 | 0                 | 3                |                    |                             |
| Short-acting beta-adrenergic–<br>receptor agonist | 0                    | 0                  | 1                 | 2                 | 6                |                    |                             |
| Theophylline                                      | 0                    | 0                  | 0                 | 0                 | 0                |                    |                             |

\* COPD denotes chronic obstructive pulmonary disease, FEV<sub>1</sub> forced expiratory volume in one second, FVC forced vital capacity, K<sub>CO</sub> carbon monoxide gas transfer corrected for alveolar volume, pack-year the number of cigarettes smoked per day, or 20 cigarettes, multiplied by the number of years of smoking, and ND not determined. Stages 0 through 4 denote severity of disease according to the grading of the Global Initiative for Obstructive Lung Disease,<sup>9</sup> with higher numbers indicating greater severity. Plus–minus values are means ±SE.

in size with the use of an RNeasy kit (Qiagen). Reverse transcription was performed with the use of an Omniscript RT Kit (Qiagen). Gene-transcript levels of HDAC1 through HDAC8, housekeeping (unchanging) genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and interleukin-8 were quantified by real-time polymerase chain reaction (PCR) with the use of a QuantiTect SYBR Green PCR kit (Qiagen) on a Rotor-Gene 3000 (Corbett Research). Variation in the amount of transcript in different samples was corrected for by GAPDH expression. Sequences of the primer pairs used for the PCR to identify HDAC1 through HDAC8, interleukin-8, and GAPDH are given in Supplement 1 of the Supplementary Appendix (available with the full text of this article at [www.nejm.org](http://www.nejm.org)).

#### Chromatin Immunoprecipitation Assay

Samples of lung tissue (three pieces approximately 0.5 cm by 0.5 cm by 0.5 cm in size) were immersed in a 3 percent solution of formaldehyde for one hour in order to fix protein-DNA complexes. The tissue was cut into small pieces and homogenized in a Dounce homogenizer. After removing debris with

the use of centrifugation (at 3000 rpm for 30 seconds), chromatin immunoprecipitation was performed with the use of antiacetylated histone-4 antibody and a chromatin immunoprecipitation assay kit (Upstate Biotechnology). Quantitative PCR was performed (QuantiTect SYBR Green PCR kit, Qiagen). Primer pairs for the nuclear factor- $\kappa$ B (NF- $\kappa$ B) binding site in the interleukin-8 promoter were 5GGCCATCAGTTGCAAATC3 (forward primer) and 5TTCCTCCGGTGGTTTCTTC3 (reverse primer). Variation in the amount of PCR product in the samples of immunoprecipitated DNA was corrected for by measuring total DNA before immunoprecipitation was performed.

#### Western Blotting

Tissue extracts were analyzed by sodium dodecyl sulfate-polyacrylamide-gel electrophoresis and Western blot analysis with the use of an immunoblot apparatus (XCell SureLock Mini-Cell and Blot Module Kit, Invitrogen). Immunoreactive bands were detected by enhanced chemiluminescence (ECL solution, Amersham Biosciences [GE Healthcare]) with the use of specific antibodies (Santa Cruz Biotechnology) as previously described.<sup>7</sup> Vari-

**Table 2. Characteristics of Subjects Who Provided Specimens of Alveolar Macrophages and Underwent Bronchial Biopsy.\***

| Characteristic                                 | COPD<br>(Stage 2 or 3) | Smokers<br>(Group 1) | Nonsmokers | Smokers<br>(Group 2) | Mild Asthma |
|--|------------------------|----------------------|------------|----------------------|-------------|
| <b>Alveolar macrophages</b>                    |                        |                      |            |                      |             |
| No. of patients                                | 7                      | 6                    | 6          |                      |             |
| Age (yr)                                       | 65.9±4.0               | 53.8±6.8             | 52.2±6.4   |                      |             |
| Sex (M/F)                                      | 4/3                    | 2/4                  | 3/3        |                      |             |
| FEV <sub>1</sub> (% of predicted normal value) | 60.5±4.3               | 87.1±4.6             | 106.2±6.9  |                      |             |
| FEV <sub>1</sub> :FVC (%)                      | 59.7±2.8               | 76.4±0.9             | 84.2±1.5   |                      |             |
| Smoking (pack-years)                           | 39.3±6.4               | 29.5±6.6             | 0          |                      |             |
| Former smoker (no.)                            | 3                      | 0                    | 0          |                      |             |
| <b>Bronchial biopsy</b>                        |                        |                      |            |                      |             |
| No. of patients                                | 7                      | 10                   | 14         | 13                   | 10          |
| Age (yr)                                       | 71.9±1.2               | 70.1±1.9             | 28.5±2.5   | 29.0±1.6             | 35.0±3.2    |
| Sex (M/F)                                      | 7/0                    | 8/2                  | 9/5        | 6/7                  | 6/4         |
| FEV <sub>1</sub> (% of predicted normal value) | 70.1±5.0               | 102.0±6.8            | 103.4±2.9  | 98.8±2.1             | 92.4±4.5    |
| FEV <sub>1</sub> :FVC (%)                      | 61.6±2.2               | 77.2±1.3             | 95.2±2.4   | 97.2±1.3             | 80.3±4.3    |
| Smoking (pack-years)                           | 40.1±12.2              | 54.0±9.8             | 0          | 12.3±1.8             | 0           |
| Former smoker (no.)                            | 1                      | 0                    | 0          | 0                    | 0           |

