

Identification of Novel Diagnostic Biomarkers for Asthma and Chronic Obstructive Pulmonary Disease

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Rationale: Proteomics may identify a useful panel of biomarkers for identification of asthma and chronic obstructive pulmonary disease (COPD).

Objectives: To conduct an unsupervised analysis of peripheral blood proteins in well-characterized subjects with asthma and COPD, and identify and validate a biomarker panel for disease discrimination.

Methods: Two-dimensional difference gel electrophoresis was used to separate plasma proteins from healthy control subjects, stable patients with asthma, and individuals with COPD. Candidate protein markers were identified by matrix assisted laser desorption ionization time of flight mass spectrometry and subsequently validated in two populations via immunoassay. A panel of four biomarkers was selected and their ability to distinguish between groups was assessed in isolation and in combination in two separate validation populations.

Measurements and Main Results: Seventy-two protein spots displayed significantly different expression levels between the three subject groupings ($P < 0.05$). Fifty-eight were positively identified, representing 20 unique proteins. A panel of four biomarkers (α_2 -macroglobulin, haptoglobin, ceruloplasmin, and hemopexin) was able to discriminate with statistical significance between the clinical groups of patients with asthma, patients with COPD, and control subjects, and these results were confirmed in a second clinical population of older adults with airflow obstruction.

Conclusions: Proteomics has identified novel biomarkers for asthma and COPD, and shown that the iron metabolism pathways and acute-phase response may be involved in the pathogenesis of airway disease. The panel of peripheral blood biomarkers has the potential to become an extremely useful addition to the clinical diagnosis and management of respiratory disease.

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The respiratory diseases asthma and chronic obstructive pulmonary disease (COPD) have many similar symptoms and are typically diagnosed by performing a number of clinical tests to assess an individual's lung function and response to reliever medication. To date, some studies have attempted to identify biomarkers of COPD or asthma; however, no study has attempted to identify noninvasive, blood-based, diagnostic biomarkers that can discriminate between healthy control subjects, patients with asthma, and individuals with COPD.

What This Study Adds to the Field

Using a proteomics approach, we have identified a panel of four blood-based biomarkers which, when used in combination, can discriminate between healthy control subjects, patients with asthma, and individuals with COPD and has the potential to be a valuable tool in the clinical diagnosis of respiratory disease.

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The obstructive airway diseases asthma and chronic obstructive pulmonary disease (COPD) are significant and increasing health problems throughout the world. The management of these diseases could be improved by better diagnosis and recognition, and better understanding of their pathogenesis. New technologies for investigating human diseases now offer significant potential to address the need for better diagnosis and improved understanding of asthma and COPD. Proteomics can simultaneously identify multiple proteins associated with different disease states (1) and potentially discover novel proteins not previously associated with particular disease states. Several studies have used proteomics for the discovery of protein changes in lung tissue from mouse models of asthma (2–6), and bronchoalveolar lavage fluid (7) and CD3⁺ T lymphocytes (8) from patients with asthma and healthy subjects. However, for diagnostic and prognostic purposes, identification of biomarkers in readily obtainable samples, such as blood, is preferred. A selected analysis of multiple known blood markers in COPD, using protein array methodology, has shown the potential for proteomics (9), whereby a panel of biomarkers was found to associate with patients with COPD versus healthy control subjects. This type of study used a predefined array of markers, and is potentially limited by the pool of analytes available on the protein array and by our current knowledge of disease pathology. An open,

unsupervised proteomic study should identify a broader panel of candidate markers, including proteins not previously associated with respiratory disease. The need for such unbiased approaches has been emphasized and called for in the chronic inflammatory diseases of asthma and COPD (10).

There is also general agreement that a panel of independent disease-related proteins considered in aggregate should be less prone to the influence of genetic and environmental “noise” than is the level of a single marker protein (11), and that proteomics has the power to identify such panels of proteins in a high-throughput manner. There remains, however, a need to apply methods to quantify the added benefit of biomarker panels for disease assessment. Thus identification of a panel of biomarkers that are differentially expressed between patients with asthma and patients with COPD, and between patients with and without these airway diseases, is required. In this study we have used two-dimensional difference gel electrophoresis (2D-DIGE) to conduct an unsupervised analysis of circulating proteins in well-characterized subjects with asthma and COPD, and applied logistic regression to evaluate the power of combining markers compared with the markers in isolation. The biomarker panel was then applied to a second validation

population with obstructive airway disease to demonstrate the power of this approach.

Some of the results of this study have been previously reported (12).

METHODS

Subjects

We confirmed 2D-DIGE reproducibility (*see* Figure E1 in the online supplement) and conducted a proteomic discovery study and two validation studies. The discovery study assessed mutually exclusive clinical groups ($n = 43$; Table 1 and Table E3) for stable nonsmoking asthma ($n = 21$) and COPD ($n = 5$), and matched healthy control subjects ($n = 17$). The biomarkers were then validated by immunoassay in this population, supplemented by seven patients with asthma and nine patients with COPD ($n = 58$; Table E6). A clinical validation study then assessed the biomarker panel in older adults (>55 yr) with obstructive airway disease ($FEV_1 < 80\%$ predicted and $FEV_1/FVC < 70\%$) consecutively recruited from the clinic ($n = 50$) (Table 2: asthma [$n = 14$], COPD [$n = 22$], asthma–COPD overlap [$n = 14$]), and in age-matched control subjects ($n = 23$) recruited by advertisement (12). The study was approved by institutional ethics committees and subjects gave written informed consent.

