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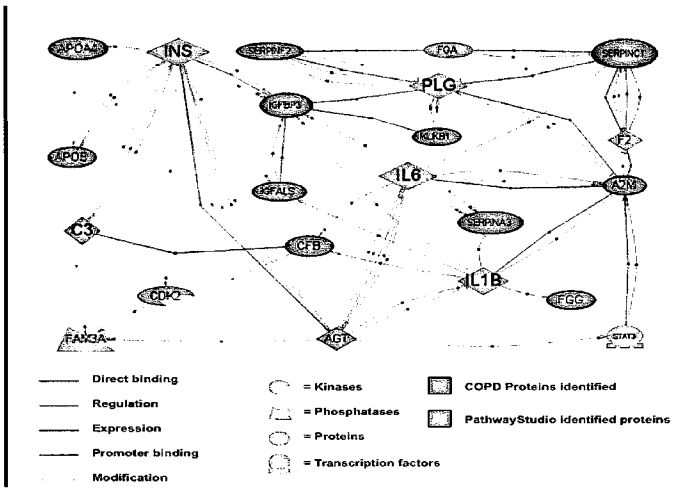
(54) Title: BIOMARKERS OF LUNG FUNCTION

Figure 1




(57) Abstract: Unique proteins associated with COPD capable of differentiating subjects likely to experience rapid (RPO) or slow (SLW) decline in lung function have been identified using comprehensive high-throughput proteomic approaches. Thirty peptides, which mapped to 21 unique proteins, were linearly associated with annualized rates of lung function decline among smokers with COPD characterized as having rapid or slow decline and smokers without COPD. Using three different statistical approaches to assess the data, the RPO and SLW groups are differentiated by 55 peptides, which mapped to 33 unique proteins. A number of the identified peptides are proteolytic fragments of proteins that are involved in the complement and/or coagulation systems or have anti-protease or metabolic functions.

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# BIOMARKERS OF LUNG FUNCTION

This application claims the benefit of U.S. Provisional Application No. 61/292,151, filed January 4, 2010, entitled: "BIOMARKERS OF LUNG FUNCTION", the entirety of which is hereby incorporated by reference.

## BACKGROUND

Lung disease, including airway diseases that affect lung function, includes asthma, obstructive pulmonary disease, emphysema, pneumonia, tuberculosis, lung cancer, pulmonary fibrosis, sarcoidosis, HIV/AIDS-related lung disease, alpha-1 antitrypsin deficiency, respiratory distress syndrome, bronchopulmonary dysplasia, embolism, and chronic obstructive pulmonary disease (COPD), among others.

COPD is the fourth leading cause of morbidity and mortality in the United States and is expected to rank third as the cause of death worldwide by 2020 (1). Cigarette smoking is widely recognized as a primary causative factor of COPD and accounts for approximately 80–90% of all cases in the United States (2). It has been estimated that up to 25–50% of cigarette smokers may develop COPD, and its prevalence increases with age (1-4).

The pulmonary component of COPD is primarily characterized by chronic airway inflammation and incompletely reversible, usually progressive, airflow obstruction (5, 1). The operational diagnosis of COPD has traditionally been made by spirometry, as a ratio of the forced expiratory volume in one second ($FEV_1$) to the forced vital capacity (FVC) below 70% (1). Pathophysiological mechanisms believed to underlie COPD include an imbalance between proteinase and anti-proteinase activity in the lung, dysregulation of anti-oxidant activity and chronic abnormal inflammatory response to long-term exposure to noxious gases or particles leading to the destruction of the lung alveoli and connective tissue (5, 1). However, COPD is increasingly recognized as a syndrome associated with significant systemic effects which are attributed to low-grade, chronic systemic inflammation (6, 7, 8, 9).

Conventional methods of diagnosing lung disease such as COPD employ diagnostic tests which rely on the presumed correlation of decreased pulmonary function with the presence of lung disease such as COPD, asthma, fibrosis, emphysema and others. Spirometry, which is the most commonly performed lung function test measures the quantity of air that a subject can exhale and the speed with which the air is exhaled. While lung function tests can provide a general assessment of the functional status of a subject's lungs, they do not distinguish between the different types of lung diseases that may be present. Certain lung related diseases cannot be confirmed based on functional tests alone. In addition, such tests assist in the diagnosis of lung disease only when an abnormality in lung function already exists. Functional diagnostic methods at a single time point also do not predict the rate of progression of the disease.

In contrast to functional diagnostic methods, assessment of protein/peptide biomarkers can be used as diagnostic as well as prognostic indicators of the progression (*e.g.*, predicted rate of progression) of a disease. Thus, the identification of proteins, such as those found in plasma, whose abundance and/or structure is altered in individuals with lung disease can be used to diagnose the presence of disease, provide a prognosis for an individual with lung disease (i.e., predicted rate of progression), and provides a better understanding of biological mechanisms underlying a disease.

## SUMMARY

Although cigarette smoking is recognized as the most important environmental cause of COPD, the pathophysiological mechanisms underlying cigarette smoking-related lung function decline are not well understood. The present disclosure provides information regarding the mechanisms involved in COPD, particularly cigarette

smoking-related COPD, by identifying a number of plasma peptides and proteins and genes encoding plasma proteins that correlate with lung function or decline in lung function. The present disclosure also describes the use of those peptides and proteins or genes encoding such proteins as biomarkers of lung function decline. The present disclosure also provides information regarding the mechanisms underlying lung function or the rate of lung function decline among subjects with COPD, including adult cigarette smokers with COPD. The plasma peptides and proteins have been identified by the utilization of robust plasma proteomic techniques, statistical analysis and biological pathway analysis. The peptides, proteins and genes encoding such proteins may be used as biomarkers in the diagnosis and prognosis of diseases including lung diseases such as COPD.