\* Patients in Group 1 who were smokers were age-matched with patients with COPD, and patients in Group 2 who were smokers were age-matched with patients with asthma. FEV<sub>1</sub> denotes forced expiratory volume in one second, FVC forced vital capacity, and pack-year the number of cigarettes smoked per day, or 20 cigarettes, multiplied by the number of years of smoking. Plus-minus values are means ±SE.

ation of the band density of HDAC2 in different samples was corrected for with use of the band density of the nuclear membrane proteins lamin A and lamin C (lamin A/C) or the total DNA content in the tissue sample.

#### STATISTICAL ANALYSIS

Results are expressed as means  $\pm$ SE. Other measures (medians and standard deviations) are provided in the Supplementary Appendix. Analysis of variance was performed with the use of the non-parametric Kruskal–Wallis test. When the result was significant, the Mann–Whitney U test was performed for comparisons between groups (SPSS software). Correlation coefficients were calculated with the use of Spearman's rank method. A P value of less than 0.05 was considered to indicate statistical significance. All reported P values are two-sided.

## RESULTS

#### PERIPHERAL LUNG TISSUE

Levels of interleukin-8 messenger RNA (mRNA), normalized according to the amount of GAPDH, were higher in samples of peripheral lung tissue from patients with increasing severity of COPD than in samples from nonsmokers. There were no significant differences in the levels of interleukin-8 mRNA between samples from patients with stage 0 disease ( $0.45\pm 0.24$ ) or stage 1 disease ( $0.36\pm 0.17$ ), and samples from nonsmokers ( $0.12\pm 0.03$ ); however, the differences were significant between samples from nonsmokers and those from patients with stage 2 disease ( $0.59\pm 0.14$ ,  $P=0.005$ ) or stage 4 disease ( $0.86\pm 0.13$ ,  $P=0.001$ ) (Fig. 1A). A similar trend was noted in the degree of histone-4 acetylation at the NF- $\kappa$ B binding site of the interleukin-8 promoter, and these differences reached significance in samples from patients with stage 2 disease (ratio to total immunoprecipitated DNA,  $0.0014\pm 0.00034$ ) and patients with stage 4 disease (ratio to total immunoprecipitated DNA,  $0.0019\pm 0.00044$ ), as compared with those from nonsmokers (ratio to total immunoprecipitated DNA,  $0.00020\pm 0.00008$ ) (Fig. 1A). Expression of interleukin-8 mRNA and of histone-4 acetylation in the interleukin-8 promoter was correlated with increasing clinical severity of disease (stages 0, 1, 2, and 4) in the Spearman correlation analysis ( $P=0.05$  and  $P=0.01$ , respectively).

Total HDAC activity (expressed as micromolar values of the deacetylated HDAC substrate standard per microgram of protein) in samples of lung tissue