TABLE 1. CLINICAL CHARACTERISTICS OF PEOPLE IN THE TWO-DIMENSIONAL DIFFERENCE GEL ELECTROPHORESIS MARKER DISCOVERY STUDY

Description	Group 1: Healthy Control Subjects	Group 2: Stable Asthma	Group 3: COPD	P Value
Number of patients	17	21	5	
Age, yr*	44.2 ± 14.4	48.1 ± 12.7	65.7 ± 10.6 ^{†‡}	0.012
Sex, male/female [§]	8/9	11/10	2/3	1.0
Smoking, n (%) [§]				0.127
Never	8 (47%)	11 (52%)	0	
Ex	9 (53%)	10 (48%)	5 (100%)	
Pack-years*	20.3 ± 17.4	20.7 ± 13.3	72.7 ± 36.9 ^{†‡}	0.003
Atopy, n (%) [§]	6 (35.3%)	19 (90.5%) [‡]	1 (20.0%) [†]	<0.0001
% predicted FEV_1 *,	97.7 ± 9.0	81.3 ± 16.7 [‡]	65.0 ± 17.5 [‡]	0.0001
% predicted FEV_1 *,	101.0 ± 8.7	85.9 ± 14.4 [‡]	69.0 ± 18.3 [‡]	<0.0001
% predicted FVC *,	101.3 ± 11.3	95.1 ± 14.9	79.9 ± 10.9 [‡]	0.010
FEV_1/FVC , %*,	79.5 ± 8.0	70.1 ± 7.9 [‡]	63.8 ± 10.0 [‡]	0.0003
PD_{15} , ml**	N/A	5.3 (3.6, 15.1)	4.8 (4.7, 8.4)	0.926
DL_{CO} *	N/A	85.9 ± 15.0	65.6 ± 11.5	0.012
ICS use, n (%) [§]	N/A	21 (100%)	0	<0.0001
ICS, μ g beclomethasone equivalents/d**	N/A	1,464 ± 1,228	N/A	
ACQ score	N/A	1.1 ± 0.8	N/A	
GOLD classification, n (%)				
I	N/A	N/A	0 (0%)	
II	N/A	N/A	5 (100%)	
III	N/A	N/A	0 (0%)	
IV	N/A	N/A	0 (0%)	
Induced sputum cell counts				
Quality**	19 (17, 20)	19 (17, 20)	19 (18, 20)	0.976
Total cell count, $\times 10^6$ /ml**	3.96 (2.4, 6.7)	3.47 (1.6, 5.1)	6.03 (3.7, 10.1)	0.3224
Neutrophils, %**	34.5 (17.8, 61.0)	42.0 (27.5, 49.3)	64.5 (57.8, 67.8) [†]	0.028
Eosinophils, %**	0 (0, 0.25)	1.0 (0.25, 6.5) [‡]	1.25 (0.25, 1.5)	0.002
Macrophages, %**	59.8 (36.8, 79.8)	51.5 (41.3, 62.8)	31.8 (31.3, 32.0) ^{†‡}	0.032
Lymphocytes, %**	0.75 (0.25, 1.25)	0.5 (0.25, 0.75)	0.75 (0.5, 1.25)	0.551
Columnar epithelial, %**	2.0 (0.5, 5.5)	2.25 (1.0, 6.5)	1.75 (0.25, 2.25)	0.442
Squamous, %**	2.7 (1.7, 5.7)	4.5 (0.7, 7.2)	2.2 (0.99, 3.9)	0.453

Definition of abbreviations: ACQ = Asthma Control Questionnaire; COPD = chronic obstructive pulmonary disease; DL_{CO} = carbon monoxide diffusing capacity; GOLD = Global Initiative for Chronic Obstructive Lung Disease; ICS = inhaled corticosteroids; N/A = not applicable; PD_{15} = provocation dose resulting in a 15% drop in baseline FEV_1 expressed as a geometric mean (log SD).

* Values represent means ± SD, one-way analysis of variance.

[†] Bonferroni post hoc test significant compared with stable asthma. Asthma: symptoms with fully reversible variable airflow obstruction (airway hyperresponsiveness and/or increased bronchodilator reversibility). COPD: symptoms, incomplete reversibility of airflow obstruction (postbronchodilator $FEV_1 < 80\%$ predicted).

[‡] Bonferroni post hoc test significant compared with healthy control subjects.

[§] Chi square or Fisher exact test.

^{||} FEV_1 prebronchodilator.

[¶] FEV_1 postbronchodilator.

** Values represent medians (interquartile range), Kruskal-Wallis test.

TABLE 2. CLINICAL DATA FOR CLINICAL VALIDATION POPULATION

Description	Group 1: Healthy Control Subjects	Group 2: Stable Asthma	Group 3: COPD	Group 4: Asthma–COPD Overlap	P Value
Number of patients	23	14	22	14	
Age, yr*	49.9 ± 17.6	67.7 ± 6.7†	68.0 ± 7.9†	68.7 ± 9.0†	<0.0001
Sex, male/female‡	11/12	8/6	8/14	5/9	0.566
Smoking, n (%)‡					0.759
Never	12 (52.2%)	7 (50%)	8 (36.4%)	5 (35.7%)	
Ex	11 (47.8%)	7 (50%)	13 (59.1%)	8 (57.1%)	
Current	0	0	1 (4.6%)	1 (7.1%)	
Pack-years*	13.9 ± 15.6	39.5 ± 28.6	39.9 ± 36.9	37.2 ± 37.9	0.151
Atopy, n (%)‡	6 (26.1%)	11 (78.6%)†	8 (36.4%)	9 (64.3%)	0.006
% predicted FEV ₁ *§	103.9 ± 13.6	56.9 ± 22.4†	54.1 ± 21.5†	50.6 ± 14.9†	<0.0001
% predicted FEV ₁ *	109.0 ± 13.8	61.6 ± 23.8†	60.6 ± 22.7†	55.0 ± 17.1†	<0.0001
% predicted FVC*§	110.10 ± 14.3	75.1 ± 18.6†	75.1 ± 22.6†	76.6 ± 14.8†	<0.0001
FEV ₁ /FVC, %*§	77.5 ± 5.3	59.2 ± 15.2†	55.9 ± 13.6†	52.1 ± 12.9†	<0.0001
PD ₁₅ , ml¶	N/A	7.4 (3.5, 19.6)	5.9 (4.9, 8.7)	6.3 (2.9, 11.2)	0.935
DL _{CO} *§	N/A	73.3 ± 17.3	72.5 ± 23.2	84.1 ± 21.2	0.323
ICS use, n (%)‡	0	11 (78.6%)	21 (95.5%)	14 (100%)	0.08
ICS, µg beclomethasone equivalents/d¶	N/A	1,600 ± 780	1,460 ± 646	1,657 ± 511	0.659
GOLD classification, n (%)					
I	N/A	N/A	4 (20%)	N/A	
II	N/A	N/A	9 (45%)	N/A	
III	N/A	N/A	4 (20%)	N/A	
IV	N/A	N/A	3 (15%)	N/A	
Sputum cell counts					
Quality¶	18 (16, 19)	17 (16, 19)	18.5 (17.5, 19)	17.5 (16, 18)	0.250
Total cell count, × 10 ⁶ /ml¶	1.7 (1.3, 3.1)	4.0 (2.8, 8.5)	3.3 (1.9, 7.6)	3.7 (1.4, 9.5)	0.039
Neutrophils, %¶	28.5 (13.3, 54.5)	62.9 (45.5, 78.5)	56.0 (35.5, 88.3)†	77.0 (45.8, 85.5)†	0.003
Eosinophils, %¶	0.25 (0, 0.5)	1.65 (0.5, 3.25)†	2.0 (0.75, 7.25)†	1.0 (0.25, 5.5)†	0.0001
Macrophages, %¶	63.8 (41.5, 77.8)	29.9 (16.8, 52.0)	15.0 (8.0, 44.0)†	17.5 (7.3, 37.3)†	0.0003
Lymphocytes, %¶	0.75 (0.25, 1.75)	0 (0, 0.5)	0.25 (0, 1.0)	0.25 (0, 1.0)	0.062
Columnar epithelial, %¶	1.0 (0.25, 7.5)	1.5 (0.5, 2.0)	0.75 (0.25, 2.25)	1.0 (0.5, 2.25)	0.659
Squamous, %¶	4.5 (1.96, 8.3)	1.6 (0.7, 18.4)	2.7 (0.99, 8.9)	3.9 (2.2, 8.5)	0.725

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; DL_{CO} = carbon monoxide diffusing capacity; GOLD = Global Initiative for Chronic Obstructive Lung Disease; ICS = inhaled corticosteroids; N/A = not applicable; PD₁₅ = provocation dose resulting in a 15% drop in baseline FEV₁ expressed as a geometric mean (log SD).

* Values represent means ± SD, one-way analysis of variance.