The plasma peptides and proteins provided in this disclosure were identified by two proteomic investigations (described herein below as Example 1 and Example 2). The first proteomic investigation discussed in this disclosure used offline strong cation exchange (SCX) fractionation of samples with reverse phase liquid chromatography coupled to a mass spectrometer fitted with electrospray ionization (RP-LC-ESI-MS). Following robust statistical analysis (using two approaches) and database searching, 1,758 peptides were identified in plasma samples from cigarette smokers. Thirty of those peptides mapped to 21 unique proteins and were linearly associated with annualized rates of lung function decline over 5 years among smokers with COPD who were characterized as having rapid or slow (or absent) decline and smokers without COPD. A number of the identified peptides are proteolytic fragments of proteins that are involved in the complement or coagulation systems or which have anti-protease or metabolic functions.

The second proteomic investigation involved the examination of the plasma proteomes of middle-aged or older adult smokers with mild to moderate COPD, with $FEV_1$ decline characterized as either rapid or slow (or absent), using a comprehensive high-throughput proteomic approach, and accurate mass and time (AMT) tag technology. Proteomic data were analyzed using three statistical approaches that permitted the rapid and slow decline groups to be differentiated by 55 peptides that map to 33 unique proteins. Twelve of the proteins have known roles in the complement or coagulation cascade and suggest potential mechanistic biomarkers associated with the rate of lung function decline in COPD.

The present disclosure provides in one aspect a method of diagnosing the presence of, or predicting the rate of lung function decline in a subject with lung disease, comprising determining the level of one or more proteins in Table 7, Table 2 or Table 4, or one or more peptide fragments of one or more proteins in Table 7, Table 2 or Table 4, in a biological sample from said subject. In one embodiment, a determination of the level of one or more proteins in Table 7, Table 2 or Table 4, or one or more peptide fragments of one or more proteins in Table 7, Table 2 or Table 4, are used as an indicator of the presence of lung disease in an individual subject and/or its rate of progression. Determinations of the levels of one or more proteins in Table 7, Table 2 or Table 4, or one or more peptide fragments of one or more proteins in Table 7, Table 2 or Table 4, may also be used to assign individuals to one or more subpopulations (*e.g.*, subpopulations of individuals having a higher risk for COPD with rapid progression or slower progression of lung function decline). In another embodiment, the level of expression of one or more genes encoding one or more proteins in Table 7, Table 2 and/or Table 4 may be determined (*e.g.*, by reverse transcription-polymerase chain reaction or real time PCR) in place of determining the level of protein or peptides translated from the gene products.

Determinations of proteins, peptides, or genes may be made relative to a sample from an individual or a population of individuals not having lung disease, or relative to an added external standard or internal standard such as a different protein.

The present disclosure provides in another aspect a method of diagnosis of or prognosis for a subject having, or suspected of having, a disease (*e.g.*, lung disease such as COPD), comprising determining the level of one or more proteins in Table 2 or Table 4, or one or more peptide fragments of one or more proteins in Table 2 or Table 4, in a biological sample from said subject. In one embodiment the disease is selected from the group consisting of, but not limited to, obstructive pulmonary disease, chronic systemic inflammation, emphysema, asthma, pulmonary fibrosis, cystic fibrosis, obstructive lung disease, COPD, and pulmonary inflammatory disorder. In one embodiment, the disease is COPD.

The methods of providing a diagnosis or prognosis provided herein may advantageously employ samples of biological fluids from a variety of sources, including, but not limited to blood, plasma, serum, lymphatic fluid, sputum, saliva, and/or urine for the direct determination of levels of proteins or peptides, or the indirect determination of levels of proteins or peptides through a measurement of the levels of nucleic acids encoding them. In one embodiment the biological fluid is plasma.

The methods of providing a diagnosis or prognosis provided herein may advantageously employ analytical methods of determining protein or peptide levels in biological fluids including, but not limited to, liquid chromatography separation with mass spectroscopic analysis (LC-MS) where the MS techniques include, but are not limited to, multistage mass spectrometric analysis, data dependent scanning, product ion scans, single ion monitoring, single reaction monitoring, and multiple reaction monitoring. Other methods/techniques of determining the level of proteins/peptides present in samples may also be used such as, for example, immunological detection and immunoaffinity techniques (e.g., ELISA, Western blotting, and various forms of immunological sandwich assays).

Also provided herein are compositions comprising two, three, four, five, six, seven or more proteins or peptide fragments that may be employed in methods of providing a diagnosis or prognosis of a subject having, or suspected of having, a disease (*e.g.*, lung disease such as COPD). In one embodiment, the compositions may comprise proteins or fragments of proteins identified in Table 2, Table 4, or combinations thereof.

In other embodiments, the present disclosure provides compositions comprising two, three, four, five, six, seven or more nucleic acids encoding the proteins and/or peptides identified in Table 2 or Table 4, and optionally comprising at least one promoter operatively coupled to at least one of said nucleic acids. In one embodiment such composition may comprise one, two, three, four, five, six, or more oligonucleotides having at least 80-90 percent, 80-95 percent, 85-95 percent, or 95-100 percent nucleic acid sequence identity to a contiguous sequence of 21 or more nucleotides of a nucleic acid sequence encoding the proteins identified in any of Tables 7, 2 or 4 or fragments thereof.