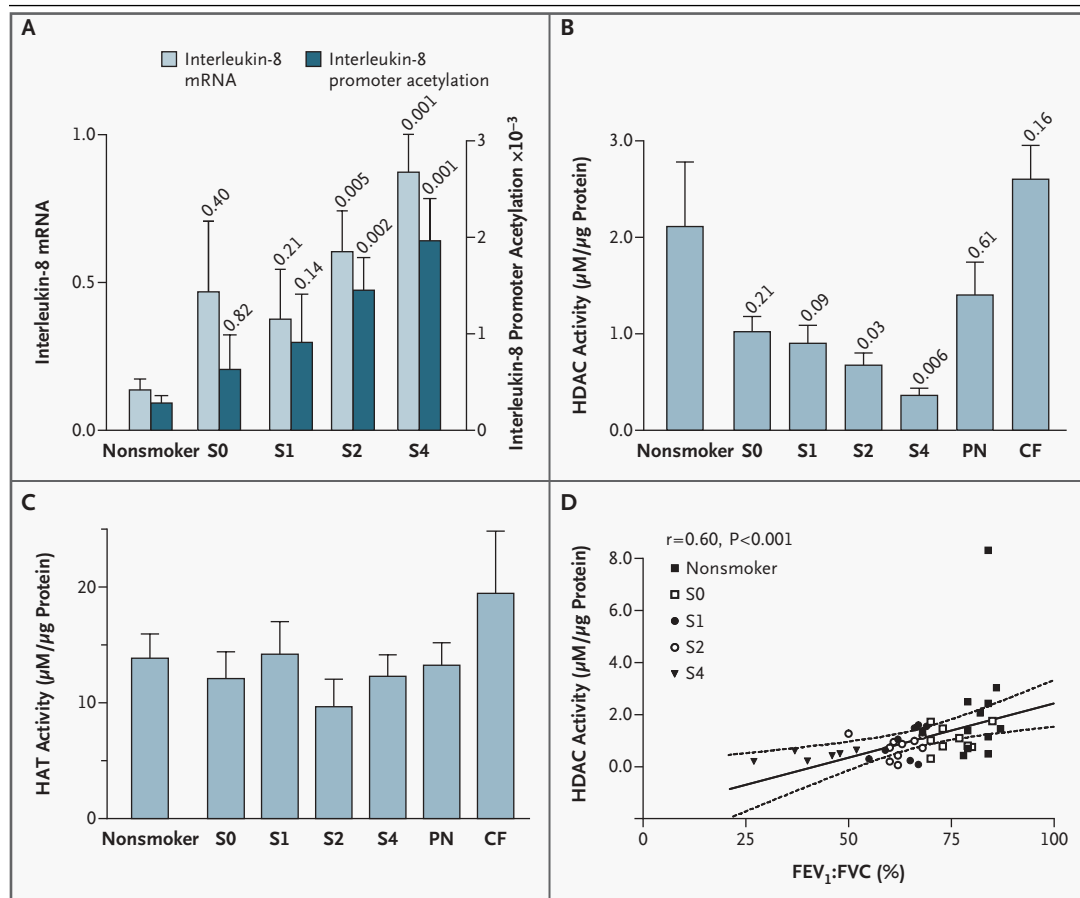
from nonsmokers was  $2.1\pm 0.67$   $\mu$ M of the HDAC standard, and the activity was reduced in patients with more advanced stages of COPD; as compared with the HDAC activity in samples from nonsmokers, this difference reached statistical significance in samples from patients with stage 2 disease (HDAC activity,  $0.67\pm 0.13$   $\mu$ M,  $P=0.03$ ) and patients with stage 4 disease (HDAC activity,  $0.36\pm 0.077$   $\mu$ M,  $P=0.006$ ) (Fig. 1B). Furthermore, in samples from patients with stage 4 disease, the level of HDAC activity was significantly lower than that among patients with stage 0 disease (HDAC activity,  $1.0\pm 0.16$   $\mu$ M,  $P=0.007$  for the comparison between patients with stage 0 disease and those with stage 4 disease). By contrast, there was no difference in total tissue HAT activity among these groups according to analysis of variance ( $P=0.46$ ) (Fig. 1C). Samples of lung tissue obtained from patients with pneumonia and patients with cystic fibrosis did not show any significant difference in HDAC activity or HAT activity from activity in samples from healthy nonsmokers. (These data are shown in Supplement 2 of the Supplementary Appendix.)

HDAC activity was significantly correlated with FEV<sub>1</sub> ( $r=0.45$ ,  $P=0.02$ ), the FEV<sub>1</sub>:FVC ratio ( $r=0.60$ ,  $P<0.001$ ) (Fig. 1D), histone-4 acetylation ( $r=-0.61$ ,  $P<0.001$ ), and interleukin-8 gene expression ( $r=-0.46$ ,  $P=0.01$ ) when all subjects were included. Significant correlations were also seen in samples from patients with COPD (stages 0, 1, 2, and 4) between HDAC activity and FEV<sub>1</sub> ( $r=0.43$ ,  $P=0.001$ ), FEV<sub>1</sub>:FVC ratio ( $r=0.55$ ,  $P<0.001$ ), histone-4 acetylation ( $r=-0.51$ ,  $P=0.003$ ), and interleukin-8 gene expression ( $r=-0.41$ ,  $P=0.02$ ). However, there was no correlation of HDAC activity with age ( $P=0.06$  for all subjects, and  $P=0.1$  for patients with COPD) or with carbon monoxide diffusion corrected for alveolar volume ( $K_{CO}$ ) ( $P=0.08$  for all subjects, and  $P=0.15$  for patients with COPD). There was no significant correlation between HDAC activity and exposure to cigarettes measured in pack-years ( $P=0.69$ ) among patients with COPD (stages 0, 1, 2, and 4). (These data are summarized in Supplement 3 of the Supplementary Appendix, and individual figures for correlation are given in Supplement 4 of the Supplementary Appendix.)