† Bonferroni post hoc test significant compared with healthy control subjects. Asthma: symptoms and reversible variable airflow obstruction (airway hyperresponsiveness and/or increased bronchodilator reversibility). COPD: symptoms, incomplete reversibility of airflow obstruction (postbronchodilator FEV₁ < 80% predicted); asthma–COPD overlap: symptoms with increased variability and incomplete reversibility of airflow obstruction.

‡ Chi square or Fisher exact test.

§ FEV₁ prebronchodilator.

|| FEV₁ postbronchodilator.

¶ Values represent medians (interquartile range), Kruskal-Wallis test.

The proteomic discovery study and immunoassay validation study recruited subjects from mutually exclusive clinical groups of asthma, COPD, and control subjects without airway disease. None of the subjects were current smokers. Asthma was defined as a compatible history of episodic symptoms responsive to asthma treatment, together with airway hyperresponsiveness to hypertonic (4.5%) saline. Patients with asthma had fully reversible airflow obstruction and DL_{CO} (carbon monoxide diffusing capacity) was greater than 70% predicted. Subjects in the COPD group were selected to have COPD (GOLD [Global Initiative for Chronic Obstructive Lung Disease] stage II or greater) with predominant airway disease with minimal emphysema (DL_{CO} > 60% predicted) and no asthma. They were ex-smokers with a significant smoking history, compatible symptoms, and a doctor's diagnosis of COPD, and incompletely reversible airflow obstruction (FEV₁ < 80% predicted, FEV₁/FVC < 0.7 postbronchodilator) and a negative bronchodilator response. The clinical validation study applied these same definitions, and subjects with features of both asthma (variable airflow obstruction) and COPD (incomplete reversibility of airflow obstruction) were labeled as having mixed asthma–COPD.

Sample Preparation and 2D-DIGE and Mass Spectrometry

Platelet-depleted plasma was processed on a ProteomeLab IgY-12 LC2 column (Beckman Coulter, Fullerton, CA), precipitated via trichloroacetic acid–acetone and protein quantified (2-D Quant; GE Biosciences, Australia). Protein (100 µg), labeled with cyanine 3 (Cy3) or Cy5 dyes (GE Biosciences) and separated on 24-cm pH 4–7 IPG

(immobilized pH gradient) strips followed by 4–18% polyacrylamide gel electrophoresis in the second dimension, was visualized on a Typhoon 9410 variable mode imager (GE Healthcare, Australia) as described (13). A pooled internal control consisting of individuals from each clinical group (n = 10) was labeled with Cy2 and included on every gel. Image analysis and comparison were performed with the Batch Processor and Biological Variation Analysis modules of DeCyder software version 6.5 (GE Healthcare, Australia). Average expression ratios of individual protein spots were compared by one-way analysis of variance and proteins with an expression ratio of ±1.2 (assigned after reproducibility analysis) and *P* < 0.05 were then manually inspected for densitometric Gaussian distribution and match quality. Proteins of interest were excised from preparative 2D gels, trypsin digested, and analyzed on an Ettan MALDI-ToF/Pro (GE Healthcare) or an Applied Biosystems 4700 proteomics analyzer with TOF/TOF optics (Applied Biosystems, Foster City, CA) in MS mode as previously described (13). Data were submitted to the database search program Mascot (Matrix Science Ltd, London, UK) for protein identification as described (13).

Immunoassay

Serum IgA, complement factor H, haptoglobin, α₂-macroglobulin, ceruloplasmin, hemopexin, and antithrombin III concentrations were assayed with commercially available ELISAs. For Western blotting, proteins were separated on 10 or 4–18% polyacrylamide gels, transferred to nitrocellulose, and probed with anti-prothrombin, anti-fibrinogen γ

TABLE 3. DIFFERENTIALLY EXPRESSED PROTEINS IDENTIFIED BY TWO-DIMENSIONAL DIFFERENCE GEL ELECTROPHORESIS AND MASS SPECTROMETRY IN THREE DISEASE GROUPS: HEALTHY CONTROL SUBJECTS, PATIENTS WITH ASTHMA, AND PATIENTS WITH COPD

Master Spot No.	ANOVA P Value	Protein ID	Accession No.	Differential Expression Ratio		
				Patients with Asthma/ Control Subjects	Patients with COPD/ Control Subjects	Patients with COPD/ Patients with Asthma
Iron Metabolism						
480	0.002	Ceruloplasmin (ferroxidase)	P00450	1.01	-1.34*	-1.35*
1437	0.0066	Haptoglobin	P00738	1.94*	1.48	-1.31
1394	0.016	Haptoglobin	P00738	2.13*	1.59	-1.34
1444	0.022	Haptoglobin	P00738	1.83*	1.37	-1.33
1403	0.027	Haptoglobin	P00738	1.88*	1.59	-1.18
1381	0.03	Haptoglobin	P00738	2.13*	1.37	-1.55
968	0.024	Hemopexin	P02790	-1.2*	NA	NA
965	0.029	Hemopexin	P02790	-1.12	-1.23*	-1.1
977	0.033	Hemopexin	P02790	-1.34*	-1.2	-1.12
Coagulation Cascade						
822	0.04	Prothrombin	P00734	-1.08	-1.24*	-1.15
824	0.0054	Prothrombin	P00734	-1.1	-1.31*	-1.19
818	0.016	Prothrombin	P00734	-1.14*	-1.26*	-1.1
819	0.018	Prothrombin	P00734	-1.13*	-1.19*	-1.05
1242	0.0019	Fibrinogen γ	P02679	1.06	1.31*	1.24*
1235	0.03	Fibrinogen γ	P02679	1.18	1.48*	1.26
1153	0.011	Fibrinogen γ	P02679	1.25*	1.28*	1.02
859	0.0041	Heparin cofactor II	P05546	1.18*	-1.25	-1.47*
865	0.0097	Heparin cofactor II	P05546	1.1	-1.2*	-1.32*
611	0.037	Heparin cofactor II	P05546	1.31*	1.05	-1.24
911	0.0028	Protein S precursor	P07225	-1.28*	-1.19	1.07
1152	0.0084	Fibrin β		1.2*	1.51*	1.26
559	0.025	ITI heavy chain H4	Q14624	-1.24*	-1.15	1.08
557	0.031	ITI heavy chain H4	Q14624	-1.14*	-1.25*	-1.09
558	0.049	ITI heavy chain H4	Q14624	-1.14	-1.29*	-1.13
1284	0.036	α_2 -Antiplasmin	P08697	1.19	1.27*	1.07
967	0.0085	α_2 -Antiplasmin	P08697	1.18	2.07*	1.75*
857	0.041	Histidine-rich glycoprotein	P04196	1.07	-1.22	-1.3*
1116	0.048	Antithrombin III	P01008	-1.23*	-1.15	1.08
1106	0.019	Antithrombin III	P01008	-1.18*	-1.22*	-1.04
737	0.042	Gelsolin	P06396	-1.19	-1.43*	-1.21
236	0.0034	α_2 -Macroglobulin	P01023	-1.31*	NA	NA
495	0.022	α_2 -Macroglobulin	P01023	-1.23*	-1.02	1.2
498	0.032	α_2 -Macroglobulin	P01023	-1.34*	-1.17	1.14
249	0.041	α_2 -Macroglobulin	P01023	-1.3*	NA	NA
Complement Pathways						
349	0.028	Complement factor H	P08603	1.3*	1.34	1.03
338	0.00017	Complement factor H	P08603	-1.53*	1.19	1.82*
336	0.0031	Complement factor H	P08603	-1.24*	1.59*	1.97*
340	0.0045	Complement factor H	P08603	-1.3*	1.27	1.66*
339	0.0078	Complement factor H	P08603	1.16	1.81*	1.56*
680	0.015	Complement factor B	P00751	-1.25	-1.5*	-1.2
688	0.029	Complement factor B	P00751	1.12	-1.2	-1.34*
547	0.032	Complement 3	P01024	-1.18	-1.76*	-1.49
691	0.0034	Complement component C4-A	P0C0L4	-1.29*	-1.59*	-1.23*
Immunoglobulins						
1001	0.0019	IgA	P01876	1.45*	2.38*	1.64*
992	0.0055	IgA	P01876	1.19	1.98*	1.66*
1009	0.0084	IgA	P01876	1.24	1.85*	1.49*
1005	0.016	IgA	P01876	1.33	2.11*	1.58
1017	0.017	IgA	P01876	1.2	1.94*	1.62*
1018	0.022	IgA	P01876	1.11	1.86*	1.67*
1022	0.022	IgA	P01876	1.16	1.86*	1.61*
996	0.024	IgA	P01876	-1.36	1.3	1.77*
1011	0.034	IgA	P01876	1.22	1.92*	1.57
1010	0.039	IgA	P01876	1.3	1.99*	1.53
806	0.011	IgM heavy chain	P01871	-2.17*	-2.04	1.07
796	0.02	IgM heavy chain	P01871	-1.8*	-2.68*	-1.49
794	0.026	IgM heavy chain	P01871	-2.09*	-2.2	-1.06
811	0.018	IgM heavy chain	P01871	-1.74*	-2.22*	-1.27
804	0.04	IgM heavy chain	P01871	-1.87*	-2.42	-1.3