Also provided are a compositions comprising one or more, two or more, three or more, four or more, five or more, or ten or more different antibodies or fragments thereof, wherein said different antibodies, or antigen binding fragments thereof, are specific to two or more different proteins or peptide fragments identified in any of Table 7, Table 2 or Table 4. In some embodiments, the compositions comprise three, four, five, six seven or more different antibodies, or antigen binding fragments thereof, each specific for a different protein or peptide fragment identified in Table 2 or Table 4.

In another embodiment, the compositions described herein are in the form of: an array having two or more proteins or peptide fragments covalently attached to two or more different spatially addressable locations; an array having two or more antibodies or antigen binding fragments thereof covalently attached to two or more different

spatially addressable locations; or an array having two or more nucleic acids covalently attached to two or more spatially addressable locations.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram prepared using Pathway Studio™ software by Ariadne Genomics, showing nine proteins having multiple relationships (as determined via natural language processing of published abstracts accessible in pubmed.gov) to 21 of the proteins identified as differentially expressed across the three groups of subjects described in Example 1. The nine proteins identified by Ariadne Genomics' Pathway Studio™ are Insulin 2 (proinsulin) (INS), Plasminogen (PLG), Fibrinogen, alpha polypeptide (FGA), Interleukin 6 (IL6), Coagulation factor II (F2), Interleukin 1 beta (IL1B), Signal transducer and activator of transcription 3 (STAT3), Cyclin dependent kinase 2 (CDK2), and FAM3A (family with sequence similarity 3, member A). Those nine proteins are shown in relation to other proteins identified as differentially expressed.

Figure 2 is a Venn diagram representing an overlap of the peptides (lower number in each pair in *italics*) and associated non-redundant proteins (upper number in each set not italicized) identified using the three different statistical methods described in Example 2.

Figure 3 is a representation of those proteins identified in Example 2 that are known to be part of the coagulation or complement cascade, and their association with other proteins/peptides.

## DETAILED DESCRIPTION

The present disclosure describes methods of analyzing the protein, peptide, and/or polypeptide content of biosamples to aid in the understanding of molecular mechanisms involved in the development, progression, and/or prognosis of diseases (*e.g.*, lung disease such as COPD) in a subject. Methods are provided for using the abundance of proteins and peptides as biomarkers for diagnostic, prognostic and/or predictive measures of a subject's disease, management of the subject's disease, and/or prediction of the subject's response to clinical treatments for the lung disease. In one embodiment, the disease includes cigarette smoking-related COPD which is assessed by identifying plasma proteins that are differentially expressed and correlate with different rates of decline in lung function ($FEV_1$). Measurements of the abundance of expressed nucleic acids encoding proteins, peptides, and polypeptides may also be used as surrogates for the measurement of these proteins, peptides and polypeptides in the methods described herein.

In addition to providing information such as the name of the protein and the name of the gene encoding the proteins identified herein, the NCBI accession number and version and/or the GI number (aka "gi number") is provided for each protein. The NCBI accession/version numbers and GI numbers uniquely identify nucleic acid and/or protein sequences present in the NCBI database (NCBI, U.S. National Library of Medicine, 800 Rockville Pike, Bethesda, MD, 20894 USA), and are publicly available, for example, on the word wide web at www.ncbi.nlm.nih.gov. Where an NCBI accession number is provided for a precursor protein it is understood that the corresponding mature protein is also available in the NCBI database and considered part of this disclosure unless expressly stated otherwise. In addition, recitation of the protein sequences provided herein indicates that the corresponding gene sequence(s) encoding each protein are also available in the NCBI database at the time of this disclosure and its priority document. Where any accession number does not recite a specific version, the version is taken to be the most recent version of the sequence associated with that accession number at the time the earliest priority document for the present application was filed.

For each proteins recited herein it is understood that the NCBI accession numbers and GI numbers only refer to a sequence that is exemplary of the proteins (and their peptides) encompassed by this disclosure. Unless

recited otherwise, the present disclosure includes all isoforms of the proteins identified herein. Isoforms include, but are not limited to: proteins encoded by alternate alleles and haplotypes of the same gene; and/or proteins produced by alternate splicing of transcripts from one or more alleles of the same gene or other forms of alternative processing, including changes due to epigenetic influences. In some embodiments, isoforms include proteins/polypeptides that share greater than 70, 80, 85, 90, 95, 97, 98, or 99% sequence identity over the length of the shorter of the two proteins/polypeptides. In one embodiment, the isoforms of proteins share the amino acid sequence of the peptides recited for the proteins listed in Tables 2, 4 and 7.

In one embodiment an individual or a population of individuals may be considered as not having lung disease or impaired lung function when they do not have clinically relevant signs, symptoms, and/or measures of lung disease. Thus, in various aspects, an individual or a population of individuals may be considered as not having chronic obstructive pulmonary disease, chronic systemic inflammation, emphysema, asthma, pulmonary fibrosis, cystic fibrosis, obstructive lung disease, pulmonary inflammatory disorder, or lung cancer when they do not manifest clinically relevant signs, symptoms and/or measures of those disorders. In another embodiment, an individual or a population of individuals may be considered as not having lung disease or impaired lung function, such as COPD, when they have a $FEV_1/FVC$ ratio greater than or equal to about 0.70 or 0.72 or 0.75. In another embodiment, an individual or population of individuals that may be considered as not having lung disease or impaired lung function are sex- and age-matched with test subjects (e.g., age matched to 5 or 10 year bands) that are current or former cigarette smokers without apparent lung disease who have an $FEV_1/FVC \geq 0.70$ or $\geq 0.75$. Individuals or populations of individuals without lung disease or impaired lung function may be employed to establish the normal range of proteins, peptides or gene expression. Individuals or populations of individuals without lung disease or impaired lung function may also provide samples against which to compare one or more samples taken from a test subject (e.g., samples taken at one or more different first and second times) whose lung disease or lung function status may be unknown. In other embodiments, an individual or a population of individuals may be considered as having lung disease or impaired lung function when they do not meet the criteria of one or more of the above mentioned embodiments.