We next determined whether reduced HDAC activity was a result of attenuated expression of the genes for specific HDAC isoforms or a global suppression of all isoforms. The relative expression of HDAC2 mRNA normalized for GAPDH was reduced in samples of lung tissue from patients with more

severe COPD. The significant reduction in the level of HDAC mRNA was found in tissue from patients with stage 2 disease ( $0.024 \pm 0.0075$ , 35 percent of the value in nonsmokers), as compared with samples from nonsmokers ( $0.068 \pm 0.017$ ,  $P=0.03$ ). A further significant reduction was seen in samples from patients with stage 4 disease ( $0.016 \pm 0.0080$ , 24 percent of the value in nonsmokers), as compared with samples from nonsmokers ( $P=0.01$ ) and from patients with stage 0 disease ( $0.048 \pm 0.0095$ ,  $P=0.02$ ) (Fig. 2A). The HDAC5 and HDAC8 mRNA

expression was also significantly reduced in samples from patients with stage 2 disease and those with stage 4 disease, as compared with levels in those from nonsmokers or from patients with stage 0 disease, whereas the expression of HDAC1, 3, 4, 6, and 7 was not different (Fig. 2A). When HDAC2 mRNA expression was compared with large ribosomal protein expression instead of expression of GAPDH, the results were similar (the data are provided in Supplement 5 of the Supplementary Appendix).

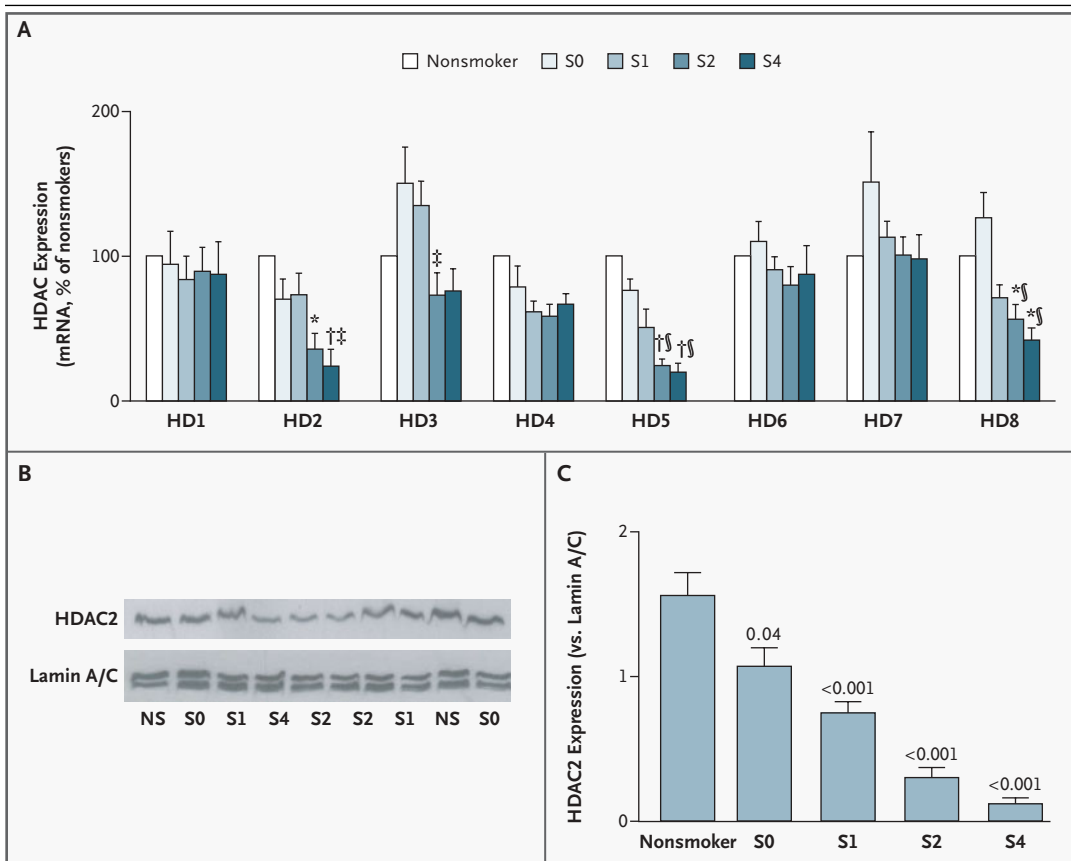


**Figure 1. Interleukin-8 Gene Expression and Histone Deacetylase (HDAC) in Peripheral Lung Tissue from Patients with COPD.**