Definition of abbreviations: ANOVA = analysis of variance; COPD = chronic obstructive pulmonary disease; IgA = immunoglobulin A; IgM = immunoglobulin M; ITI heavy chain H4 = inter- α -trypsin inhibitor heavy chain H4; NA = not applicable.

* $P < 0.05$, t test.

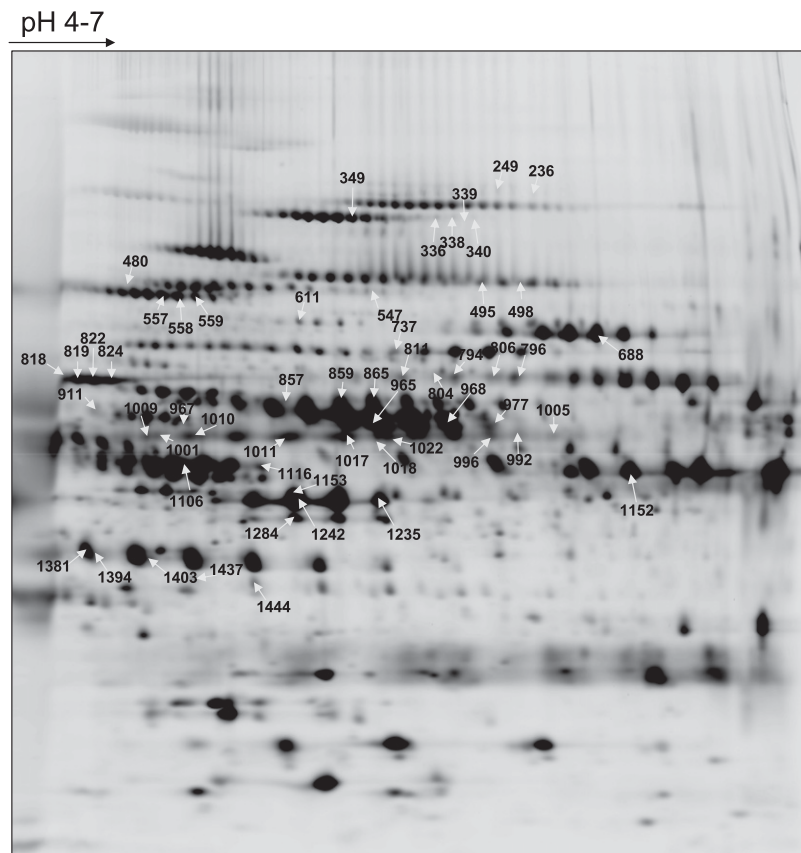


Figure 1. Representative cyanine 3 (Cy3)-labeled two-dimensional gel of immunodepleted human plasma proteins from a healthy control subject. Proteins were separated on pH 4–7 IPG (immobilized pH gradient) strips in the first dimension, and by 4–18% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in the second dimension. Numbered spots are differentially expressed ($P < 0.05$; one-way analysis of variance) across the three disease groups (healthy control subjects, patients with asthma, and patients with COPD) and were positively identified by mass spectrometry (Table 3).

chain, or anti-inter- α -trypsin inhibitor heavy chain H4 (anti-ITI-H4) primary antibodies followed by appropriate horseradish peroxidase-linked secondary antibodies. Reactive bands were visualized using enhanced chemiluminescence and a Fujifilm luminescent image analyzer (LAS-3000; Fujifilm, Tokyo, Japan) and quantified with Multi Gauge V3.0 software (Fujifilm). The chemiluminescence intensity was normalized to a healthy control serum sample run on every gel.

Statistical Analysis

Differential expression between groups was assessed by Student *t* test (GraphPad Prism 4 for Windows; GraphPad Software Inc., La Jolla, CA) with Bonferroni-corrected *P* values. Logistic regression (Stata 9; StataCorp, College Station, TX) was used to calculate the predicted value of an individual having the disease, based on their level of a single marker or combination of markers. Receiver-operating characteristic curves were generated, and sensitivity, specificity, and the area under the curve were calculated. Significance was accepted when $P < 0.05$. The possibility of biomarker serum concentrations varying with inhaled corticosteroid (ICS) use and age was assessed by either simple logistic regression or Spearman's rank correlation, as appropriate.

RESULTS

Differential Protein Expression between Healthy Control Subjects, Patients with Asthma, and Patients with COPD

Plasma proteins were separated by 2D-DIGE for 43 subjects across the three clinical groups: healthy control subjects ($n = 17$), asthma ($n = 21$), and COPD ($n = 5$). More than 1,900 proteins were detected on each gel, 72 proteins displayed significantly altered expression ($P < 0.05$, one-way analysis of variance), and 58 were identified by mass spectrometry (Table 3 and Figure 1). Although 58 individual protein spots were differentially expressed, a number of these represented variant charge and/or molecular weight isoforms of the same protein

(e.g., 10 isoforms of IgA; Table 3), and thus a total of 20 unique proteins were identified as candidate markers. These proteins clustered in groups of known function including iron metabolism (ceruloplasmin, haptoglobin, hemopexin), the coagulation cascade (α_2 -macroglobulin, prothrombin, fibrinogen γ , fibrin β), immunoglobulins (IgA, IgM), and complement pathways (complement factor H, complement factor B).