**Identification of Protein(s) and/or Peptide(s) Associated with COPD or its progression at a Slow or Rapid Rate.**

The present disclosure provides in one embodiment a method for identifying protein or peptide biomarkers of a disease that are associated with either the presence of a lung disease, or a slow or a rapid decline in lung function, as measured by a decline in $FEV_1$, in subjects with a lung disease. In one embodiment the lung disease is COPD, which affects the lungs and also the tissues of other organs.

In one embodiment, proteins and/or peptides are identified using expression profiling of samples of a tissue, cells or fluids (e.g., biofluids such as serum, plasma, urine, sputum, saliva, lymph, and the like) from subjects with a lung disease as compared to a profile of peptides in subjects without the disease. In another embodiment, the present disclosure provides polypeptide-based biomarkers that are differentially present in subjects with lung disease versus individuals without lung disease.

In another embodiment, a method for identifying protein and/or peptide biomarkers of a disease that is associated with a decrease in lung function comprises:

a) obtaining an expression profile of proteins and/or peptides in a biological sample from at least one subject (case sample) diagnosed as having a preselected disease that is associated with a decline in lung function (case);

b) obtaining an expression profile of proteins and/or peptides in a biological control sample from at least

one subject identified as not having the disease (control);

c) identifying one or more proteins and/or peptides that are differentially expressed in the sample from the subject (case sample) as compared to the control sample; and

d) optionally performing a statistical analysis on abundance values of the one or more identified proteins and/or peptides, wherein a statistically significant difference in abundance of the identified peptide(s) and/or protein(s) in the case sample as compared to the control sample identifies the peptide or protein as a biomarker of the preselected disease.

The profiling of proteins and/or peptides may be conducted by any method known in the art including, but not limited to, various mass spectroscopic methods. In some embodiments, proteins/peptides profiles are obtained by liquid chromatography separation of a sample coupled to mass spectroscopic analysis (LC-MS), where the mass spectroscopic analysis techniques including, but are not limited to, multistage mass spectrometric analysis, data dependent scanning, product ion scans, single ion monitoring, single reaction monitoring, and multiple reaction monitoring. Other techniques/instrumentation that may be employed for the analysis of proteins and peptides, include, but are not limited to, FT-ICR MS, LC FT-ICR MS, accurate mass and time (AMT) technology, putative mass and time (PMT) technology, high resolution LC separations and high mass accuracy measurements, MALDI, ESI, offline SCX fractionation with RP-LC-ESI-MS/MS, two-dimensional gel electrophoresis, immunoaffinity methods (e.g., ELISA, Western blotting, *in situ* immunohistochemistry) and protein array analysis.

In another embodiment, the present disclosure provides a method of using one, two, three, four, five, six seven, eight, ten, fifteen or more different proteins and/or peptides for diagnosing the presence of a lung disease or for developing a prognosis of the rate of lung function decline in a subject.

In another embodiment, the present disclosure provides a method of using one, two, three, four, five, six seven, eight, ten, fifteen or more different proteins and/or peptides for evaluating lung function in the presence of a lung disease or in the absence of a lung disease, or for developing a prognosis of lung function in a subject.

In one aspect, this disclosure also provides methods for comparison of differential protein/peptide expression in one or more subjects with lung disease relative to one or more individuals without lung disease, or in subjects having lung disease such as COPD with little or no decline in lung function, such as by measurement of $FEV_1$, compared with subjects having lung disease such as COPD with rapid decline in lung function. In one embodiment such methods comprise determining the level of one or more proteins that are set forth in Tables 2 and/or 4, or peptide fragments of those proteins or the level of expression of genes encoding those proteins.

This disclosure also provides methods for comparing differential protein expression in subjects with lung disease. Such individuals may be divided into groups having rapid or slow rates of decline in lung function by determining the annualized rate of lung function decline for a subject as the slope of the linear regression of $FEV_1$% predicted (i.e., adjusted for age, sex, and height). Subjects with the steepest rate of decline in annualized $FEV_1$% predicted (greater than the average annual decline) are considered to have COPD with "rapid decline" (RPD). Those individuals with the least steep or no annualized rate of decline in $FEV_1$% predicted (less than the average) are considered to have COPD with slow decline (SLW).

In one embodiment proteins present in a biological sample obtained from one or more cigarette smokers having COPD with rapid decline in lung function may be compared to proteins present in biological samples obtained from cigarette smokers having COPD with slow decline in lung function (SLW), or may be compared to proteins present in biological samples obtained from smokers without COPD or from non-smokers, to identify proteins or peptides differentially expressed in those groups.

Comparison of the differentially expressed proteins identifies potential protein/peptide biomarkers useful for classifying the lung condition or disease (*e.g.*, as slow- or rapid-decline COPD) presented by a subject. Protein/peptide biomarkers may also be identified by analysis of proteins differentially expressed by a subject with a lung disease as compared to proteins expressed by a gender-matched subject without lung disease. Identification of proteins that are differentially abundant among different groups of subjects with lung disease (*e.g.*, age and gender matched subjects) allows an understanding of the mechanisms (e.g., molecular changes) underlying a lung disease and the related decline in lung function. Such proteins are useful as molecular biomarker(s) for diagnosis, determining prognosis, and/or management of a subject's lung disease. For example, the proteins/peptides provided herein can be used for diagnosis and/or prognosis of rate of lung function decline in a subject with a lung disease.