Panel A shows interleukin-8 messenger RNA (mRNA) expression and histone-4 acetylation at the interleukin-8 promoter. Panel B shows HDAC activity. Panel C shows histone acetyltransferase (HAT) activity. The data were plotted as means  $\pm$  SE, with the number of subjects in each group ranging from 5 to 11. (The complete data are given in Supplements 1, 2, and 3 of the Supplementary Appendix.) Panel D shows the correlations between HDAC activity and the ratio of forced expiratory volume in one second ( $FEV_1$ ) to forced vital capacity (FVC) in specimens obtained from normal nonsmokers and from patients with COPD at different stages. The dotted line indicates the 95 percent confidence interval of the regression line. When the single outlier point shown at the top right in this panel was omitted, there was still a significant positive correlation between HDAC activity and  $FEV_1:FVC$  ( $r=0.60$ ,  $P<0.001$ ). S0, S1, S2, and S4 denote severity of disease according to the grading of the Global Initiative for Obstructive Lung Disease, with higher numbers indicating greater severity; PN pneumonia; and CF cystic fibrosis.

Levels of HDAC2 protein measured by Western blotting and normalized to the lamin A/C proteins were also related to the clinical stage of COPD. There was a graded reduction in HDAC2 expression in samples from nonsmokers ( $1.60 \pm 0.16$ ) and patients with stage 0 disease ( $1.10 \pm 0.13$ ), stage 1 disease ( $0.750 \pm 0.077$ ), and stage 2 disease ( $0.300 \pm 0.073$ ), reaching a maximal reduction of 92 percent in the HDAC2 protein levels in samples from patients with stage 4 disease ( $0.120 \pm 0.042$ ;  $P=0.001$  for the comparison with nonsmokers and for the comparison with patients with stage 0 disease) (Fig. 2B and 2C and Supplement 2 of the Sup-

plementary Appendix). When normalized according to DNA content instead of lamin A/C, HDAC2 expression also decreased with the increasing severity of COPD (in samples from nonsmokers,  $250 \pm 26$  band density per microgram of DNA; in those from patients with stage 0 disease,  $173 \pm 26$  [ $P=0.06$ ] for the comparison with nonsmokers; in those from patients with stage 1 disease,  $147 \pm 27$  [ $P=0.04$ ]; in those from patients with stage 2 disease,  $110 \pm 33$  [ $P=0.004$ ]; in those from patients with stage 4 disease,  $76 \pm 14$  [ $P=0.001$ ]), indicating that these changes cannot be explained by loss of lung tissue as a result of emphysema.