Validation of Candidate Markers in the Diagnostic Immunoassay Validation Assessment Group

To assess the performance of the markers identified by 2D-DIGE analysis, we determined the relative expression of selected marker candidates in serum by immunoassay (ELISA or immunoblotting). Markers were prioritized for validation according to three criteria: (1) multiple isoforms identified with consistent trends observed, (2) biologically plausible in the context of respiratory disease, and (3) the availability of commercially available ELISA kits or primary antibodies. Serum concentrations of ceruloplasmin ($P = 0.0002$), haptoglobin ($P = 0.0003$), and antithrombin III ($P = 0.0140$) were significantly higher in patients with asthma compared with healthy control subjects. In addition, serum levels of prothrombin (high molecular weight isoform) were lower in patients with COPD than in patients with asthma ($P = 0.0024$) and healthy control subjects ($P < 0.0001$) (Figure 2).

Validation of Candidate Markers in the Clinical Validation Population

We then proceeded to validate the candidate biomarkers in an unselected clinical population consisting of older adults with asthma, COPD, and asthma–COPD overlap, and who also exhibited significant comorbidity. Serum concentrations of

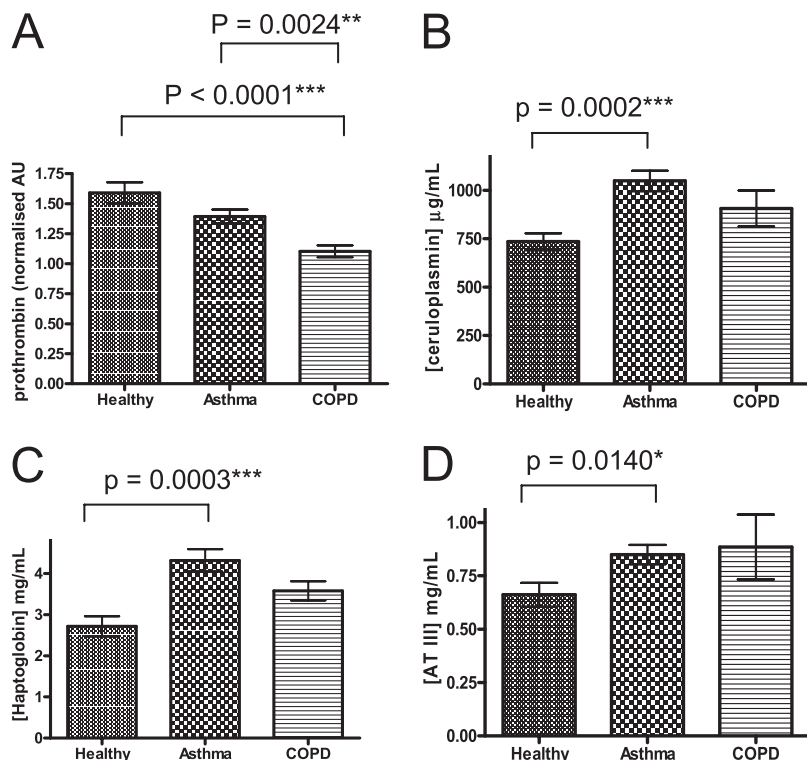


Figure 2. Validation data for (A) prothrombin, (B) ceruloplasmin, (C) haptoglobin, and (D) antithrombin III (AT III) in the diagnostic immunoassay validation group. Statistical significance was determined by unpaired, two-tailed Student *t* test with multiple comparisons correction by the Bonferroni method (significant; $P < 0.017$). Triplicate analyses were performed by either two-site ELISA or immunoblotting for each patient, and the mean value was used for analysis. Error bars represent $\pm$ SEM.

ceruloplasmin ($P = 0.0010$), haptoglobin ($P = 0.0073$), and hemopexin ($P = 0.0004$) were significantly higher in patients with asthma compared with healthy control subjects. When compared with control subjects, the patients with COPD showed trends for elevations in ceruloplasmin, α_2 -macroglobulin, haptoglobin, and hemopexin. Haptoglobin ($P = 0.0014$) and hemopexin ($P = 0.0024$) exhibited significantly elevated serum levels in individuals with asthma–COPD overlap compared with healthy control subjects. None of the markers investigated showed statistically significant differences between patients with COPD and individuals with asthma–COPD overlap and patients with asthma (Figure 3).

Analysis of a Panel of Proteomic Biomarkers

The performance of a biomarker panel was evaluated in the two populations and the candidate markers ceruloplasmin, α_2 -macroglobulin, haptoglobin, and hemopexin were selected for use in a combinatorial analysis because of their statistically significant differences in serum marker levels between disease groups and their consistent performance over the two distinct populations.

Diagnostic immunoassay validation assessment group. Several biomarker combinations were capable of distinguishing between all three clinical groups (asthma, COPD, healthy control) in a manner superior to any one marker in isolation (Table 4 and Table E4). The combination of ceruloplasmin and haptoglobin was the best for discriminating between patients with asthma and healthy control subjects, whereas serum ceruloplasmin and hemopexin concentrations in combination best distinguished between patients with COPD and healthy control subjects. For differential diagnosis between patients with COPD and patients with asthma several marker combinations were able to discriminate between the two disease states. The combination α_2 -macroglobulin and either haptoglobin or hemopexin provided a good balance of sensitivity and specificity whereas α_2 -macroglobulin, haptoglobin, and hemopexin delivered the

best sensitivity (92%) and α_2 -macroglobulin, hemopexin, and ceruloplasmin gave the best specificity (89.3%). It is interesting to note that the serum concentration of α_2 -macroglobulin alone performed almost as well, indicating that it was the predominant contributor to the power of the combinations tested.

Clinical validation population. The combination of haptoglobin and hemopexin was the best for discriminating between patients with asthma and healthy control subjects in the clinical validation population (Table 5, Table E5, Figure 4). Ceruloplasmin either alone, or combined with haptoglobin, also performed well. α_2 -Macroglobulin and hemopexin concentrations in combination best distinguished between patients with COPD and healthy control subjects. The combination of ceruloplasmin and hemopexin also demonstrated statistically significant discrimination for patients with COPD and control subjects, in agreement with the analysis of the primary validation group.

For differential diagnosis between patients with COPD and patients with asthma the combination α_2 -macroglobulin, haptoglobin, and ceruloplasmin delivered the best sensitivity whereas α_2 -macroglobulin, haptoglobin, and hemopexin provided the best specificity. The combination of the markers α_2 -macroglobulin, haptoglobin, and hemopexin was the best for discriminating between individuals with asthma–COPD overlap and healthy control subjects. α_2 -Macroglobulin and ceruloplasmin serum levels in combination could discriminate patients with asthma and individuals with asthma–COPD overlap. No marker combinations or markers in isolation were able to discriminate between patients with COPD and individuals with asthma–COPD overlap with statistical significance.