In one embodiment, protein expression among one or more groups of adult cigarette smokers with mild to moderate COPD, but different rates of lung function decline, such as rapid- or slow-decline, may be compared to gender-matched smokers without COPD. Identification of proteins that are differentially abundant among the groups reflects the mechanisms underlying cigarette smoking-related lung function decline. Such proteins/peptides are molecular biomarkers for COPD and are useful in diagnosis, prognosis and/or management of COPD.

In another embodiment a method for identifying protein and/or peptide biomarkers of a disease that is associated with a rapid or slow decrease (decline) in lung function comprises:

a) obtaining an expression profile of proteins and/or peptides in a biological sample from at least one subject diagnosed as having a preselected disease that is associated with a decline in lung function (case);
b) obtaining an expression profile of proteins and/or peptides in a biological control sample from at least one subject identified as not having the disease (control);
c) identifying one or more proteins and/or peptides that are differentially expressed in the case sample as compared to the control sample; and
d) optionally performing a statistical analysis on abundance values of the one or more identified proteins and/or peptides, wherein a statistically significant difference in abundance of the identified peptide(s) and/or protein(s) in the case sample as compared to the control sample identifies the peptide or protein as a biomarker of the preselected disease.

For the purpose of this disclosure, the term "peptides" includes peptides prepared synthetically, or by any form of proteolysis including, but not limited to, enzymatic proteolysis. Such peptides may be limited to those peptides with a length greater than seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen, seventeen, eighteen, twenty, twenty two, twenty five, thirty, or thirty five amino acids. Such peptides may also be less than 40, 50, 60, 70, 80, or 100 amino acids in length. Alternatively, such peptides may have a range from about 7 to 50, 9-25, 10 to 20, 8 to 24, 9 to 18, 12 to 24, 15 to 45, 18 to 40, 20 to 50, or 25 to 50 amino acids in length.

**Methods of Providing a Diagnosis or Prognosis of a Subject Having, or Suspected of Having, a Lung Disease Including COPD**

Methods are provided for the diagnosis or prognosis of a subject having, or suspected of having, a lung disease, comprising making a determination of one or more proteins in Table 7, or one or more peptides of a protein in Table 7, in a biological sample from a subject. Optionally, the methods comprise making a determination of one or more proteins in Tables 2, 4, 5 and/or 6, or one or more peptides of one or more proteins in Tables 2, 4, 5 and/or 6. In such methods two or more, three or more, four or more, five or more, six or more, eight or more, ten or more,

fifteen or more, twenty or more, twenty five or more, thirty or more, or forty or more different proteins or peptides from different proteins in Tables 2, 4, 5 6 and/or 7 may be determined.

Methods are provided for the diagnosis or prognosis of a subject having, or suspected of having a lung disease, comprising determining the level of one or more proteins in Table 7 and/or in Table 2 or Table 4 or one or more peptide fragments of one or more proteins in Table 7 and/or in Table 2 or Table 4, in a biological sample from said subject. In some embodiments the disease is selected from the group consisting of but not limited to obstructive pulmonary disease, chronic systemic inflammation, emphysema, asthma, pulmonary fibrosis, cystic fibrosis, obstructive lung disease, COPD, and pulmonary inflammatory disorder.

Assessment of the level of one or more proteins found in Tables 2 and/or 4, or fragments thereof, provides information for diagnosing lung diseases such as COPD, or for providing a prognosis of lung disease (*e.g.*, COPD progression).

In one embodiment, a method of determining a prognosis of a lung disease can include determining the abundance (quantity or concentration) of one or more biomarkers present in a biological sample obtained from a subject, wherein the one or more biomarkers are selected from the group consisting of: a blood coagulation pathway component (protein), a component of the renin-angiotensin pathway, a complement system protein, a growth factor, a cytokine, a binding protein, a plasma glycoprotein, an anti-inflammatory protein, an immunoglobulin, and a lipoprotein. In another embodiment, a method of determining prognosis of a lung disease can include determining the quantity or concentration of one, two, three, four, five, six, seven, eight, nine, ten, twelve, fifteen, or more proteins listed in Table 7 and/or in Table 2 or Table 4 (or peptides of such proteins) or of transcripts from genes coding for the proteins listed in Table 7 and/or in Table 2 and 4.

In another embodiment, the present disclosure provides a method of managing a subject's lung disease, wherein a therapeutic treatment plan is customized or adjusted based on the status of the disease as determined by assessment of one or more proteins and/or peptide fragments of such proteins identified in Table 7, and/or Tables 2 and/or 4. Exemplary therapeutic treatments for lung disease include administering to the subject, one or more of: immunosuppressants, corticosteroids (*e.g.* betamethasone delivered by inhaler), b2-adrenergic receptor agonists (*e.g.*, short acting agonists such as albuterol), anticholinergics (*e.g.*, ipratropium, or a salt thereof delivered by nebuliser), and/or oxygen. In addition, where the lung disease is caused by or exacerbated by bacterial or viral infections, one or more antibiotics or antiviral agents may also be administered to the subject. In other embodiments a method of treatment comprises measuring at least one protein or peptide fragment of a protein identified in Table 7, and/or in Tables 2 and/or Table 4, during the course of the subject's lung disease. In such an embodiment, the level of expression of a protein in Table 7, and/or in Table 2 and/or Table 4, may also be assessed by measurement of the nucleic acids (mRNAs) expressed from the gene encoding the protein as surrogate for measuring the protein directly. The course of lung disease may be determined by making a first determination (e.g., taking a first measurement) at a first time, of at least one protein or peptide fragment of a protein identified in Table 7, and/or in Tables 2 and/or Table 4, or mRNA encoding a protein a protein identified in Table 7, and/or in Tables 2 and/or Table 4, in a first sample from the subject; and making a second determination of at least the same protein, peptide fragment or mRNA in a second biological sample obtained from the subject at a second time; and comparing the first determination to the second determination to determine the lung disease is in progression or regression. A method of managing a subject's treatment includes selecting an initial treatment protocol or altering a preselected treatment protocol based on the status or change in the status of the lung disease from the measurements at the first and second times. In other aspects, the method further comprises measuring two, three, four, five, six, seven, eight, ten, twelve, fifteen or more different proteins or peptide fragments of proteins listed in Tables 2 and/or