**Figure 2. Histone Deacetylase (HDAC) Expression in Peripheral Lung Tissue.**

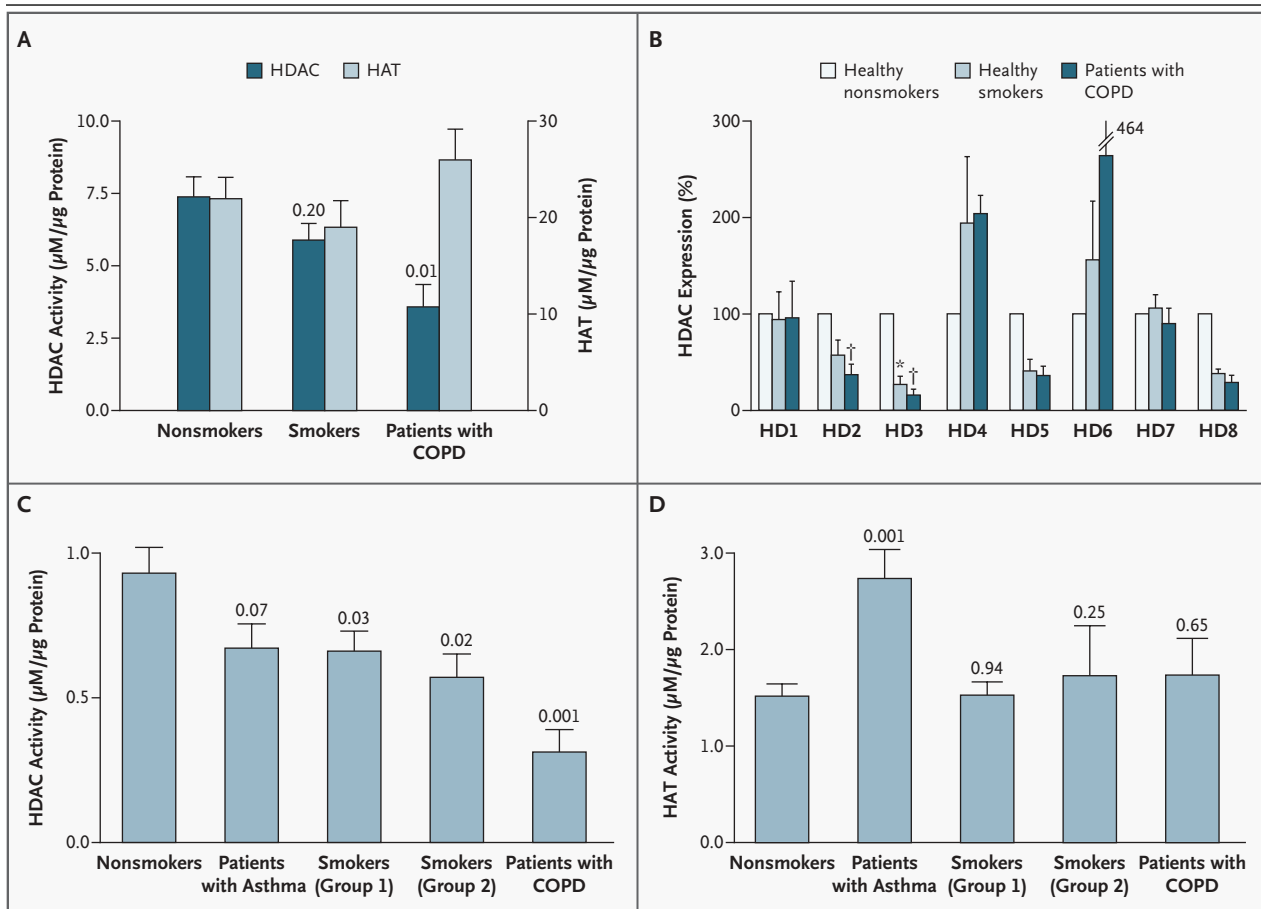
Panel A shows the expression of histone deacetylase (HD) genes 1 through 8 with the use of real-time PCR. Panel B shows HDAC2 protein expression with the use of Western blotting and lamin A and lamin C (lamin A/C) nuclear membrane proteins as an internal control for nuclear-protein loading. Panel C shows the densitometric analysis of HDAC2 protein expression in samples of peripheral lung tissue from healthy nonsmokers (NS) and from patients with COPD stage 0 (S0), stage 1 (S1), stage 2 (S2), and stage 4 (S4). The results are plotted as means  $\pm$  SE, with the number of subjects in each group ranging from 6 to 11. In Panel A, there were significant differences (asterisks indicate  $P < 0.05$  and daggers  $P < 0.01$  for the comparison with nonsmokers, and double daggers  $P < 0.05$  and section marks  $P < 0.01$  for the comparison with patients with COPD stage 0). In Panel C, P values for the comparison with healthy nonsmokers are shown above each bar. (The complete data and P values for all comparisons are given in Supplements 2 and 5 of the Supplementary Appendix.)

**ALVEOLAR MACROPHAGES**

Total HDAC activity in alveolar macrophages obtained with the use of bronchoalveolar lavage from subjects who were smokers ( $5.90 \pm 0.58 \mu\text{M}$ ) was slightly but not significantly decreased, as compared with levels in samples from nonsmokers ( $7.40 \pm 0.69 \mu\text{M}$ ). There was a significant reduction in HDAC activity in macrophages from patients with COPD stages 2 and 3 ( $3.60 \pm 0.77 \mu\text{M}$ ,  $P=0.01$ ), as compared with activity in those from nonsmokers ( $P=0.01$ ), but not as compared with activity in those from healthy smokers ( $P=0.20$ ) (Fig. 3A).

There was no evidence of a difference in total HAT activity in alveolar macrophages of patients with COPD as compared with normal smokers and nonsmokers ( $P=0.33$ ) (Fig. 3A and Supplement 6 of the Supplementary Appendix).

HDAC2 mRNA expression was reduced in alveolar macrophages from patients with COPD ( $0.00030 \pm 0.00016$ , corresponding to 38 percent of the expression in samples from nonsmokers [ $0.00080 \pm 0.00016$ ];  $P=0.01$ ) but not in samples from healthy smokers ( $0.00050 \pm 0.00013$ , corresponding to 63 percent of the expression in sam-



**Figure 3. Histone Deacetylase (HDAC) and Histone Acetyltransferase (HAT) Activity in Alveolar Macrophages and Bronchial-Biopsy Specimens.**

Panel A shows HDAC activity and HAT activity. Panel B shows expression of HDAC (HD) 1 through 8 mRNA in alveolar macrophages. Panel C shows HDAC activity. Panel D shows HAT activity in bronchial-biopsy specimens obtained from healthy nonsmokers, healthy smokers (those in group 1 are younger than those in group 2), and patients with COPD and those with mild asthma that had not previously been treated with steroids. The results are plotted as means  $\pm$ SE, with the number of subjects in each group ranging from 6 to 14. In Panels A, C, and D, P values for the comparison with nonsmokers are shown above each bar. In Panel B, the significant differences are shown by the symbols above the bars (the asterisk indicates  $P<0.05$  and the daggers  $P<0.01$  for the comparison with nonsmokers). (The complete data and P values for all comparisons are given in Supplements 6, 7, and 8 of the Supplementary Appendix.)