Effect of Comorbid Conditions in the Clinical Validation Population

A literature review identified five medical conditions as potential confounders of the blood-based marker diagnosis panel: hepatobiliary disease (14), ischemic coronary disease (15), obesity (16,

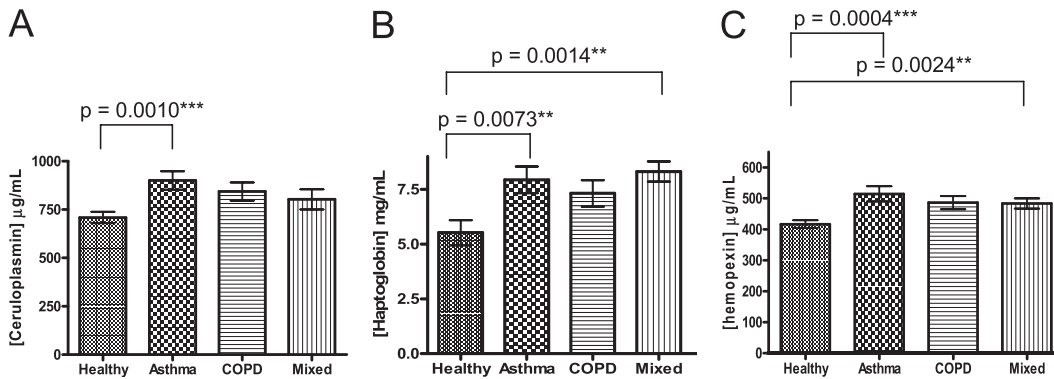


Figure 3. Validation data for (A) ceruloplasmin, (B) haptoglobin, and (C) hemopexin in the clinical validation population. Statistical significance was determined by unpaired, two-tailed Student *t* test with multiple comparisons correction by the Bonferroni method (significant; $P < 0.0083$). A single analysis was performed for this population, using either two-site ELISA or immunoblotting. Error bars represent $\pm$ SEM.

17), endocrine and metabolic disorders (18, 19), as well as psychiatric conditions (20). Table E7 summarizes a comorbidity analysis of the clinical validation population and indicates no significant effect of these comorbid conditions on the marker levels, apart from an effect of psychiatric disease on levels of α_2 -macroglobulin.

Potential Effect of ICS Use and Age on Biomarker Serum Concentrations

For the diagnostic immunoassay validation assessment (DIVA) group, stable patients with asthma (but not patients with COPD) were prescribed ICS. The potential effect of ICS use on serum marker concentrations was assessed and it was found that ICS dosage did not significantly alter serum marker concentrations and is thus unlikely to be a cause of the observed elevation of serum marker concentrations in this study (hemopexin, $P = 0.5135$; haptoglobin, $P = 0.083$; ceruloplasmin, $P = 0.6723$; α_2 -macroglobulin, $P = 0.6761$).

For the clinical validation population many patients with COPD were also using ICS. To evaluate the effect of ICS use on serum marker levels in patients with COPD, individuals from

the clinical validation and DIVA (none using ICS) groups were combined and reclassified into two groups (with and without ICS). Logistic regression analysis identified no statistically significant effect of ICS use on marker concentration.

In addition, we evaluated the potential effect of age on serum marker concentrations and found that there was no significant correlation between age and serum marker concentration for the DIGE, DIVA, and clinical validation groups ($P > 0.05$ for all panel markers).

DISCUSSION

This proteomic discovery program has identified a panel of protein markers whose serum concentrations are significantly altered in asthma and COPD compared with age- and sex-matched healthy control subjects, and whose function points to novel mechanistic pathways indicating the involvement of the iron metabolism pathways, complement pathways, and the coagulation cascade in asthma and COPD. In addition, we have identified a panel of four serum biomarkers (ceruloplasmin, haptoglobin, hemopexin, and α_2 -macroglobulin) that can be used in

TABLE 4. ANALYSIS OF DIAGNOSTIC VALUES OF MARKER AND MARKER IN COMBINATIONS IN DIAGNOSTIC IMMUNOASSAY VALIDATION GROUP

Comparison	Marker Combination	Logistic Regression Model <i>P</i> Value	Minimal False Negatives			Minimal False Positives			AUC (%)
			Cutoff Predicted Value	Sensitivity	Specificity	Cutoff Predicted value	Sensitivity	Specificity	
Asthma vs. healthy (n = 44)	Ceruloplasmin	<0.00001	≥ 0.448	92.86	75.0	≥ 0.785	67.86	93.75	89.29
	Haptoglobin								
	Ceruloplasmin	0.0006	≥ 0.442	89.29	62.5	≥ 0.753	67.86	93.75	83.04
	Hemopexin								
	Haptoglobin	0.0006	≥ 0.590	85.71	68.75	≥ 0.676	78.57	81.25	82.59
COPD vs. healthy (n = 30)	Hemopexin								
	Ceruloplasmin	0.0004	≥ 0.342	84.62	68.75	≥ 0.590	69.23	87.50	88.94
	Haptoglobin								
	Ceruloplasmin	<0.00001	≥ 0.426	92.86	75.0	≥ 0.480	85.71	87.50	91.07
	Hemopexin								
COPD vs. asthma (n = 42)	Haptoglobin	0.009	≥ 0.444	76.92	62.50	≥ 0.547	61.54	87.50	81.25
	Hemopexin								
	α_2 -Macroglobulin	0.045	≥ 0.289	84.62	64.29	≥ 0.382	61.54	78.57	74.73
	Haptoglobin								
	α_2 -Macroglobulin	0.019	≥ 0.314	78.57	67.86	≥ 0.435	57.14	78.57	75.51
	Hemopexin								
	α_2 -Macroglobulin	0.046	≥ 0.285	92.31	64.29	≥ 0.355	76.92	75.0	76.10
	Haptoglobin								
	Hemopexin								
	α_2 -Macroglobulin	0.048	≥ 0.338	78.57	71.43	≥ 0.493	50.0	89.29	75.77
	Hemopexin								
	Ceruloplasmin								

Definition of abbreviations: AUC = area under the curve; COPD = chronic obstructive pulmonary disease.

Data presented in bold typeface represent combinations that deliver the best performance for sensitivity and/or specificity.