4, or the level of gene expression (e.g., mRNA levels) for those proteins at one or more times during the management of a subject's lung disease. Any one or more of the proteins identified in Table 5 and/or Table 6, or peptide fragments thereof, may also be employed in such methods of treatment, following the course of lung disease, or managing a subject's treatment.

In another embodiment, the present disclosure provides a method for monitoring the course of progression of a lung disease in a subject comprising: (a) obtaining a first measurement of at least one protein or peptide fragment of a protein listed in Table 7, and /or Table 2 and/or Table 4, or the level of gene expression (e.g., mRNA levels) for that protein in a first biological sample from the subject; (b) obtaining a second measurement of at least the same one protein, peptide fragment or level of gene expression in a second biological sample from the subject, where the second biological sample is obtained from the subject after the first biological sample; and (c) correlating the changes in the first and second measurements with a progression, lack of progression, or regression of lung disease in the subject. . Any one or more of the proteins identified in Table 5 and/or Table 6, or peptide fragments thereof, may also be employed in such methods.

Where determinations of one or more proteins or peptide fragments indicate that a treatment administered to a subject is ineffective, the determinations may be taken to indicate that higher levels of an applied therapeutic may be required to effect treatment, the protocol for administration may need to be modified, or that a different therapeutic agent is required. Where determinations of one or more proteins indicate that a treatment administered to a subject is effective, the determinations may be taken to indicate that the course of therapy (e.g., the choice or dosage of therapeutic agent(s) and/or the protocol for administration) should be continued. Where a treatment is only marginally effective based upon the determinations, either a change in the treatment, or an increase in the dosage of a therapeutic agent already being administered to the subject may be indicated.

In embodiments where changes in the levels of the proteins identified in Tables 2, 4 or 7 are observed over time, an increase in at least one protein identified in any of Tables 2, 4 or 7 that is associated with progression of COPD at a slow or rapid rate is indicative of disease progression. In contrast the decrease in at least one protein identified in any of Tables 2, 4 or 7 may be indicative of a lack of disease progression or may be indicative of disease regression. Similarly, an increase in a protein identified in any of Tables 2, 4 or 7 associated with stable COPD is indicative of stability or regression of the disease. Determinations of proteins (e.g., changes in level or amount) may be made by obtaining measurements of the intact protein, peptide fragment(s) of the protein, or nucleic acids (e.g. mRNA) encoding the protein in samples (e.g., first and second samples) obtained from the subject at different times.

**Samples for the Identification and Determination of Protein/Peptide Profiles or the Levels of Proteins and/or Peptides**

Biological sources for detection and determination of the levels of protein/peptide biomarker(s) include any tissue of interest from a subject suspected of having, or diagnosed as having, a disease (*e.g.*, a lung disease such as COPD). In one embodiment, samples for detection of protein(s) and/or peptide(s) of interest include, but are not limited to, serum, plasma, blood, lymphatic fluid, cerebral spinal fluid, sputum or saliva. In another embodiment a protein/peptide biomarker may be detected and levels determined in plasma.

**Determination of Protein and Peptide Abundance Levels in Samples**

Protein and peptide biomarkers provided herein that are correlated with diseases such as COPD or its progression may be identified without prior knowledge of their identity. For example, a biomarker's amino acid sequence can be determined using peptides present in a sample, peptides from enzymatic digests of a protein containing sample, or peptides derived by sequencing (*e.g.*, sequencing using mass spectroscopy). A sequence for a

peptide can be compared to a database of known proteins to identify the proteins from which the peptide was derived.

For the purpose of this disclosure "determination", "determine", or "determining" means measuring or observing the quantity (e.g., mass, weight, or number of moles) of a material or substance or the concentration of a material or substance. Determinations may be made of relative amounts of a material or substance (e.g., the amount of protein in a sample is twice that of the control sample) without ascertaining an absolute amount, provided the determination permits any relevant comparison to be made or method recited herein to be conducted.

Proteins and peptides differentially expressed in subjects having lung diseases such as COPD or in patients with different rates of decline in lung function may be identified and/or their levels measured using a variety of techniques that may be applied to sample protein and/or proteomic analysis. Exemplary methodologies include, but are not limited to, the use of chromatographic separation techniques such as 2-dimensional (2-D) gel electrophoresis, intact protein fractionation, peptide fractionation, and nano-flow liquid chromatography (LC). Analysis of peptides in proteomic studies may employ mass spectrometry (MS), which is a detection technique often used in either matrix assisted laser desorption ionization (MALDI) or electrospray ionization (ESI) for peptide analysis. The MS platforms by which measurements are made include instruments configured as quadrupole, time-of-flight (TOF), ion-trap, and Fourier transform ion cyclotron resonance MS (FTMS) instruments, or hybrid instruments such as triple quadrupole, quadrupole-TOF and ion-trap-FTMS. Recent observations by the Human Proteome Organization's (HUPO) Plasma Proteome Project have shown that offline peptide separation by strong cation exchange (SXC) followed by reverse-phase (RP) LC with ESI-MS/MS can result in the identification of more proteins of low abundance. See Li, et al., 2005 (10), which is hereby incorporated by reference in its entirety. Methods that combine immunological capture and of peptides coupled with mass spectroscopic analysis may also be employed in the methods described herein. See, e.g., U.S. 7,632,686 and U.S. 6,872,575 each of which are incorporated by reference herein.