ples from nonsmokers;  $P=0.11$ ) (Fig. 3B). HDAC3 mRNA expression was also significantly reduced in samples from healthy smokers ( $P=0.02$ ) and those from patients with COPD ( $P=0.004$ ), as compared with expression in samples from nonsmokers (Fig. 3B). Despite a tendency toward lower levels of HDAC5 mRNA and HDAC8 mRNA, there was no significant difference among all subject groups (according to the Kruskal–Wallis analysis of variance  $P=0.13$  and  $P=0.67$ , respectively). (The complete data and  $P$  values are summarized in Supplement 7 of the Supplementary Appendix.)

#### BRONCHIAL BIOPSIES

To see whether the changes in HDAC activity and expression were specific to COPD or reflected some process related to airway obstruction, we compared HAT activity and HDAC activity in bronchial-biopsy specimens from patients with COPD and from patients with asthma. Because the age of populations with asthma and those with COPD differ, we also studied healthy smokers who were matched for age with patients with asthma and those with COPD (Table 2). Total HDAC activity in biopsy specimens from patients with mild asthma ( $0.670\pm 0.085\ \mu\text{M}$ ) and from age-matched healthy smokers ( $0.660\pm 0.069\ \mu\text{M}$ ) showed a slight reduction as compared with activity in biopsy specimens from healthy nonsmokers ( $0.930\pm 0.090\ \mu\text{M}$ ). Specimens from patients with COPD ( $0.310\pm 0.079\ \mu\text{M}$ ) and from age-matched smokers ( $0.570\pm 0.083\ \mu\text{M}$ ) showed a significant further reduction in HDAC activity, as compared with activity in those from healthy nonsmokers ( $0.930\pm 0.089\ \mu\text{M}$ ) (Fig. 3C). HDAC activity in specimens from patients with COPD was also significantly less than in specimens from age-matched smokers ( $P=0.04$ ) and from patients with asthma ( $P=0.01$ ). There was no significant difference ( $P=0.52$ ) between HDAC activity in specimens from younger smokers ( $0.660\pm 0.069\ \mu\text{M}$ ) and those from older smokers ( $0.570\pm 0.083\ \mu\text{M}$ ). By contrast, specimens from patients with asthma showed a significant increase in HAT activity ( $27.4\pm 3.0\ \mu\text{M}$ ,  $P=0.001$ ), as compared with those from nonsmokers ( $15.2\pm 1.3\ \mu\text{M}$ ), whereas specimens from patients with COPD ( $17.4\pm 3.8\ \mu\text{M}$ ) showed no change in HAT activity (Fig. 3D).

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#### DISCUSSION

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Our data show that total HDAC activity is decreased in samples of peripheral lung tissue, alveolar mac-

rophages, and bronchial-biopsy specimens from patients with COPD, as compared with activity in age-matched healthy nonsmokers. Increases in histone acetylation are usually associated with gene induction.<sup>6,7</sup> The overall acetylation status of histones depends on the dynamic equilibrium between HAT activity and HDAC activity. Our current data show that interleukin-8 mRNA and histone-4 acetylation at the NF- $\kappa$ B binding site of the interleukin-8 promoter were increased in samples of peripheral lung tissue from patients with COPD. HDAC activity is decreased, despite little change in HAT activity, among these patients. There was a positive correlation between histone-4 acetylation and HDAC activity ( $P<0.001$ ), indicating that the balance between HAT activity and HDAC activity is shifted toward histone hyperacetylation in the peripheral lung of patients with COPD. These changes may be relatively specific to COPD, because we could not find a reduction in total HDAC activity in samples from patients with asthma, cystic fibrosis, or pneumonia (Fig. 1 and 3). In contrast, in asthma, which also involves increased expression of inflammatory genes in the respiratory tract, we confirmed previous observations<sup>14</sup> showing increased HAT activity in bronchial-biopsy specimens. Thus, in both asthma and COPD, chromatin appears to be hyperacetylated but by means of different mechanisms, and this increased histone acetylation provides a mechanism for local unwinding of chromatin and a subsequent increase in inflammatory gene expression.<sup>6</sup>

In the present study, there was a positive correlation between HDAC activity and disease severity, as measured by the percent of predicted FEV<sub>1</sub> or the degree of airway obstruction as measured by the FEV<sub>1</sub>:FVC ratio. HDAC activity also correlated inversely with expression of the interleukin-8 gene and associated histone-4 acetylation in lung tissue. This finding suggests that the clinical stage of COPD may be related to reduced HDAC activity, and this reduced activity, in turn, could facilitate increased expression of the relevant inflammatory genes.