TABLE 5. ANALYSIS OF DIAGNOSTIC VALUES OF MARKER AND MARKER IN COMBINATIONS IN CLINICAL VALIDATION POPULATION

Comparison	Marker Combination	Logistic Regression Model P Value	Minimal False Negatives			Minimal False Positives			AUC (%)
			Cutoff Predicted value	Sensitivity	Specificity	Cutoff Predicted value	Sensitivity	Specificity	
Asthma vs. healthy (n = 36)	Ceruloplasmin	0.0002	≥0.342	85.71	72.73	≥0.561	64.29	95.45	87.01
	Haptoglobin								
	Ceruloplasmin	0.0001	≥0.226	92.31	68.18	≥0.421	69.23	81.82	89.16
	Hemopexin								
COPD vs. healthy (n = 44)	Haptoglobin	<0.00001	≥0.289	100.0	90.91	≥0.457	92.31	95.45	96.85
	Hemopexin								
	Ceruloplasmin	0.016	≥0.460	72.73	59.09	≥0.573	63.64	81.82	75.41
	Haptoglobin								
	Ceruloplasmin	0.008	≥0.521	72.73	77.27	≥0.617	63.64	90.91	76.65
	Hemopexin								
	Haptoglobin	0.014	≥0.439	72.73	63.64	≥0.567	59.09	95.45	73.97
Mixed vs. healthy (n = 36)	Hemopexin								
	α ₂ -Macroglobulin	0.006	≥0.412	86.36	68.18	≥0.631	54.55	95.45	80.17
	Hemopexin								
	Ceruloplasmin	0.002	≥0.321	92.86	59.09	≥0.488	64.29	81.82	80.84
	Haptoglobin								
	Ceruloplasmin	0.007	≥0.321	92.86	68.18	≥0.464	64.29	86.36	81.49
	Hemopexin								
	Ceruloplasmin	0.0007	≥0.277	92.86	59.09	≥0.530	71.43	95.45	86.04
	Hemopexin								
	Haptoglobin								
COPD vs. asthma (n = 36)	α ₂ -Macroglobulin	0.0002	≥0.295	100.0	77.27	≥0.435	92.86	95.45	94.16
	Hemopexin								
	Haptoglobin								
	α ₂ -Macroglobulin	0.013	≥0.585	72.73	71.43	≥0.672	54.55	92.86	71.43
	α ₂ -Macroglobulin	0.008	≥0.556	72.73	64.29	≥0.621	68.18	92.86	79.55
	Haptoglobin								
	α ₂ -Macroglobulin	0.028	≥0.523	81.82	61.54	≥0.667	59.09	84.62	74.48
	Hemopexin								
	α ₂ -Macroglobulin	0.012	≥0.585	72.73	71.43	≥0.665	68.18	92.86	78.25
	Ceruloplasmin								
	α ₂ -Macroglobulin	0.003	≥0.512	81.82	61.54	≥0.686	68.18	100.0	84.27
	Haptoglobin								
	Hemopexin								
	α ₂ -Macroglobulin	0.011	≥0.520	86.36	64.29	≥0.641	63.64	85.71	79.87
	Haptoglobin								
Mixed vs. asthma (n = 28)	Ceruloplasmin								
	α ₂ -Macroglobulin	0.024	≥0.477	81.82	61.54	≥0.684	68.18	84.62	78.67
	Hemopexin								
	Ceruloplasmin								
	α ₂ -Macroglobulin	0.025	≥0.392	78.57	64.29	≥0.544	57.14	71.43	70.92
	α ₂ -Macroglobulin	0.069	≥0.340 or >=0.450	85.71 or 78.57	64.29 or 71.43	≥0.490	57.14 or 64.29	71.43 or 78.57	77.04
	Haptoglobin								
	α ₂ -Macroglobulin	0.063	≥0.380	78.57	61.54	≥0.602	57.14	84.62	75.82
	Hemopexin								
	α ₂ -Macroglobulin	0.045	≥0.368	85.71	57.14	≥0.583 or ≥0.509	57.14 or 64.29	92.86 or 71.43	75.00
	Ceruloplasmin								
	α ₂ -Macroglobulin	0.069	≥0.390	85.71	71.43	≥0.503	71.43	78.57	78.57
	Haptoglobin								
	Ceruloplasmin								
	α ₂ -Macroglobulin	0.089	≥0.428	71.43	61.54	≥0.517	64.29	76.92	73.63
	Hemopexin								
	Ceruloplasmin								

Definition of abbreviations: AUC = area under the curve; COPD = chronic obstructive pulmonary disease.

Data presented in bold typeface represent combinations that deliver the best performance for sensitivity and/or specificity.

combination to accurately identify asthma and COPD, and have validated these results in a second clinical population of older adults with obstructive airway diseases, including asthma and COPD. The proteins in the diagnostic biomarker panel are all involved in the regulation of inflammation, and usually function as antiinflammatory proteins.

We used an unbiased analysis design in well-characterized groups of patients with asthma and COPD to discover differentially expressed proteins in these groups. Ten of the 20 candidate markers identified were subsequently validated in serum and a biomarker

panel including the markers ceruloplasmin, α₂-macroglobulin, haptoglobin, and hemopexin provided significant discrimination between subject groups in both validation studies. Although individual markers can differentiate between particular clinical groupings, logistic regression analysis has shown that the consideration of marker combination for each specific comparison yields vastly superior performance. Receiver-operating characteristic curves constructed for each comparison are of high quality (Figure 4) and specific cutoff points may be chosen to tailor the test for either maximal sensitivity or specificity, depending on the

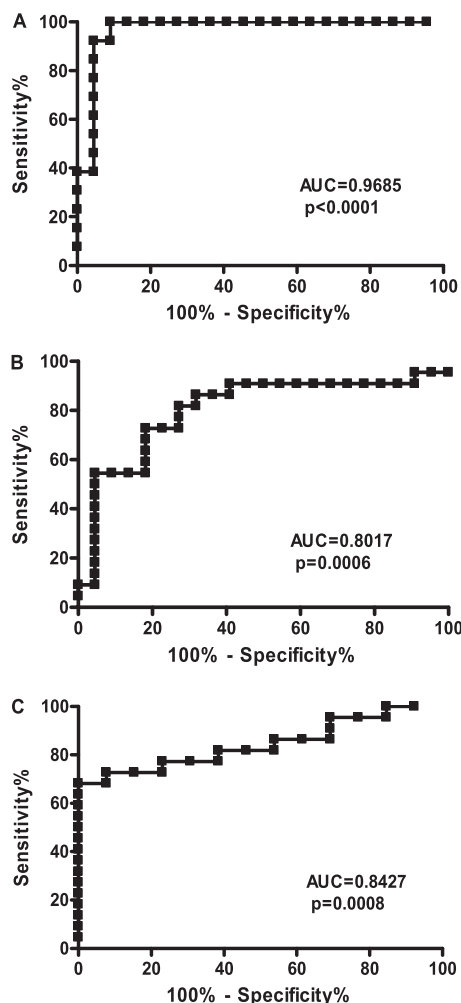


Figure 4. Selected receiver-operating characteristic curves for (A) asthma versus healthy control subjects (haptoglobin/hemopexin); (B) COPD versus healthy control subjects (α_2 -macroglobulin/hemopexin); and (C) COPD versus asthma in the clinical validation population (α_2 -macroglobulin/haptoglobin/hemopexin).

diagnostic requirements. Indeed, apart from mixed airway disease and COPD (for which no combination can differentiate) cut-points may be chosen where sensitivity and specificity are well balanced (Tables 4 and 5). Thus our study has identified a panel of highly discriminatory proteins that could be extremely useful in a clinical context.

The biomarker panel comprises three positive acute-phase proteins (α_2 -macroglobulin, ceruloplasmin, and haptoglobin) and one type II acute-phase protein (hemopexin). These are predominantly liver-synthesized proteins that can have important antiinflammatory activity through inhibition of oxidative stress, and iron sequestration resulting in antimicrobial activity. As such, they may function to modulate the systemic inflammatory response to inflammation (21) and be involved in tissue repair through fibrosis and angiogenesis.

The acute-phase response is an innate body defense observed during infection, physical trauma, malignancy, and tissue damage that aims to minimize on-going tissue damage by isolating and destroying infective agents while activating repair processes. It is an antigen-nonspecific, innate response that aims to eliminate microbes and hence prevent infection. The innate immune response involves the recruitment and activation of macrophages and leukocytes that release inflammatory cytokines on recognition of a pathogen-associated molecular pattern. These cytokines travel through the bloodstream and stimulate hepatocytes in the liver to synthesize and secrete acute-phase proteins, which we have in this case identified as differentially expressed between our clinical groupings in circulating blood.