In one embodiment, determination of a protein and/or peptide present in a biological sample can include its capture on a chromatographic resin that binds the protein and/or peptide. For example, a protein and/or peptide may be captured using a strong or weak cation exchange resin followed by elution. The eluted protein and/or peptide can then be detected by a mass spectrometry method. In another alternative, a protein and/or peptide can be fractionated on an anion exchange resin and detected directly by a mass spectrometry method. In yet another method, a protein and/or peptide can be captured on an immuno-chromatographic resin comprising antibodies that bind the protein and/or peptide followed by a specific detection method or a detection method allowing determination of a protein or peptide level or identification of the protein and/or peptide, such as ELISA or a mass spectrometry method.

Other methods/techniques of isolating, identifying and determining the level of proteins/peptides present in samples include, but are not limited to, SDS-PAGE electrophoresis, two-dimensional gel electrophoresis, intact chromatographic protein fractionation, and peptide chromatographic fractionation, quantitative ligand-binding, and nano-flow liquid chromatography (nano-flow LC).

Nucleic acids encoding proteins and/or peptides may also be measured as surrogates for measurement of the proteins or peptides themselves (e.g., gene expression). In such circumstances a variety of techniques may be employed, including, but not limited to, polymerase chain reaction, nucleic acid array analysis, quantitative RT-PCR (reverse transcriptase PCR), quantitative real time PCR, multiplex PCR, quantitative DNA arrays, quantitative hybridization, chromatography, quantitative rRNA-based amplification, fluorescent probe hybridization, fluorescent

nucleic acid sequence specific amplification, loop-mediated isothermal amplification and/or ligase amplification (*e.g.*, ligase chain reaction).

Immunoassays may also be used to identify proteins/peptides that correlate with disease function or for forming a diagnosis or prognosis based on the levels of proteins or peptides present. Such immunoassays include, but are not limited to, ELISA, immunohistochemistry, immunoelectrophoresis, analysis using arrays of immobilized antibodies, and Western blot analysis.

For the purpose of this disclosure antibodies are intended to include all type of antibodies, suitable for use in any given procedure unless specified otherwise. Antibodies include, without limitation, monoclonal antibodies, (monospecific) polyclonal antibodies, Fab(s), Fab'(s), single chain antibodies, diabodies, domain antibodies, miniantibodies, or an antigen binding fragments of any of the foregoing.

In one embodiment, a biological sample may be analyzed by use of an array technology and methods employing arrays such as, for example, a protein or nucleic acid microarray or a biochip bearing an array of proteins (*e.g.*, antibodies) or nucleic acids. A protein array or biochip generally comprises a solid substrate having a generally planar surface, to which a capture reagent is attached. Frequently, the surface of an array or biochip comprises multiple addressable locations, each bearing a bound capture reagent. In one embodiment the arrays permits the detection and/or determination (quantitation) of two, three, four, five, six seven, eight, ten, fifteen or more different biomarkers associated with COPD or its progression at a slow or rapid rate. In another embodiment the array comprises addressable locations for analysis of two, three, four, five, six seven, eight, ten, fifteen or more different proteins or peptide fragment(s) of proteins identified in any of Tables 2, 4 or 7. In another embodiment the array comprises addressable locations for analysis of two, three, four, five, six seven, proteins or fragments of proteins from the group consisting of: a blood coagulation pathway, a component of the renin-angiotensin pathway, and a complement system protein, identified in any of Tables 2, 4 or 7.

Analysis of proteins and/or peptides described herein may be conducted by detection or measurement of individual proteins and/or peptides or a combination of proteins and/or peptides. For example, methods for diagnoses, determining prognosis of a lung disease and/or management of a lung disease in a subject can include use of a composition comprising at least two proteins and/or peptides described herein. Thus, this disclosure includes embodiments of compositions comprising: at least two proteins and/or peptides; one or more nucleic acid sequence, or fragment(s) thereof, encoding proteins and/or peptides; one or more oligonucleotides having at least 80 percent identity to a contiguous sequence of at least 9, 12, 15, 18, 21, 24, 27, or 30 nucleotides of a nucleic acid sequence encoding a protein and/or peptide; or at least two antibodies or fragment(s) thereof specific to a protein or peptide described in any of Tables 2, 4 or 7.

The essential materials and reagents required for diagnosing a lung disease, for determining the prognosis of a lung disease amd/or for use in the treatment or management of lung disease in a subject may be assembled together in a kit. The kit generally will comprise components and reagents necessary for determining the level of one or more proteins or peptides (*e.g.*, the proteins or fragments of proteins identified in Tables 2 and/or 4) in a biological sample as well as in control and/or standard samples. For example, a kit may include oligonucleotide sequences, probes, and/or antibodies specific to the one or more of the aforementioned proteins or peptide fragments of those proteins for use in a quantitative assay such as RT-PCR, in situ hybridization, and/or microarray assays.