There are 11 classic human HDACs that regulate histone acetylation.<sup>15</sup> We previously reported that HDAC2 is involved in suppression of NF- $\kappa$ B-mediated inflammatory gene expression by corticosteroids.<sup>7</sup> In the present study, we have shown that HDAC2 mRNA and protein expression is significantly reduced in tissue specimens of the peripheral lung and in alveolar macrophages from patients with COPD. In addition, we have shown that HDAC3, 5, and 8 are also reduced in lung tissue

and macrophages. These HDAC isoforms are reported to be involved in the cell cycle, cell differentiation, and apoptosis.<sup>15-17</sup> Further experiments will be required to clarify how the reduced function of these HDACs influences the inflammatory process in COPD.

We speculate that our findings may have therapeutic implications, because reductions in HDAC activity may be reversible. Theophylline is an activator of HDAC,<sup>13</sup> and we have recently shown that low concentrations of theophylline completely restore HDAC activity in alveolar macrophages from patients with COPD, with reduced production of inflammatory cytokines and restoration of responsiveness to corticosteroids.<sup>18</sup> Whether this is a mechanism of the therapeutic action of theophylline in COPD is not known. We report that total HDAC activity and the expression of specific HDAC isoenzymes are decreased in peripheral lung tissue, bronchial-biopsy specimens, and alveolar macrophages of patients with COPD and that this finding is related to increasing disease severity. This finding could in part account for the increased inflamma-

tory response in the respiratory tract of patients with COPD.

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## CORRECTION

## Histone Deacetylase Activity and COPD

*To the Editor:* In their study, Ito and colleagues (May 12 issue)<sup>1</sup> observed reductions in both the activity and expression of histone deacetylases (HDACs), especially HDAC2, in patients with chronic obstructive pulmonary disease (COPD). In addition, the activity inversely correlated with the severity of COPD.

Histone-modifying enzymes play essential roles in gene regulation.<sup>2</sup> The balance between histone acetylation and deacetylation appears to be crucial to normal cell growth. Disruption of either of these molecular mechanisms has been associated with the development of cancer. Several molecules and genes have been identified or developed or both to inhibit HDACs.<sup>3</sup> Valproic acid, an antiepileptic drug that has been commercially available for decades, has been found to inhibit HDACs, including HDAC2.<sup>4</sup> However, there is no evidence that valproic acid worsens pulmonary function in patients taking the medicine.<sup>5</sup> The “chicken-and-egg” conundrum remains unresolved: Does the reduction of HDAC activity cause severe COPD, or is it a secondary event?

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*To the Editor:* The following statement in the Discussion section of the article by Ito et al. is rather confusing: “In the present study, there was a positive correlation between HDAC activity and disease severity, as measured by the percent of predicted FEV<sub>1</sub> [forced expiratory volume in one second]. . . .” The Results section, Figure 1D, and the

abstract clearly indicate that there was apparently a negative correlation between HDAC activity and disease severity. I assume that the authors meant to say that there was a positive correlation between HDAC activity and FEV<sub>1</sub>.

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*The authors reply:* Histone modification regulates many genes, including those involved in normal cell growth. Eleven classic HDACs have been identified.<sup>1</sup> In patients with COPD, we found a marked reduction in HDAC2, with lesser reductions in HDAC5 and HDAC8. Different HDACs appear to be involved in different cellular functions and presumably regulate different sets of genes. Indeed, the targeted reduction of HDAC2 through RNA interference results in reduced responsiveness to corticosteroids in a human epithelial cell line (A549), whereas this reduction is not observed when other classic HDACs are inhibited.<sup>2</sup> Valproate, a nonselective inhibitor of classic HDACs, is associated with 50 percent inhibition of HDAC activity at approximately 200 µg per milliliter (1.4 mmol per liter) in A549 cells. Steady-state plasma concentrations of valproate in patients with epilepsy are 50 to 100 µg per milliliter (0.3 to 0.7 mmol per liter), so it is possible that clinical doses may have some HDAC inhibitory effect, enhancing inflammation or reducing responsiveness to corticosteroids in patients with inflammatory diseases. There is one report of increased circulating proinflammatory cytokines in children with epilepsy treated with valproic acid.<sup>3</sup> However, we are not aware that a worsening of inflammatory diseases has been investigated or reported with valproate. We agree with Dr. Lin that it is not certain whether the reduction in HDAC activity is a consequence or a cause of severe COPD, but we would like to suggest that it is both and that it provides a molecular basis for the increasing pulmonary inflammation as COPD progresses.

We agree with Dr. Bhowmik that the sentence in the Discussion was incorrectly written. We should have stated that there was a negative correlation between HDAC activity and disease severity.

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