Ceruloplasmin, also called ferroxidase, is a multifunctional, copper protein synthesized primarily in the liver and by activated macrophages. It has important roles in iron homeostasis and inflammation, and it has antimicrobial activity via regulation of iron availability to microorganisms. Serum levels have previously been reported to be elevated (22, 23) or unchanged in children with allergic asthma (24). Engstrom and colleagues reported that ceruloplasmin and haptoglobin were weakly correlated with lung function in COPD, and were associated with an increased future risk of hospitalization for COPD (25). Our results extend these observations by showing elevated ceruloplasmin in adults with asthma and COPD, as well as asthma-COPD overlap. In addition, we show that the elevation of ceruloplasmin is part of an increase in iron metabolism proteins as part of asthma and COPD, probably as an antiinflammatory response to the airway inflammation that characterizes these conditions.

Haptoglobin and hemopexin were other iron-related proteins that were altered in this study.

The inhibition of heme release from globin by haptoglobin and sequestration of heme by hemopexin suppress hemoglobin-mediated oxidative stress, attenuate endothelial cytotoxicity, and protect cells from heme toxicity. Iron sequestration may also have antimicrobial effects by depriving microbes of essential iron. In addition, hemoglobin and its derivative heme are often released into tissue compartments where there is infection and inflammation, in the presence of degrading blood, and hemoglobin synergizes with multiple Toll-like receptor agonists to induce release of high levels of tumor necrosis factor and IL-6 from macrophages, an effect that is attenuated by hemopexin (26). Hemopexin also down-regulates LPS-induced proinflammatory cytokine release from macrophages (27). Hemopexin has not been previously been associated with asthma or COPD. However, a 2D-DIGE-based proteomic study of bronchoalveolar lavage fluid in patients with sarcoidosis, patients with chronic beryllium disease (CBD), and control subjects showed a significant difference in bronchoalveolar lavage fluid hemopexin concentration between control subjects and patients with CBD. CBD is a granulomatous disorder that can lead to chronic lung inflammation and fibrosis (28).

Haptoglobin can be expressed by eosinophils, and variable serum levels have been reported in asthma, in which both elevated (29) and reduced (30) serum haptoglobin levels are described. Increases in haptoglobin are seen in uncontrolled asthma, such as asthma exacerbation (31) and 24 hours after allergen challenge in late responders (32). In asthma, haptoglobin has also been correlated with FEV₁ (29). As part of its tissue repair function, haptoglobin can induce differentiation of fibroblast progenitor cells into lung fibroblasts (33), and angiogenesis, potentially implicating haptoglobin in remodeling and fibrosis in asthma and COPD. Haptoglobin has not previously been linked to COPD.

α_2 -Macroglobulin binds host or foreign peptides and particles, thereby serving as a humoral defense barrier against pathogens in the plasma and tissues of vertebrates. It interacts with and captures virtually any proteinase including serine, cysteine, aspartic, and metalloproteinases, for example, gelatinase (matrix metalloproteinase [MMP]-2 and MMP-9), both self and foreign, suggesting a function as a unique "pan-proteinase inhibitor" (34). Its structure contains a 35-amino acid "bait" region. Proteinases binding and cleaving the bait region become bound to α_2 -macroglobulin and the proteinase- α_2 -macroglobulin complex is recognized by macrophage receptors and cleared from the system (35). α_2 -Macroglobulin provides negative feedback on the inflammatory response by inhibiting thrombin (coagulation) and plasmin (fibrinolysis). It has been studied in airway secretions

as a marker of plasma exudation, and is increased in sputum samples in asthma and COPD (36). Plasma levels may be increased in asthma (37), and are normal in emphysema but reduced in chronic bronchitis (38).

In agreement with these proteins being positive acute-phase proteins, their serum levels were elevated in the asthmatic and COPD groups (independent of age and ICS use) in both our validation populations relative to the healthy control subjects in our study. More importantly, the differential expression of the markers in our panel makes sense in the context of asthma and COPD, which both manifest inflammatory and fibrotic components during their progression.

Some limitations to our study relate to subject selection in the discovery population. The COPD group was small in number, and selected to have airway-predominant disease. The primary reason for studying airway-predominant COPD was to rigorously test the hypothesis that there would be differential markers between asthma and COPD reflective of small airway inflammation, which is a relevant lesion in both asthma and COPD. This approach eliminates the potentially confounding effects of air-space disease. By designing the study this way, we believe that the information about potential disease mechanisms is not confounded by the site of pathology. In addition, because COPD is a heterogeneous disease, we thought it was important to minimize this heterogeneity by studying a recognized and clinically relevant group of subjects with COPD. A larger group or one with more emphysema may identify additional proteins that are associated with COPD, and this would be useful future work. Similarly, the subjects with COPD in the discovery group were not using ICS, whereas most of the patients in the clinical validation group were prescribed ICS. This has the potential to influence results. We conducted some analyses to test for these effects and none were apparent; however, this requires further study. The 2D-DIGE technology is not well suited to analysis of small molecular mass proteins, for example, less than 10 kD. This means that many cytokines would not be detected by this approach, and additional methods such as used by Pinto-Plata and colleagues are useful here (9).

The strengths of the results in this study come from the approach used and the combination of a panel of markers to use for disease discrimination. A panel of independent disease-related biomarkers, as can be identified by proteomics, is generally considered to be more powerful and less prone to the influence of genetic and environmental "noise" than a single marker protein (11). For example, Rai and colleagues identified three potential biomarkers that could differentiate ovarian cancer from healthy individuals and compared their performance against the tumor marker, cancer antigen 125 (CA125) (39). Each biomarker individually did not outperform CA125; however, the combination of two of the new biomarkers together with CA125 significantly improved their performance (39, 40). Similarly, we show here that the combination of protein biomarkers significantly improves their performance as a diagnostic marker compared with each individual protein alone.

Proteomic analysis, using high-resolution 2D-gel electrophoresis coupled with mass spectrometry, is a powerful means to identify differential protein expression between biological samples. However, a major limitation in traditional 2D-gel technology is the reproducibility, and thus statistical comparison of protein expression between individual gels is difficult. An advance in this area has come from the introduction of cyanine dye fluorophores for prelabeling of protein samples. 2D-DIGE technology adds a quantitative component to conventional two-dimensional gel analyses, allowing for comparison of protein expression changes across multiple samples simultaneously without gel-to-gel variation, and hence with statistical confidence (41,

42). Our reproducibility study (see the online supplement) clearly shows that this technology, together with immunodepletion of abundant plasma proteins, can reproducibly separate thousands of proteins. Combined with well-defined clinical groups and advanced statistical analyses, we have shown that this technology is a powerful tool for the identification of novel disease biomarkers. As the biomarkers are detectable in blood, a readily obtainable biological sample, and reagents are currently available for testing the abundance of these proteins, this panel of biomarkers has the potential to become an extremely useful addition to the clinical diagnosis and management of respiratory disease.

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