## EXAMPLES

### Example 1: Differential Protein Expression Among Two Groups Of Adult Cigarette Smokers With Mild To Moderate COPD But Different Rates Of Lung Function Decline And A Gender-Matched Group Of Smokers Without COPD

**1.1 Subjects.**

Subjects were selected from 244 University of Utah study center participants in the Lung Health Study (LHS) who also participated in the follow-on Genetics of Addiction Project (GAP). LHS enrolled male and female cigarette smokers, aged 35–60 years, with mild or moderate COPD, in a prospective, randomized, multicenter clinical study (11). GAP was a cross-sectional assessment which also enrolled 94 adult cigarette smokers without COPD as a control group. Smoking status was assessed and lung function measured by spirometry at baseline (1986-1989), annually for 5 years, once during 1998-2001 (12), and once in GAP (2003–2004). Spirometry included $FEV_1$ and $FEV_1$ adjusted for age, sex, and height (*i.e.*, as a percentage of predicted) (1). The annualized rate of lung function decline during the 5 years of LHS was calculated for each participant as the slope of the linear regression of $FEV_1$% predicted.

A subset of 54 GAP participants was selected for plasma proteomic analysis in this study: the 18 with the steepest rate of decline in $FEV_1$ (rapid decliners, RPD), the 18 with the least steep or no annualized rate of decline in $FEV_1$ (slow decliners, SLW), and 18 smokers without COPD as a control group. Characteristics of the three groups are shown in Table 1. Over the first 5 years of LHS, the rapid decliners had an average annual decrease in $FEV_1$ of 1.6 %predicted/y while the slow decliners had an average increase of 0.8 %predicted/y. At the GAP assessment approximately 17 years after baseline, 7/18 (39%) of the RPD participants and 12/18 (67%) of the SLW participants no longer smoked and in the control group, 8/18 (44%) had quit smoking in the three months before GAP participation ($\chi^2 = 3.11$, 2 d.f., $p = 0.21$).

**Table 1. Characteristics of study participants.**

| | Cigarette Smokers with COPD, Rapid Decline[a] (RPD) (n=18) | | | Cigarette Smokers with COPD, Slow Decline[a] (SLW) (n=18) | | | Cigarette Smokers without COPD (n=18) | |
|---|---|---|---|---|---|---|---|---|
| | Lung Health Study | | | Lung Health Study | | | | |
| Characteristic | Base-line | Year 5 | GAP | Base-line | Year 5 | GAP | GAP | *p*-value |
| Male, n (%) | | | 13 (72.2) | | | 10 (55.6) | 9 (50.0) | 0.369[b] |
| Age, mean (SD) | | | 64.8 (5.4) | | | 63.6 (7.3) | 57.2 (7.7) | 0.002[c] |
| Cigarettes per Day, mean (SD)[e] | 37.3 (17.2) | 21.6 (19.0) | 16.0 (17.0) | 27.7 (10.7) | 8.3 (11.4) | 5.6 (9.5) | 9.7 (12.0) | 0.167[c] |
| Years Smoked, mean (SD) | | | 42.1 (6.8) | | | 34.8 (9.2) | 30.1 (11.3) | < 0.001[c] |
| $FEV_1$ (L), mean (SD) | 2.75 (0.59) | 2.34 (0.67) | 1.70 (0.60) | 2.61 (0.57) | 2.63 (0.66) | 2.32 (0.55) | 3.20 (0.63) | |
| Δ $FEV_1$ (L), mean (SD) | | -0.40 (0.23) | | | 0.02 (0.21) | | na | < 0.001[d] |
| $FEV_1$ % predicted, mean (SD) | 76.1 (9.7) | 67.8 (13.3) | 54.6 (16.6) | 74.8 (9.7) | 78.5 (12.1) | 77.2 (14.2) | 103.1 (18.4) | |
| Δ $FEV_1$ % predicted, mean (SD) | | -8.22 (7.34) | | | 3.77 (5.73) | | na | < 0.001[d] |

GAP, Genetics of Addiction Project, an average of 17 years after Baseline at which time plasma proteomic analysis was performed; na, not applicable.

[a] Decline in lung function was assessed as the slope of a linear regression of the annualized rate of decline during the first 5 years of participation in the Lung Health Study in $FEV_1$ % predicted (*i.e.*, adjusted for age, height, and gender)

[b] $\chi^2 = 1.99$, 2 d.f. test

[c] Test of association between characteristic and lung function at GAP by linear regression

[d] Change in characteristic from Baseline to Year 5 for RPD versus SLW

[e] At the GAP time point, 7/18 (39%) of RPD, 12/18 (67%) of SLW, and 8/18 (44%) of Control subjects had quit smoking; $\chi^2 = 3.11$, 2 d.f., $p = 0.21$.

**1.2 Plasma Sampling and Processing.**

Plasma was sampled by venipuncture using a sodium citrated Vacutainer® tube at least two hours after eating. Within ten minutes of collection, blood was centrifuged for 15 minutes at 1500 g and 2-6 °C. The topmost plasma was removed and further centrifuged at 1500 g for 15 minutes. Plasma samples were shipped on dry ice, stored at -80 °C, and thawed just before analysis.

**1.3 Sample Pooling.**

In each of the 3 study groups, plasma samples from 6 subjects were pooled to reduce heterogeneity within the group, increase yield of low-abundance peptides, and minimize instrument run time. Therefore, three pools were evaluated for each of the three study groups, for a total of nine plasma sample pools. Samples were selected for each pool by applying a random number generator.

**1.4 Depletion of High–Abundance Plasma Proteins.**

All pooled plasma samples were depleted of the top 12 most abundant proteins using a Beckman Coulter IgY-12 High Capacity spin column (part #A24618) using the recommended manufacturer's procedure. In short, 20µL of plasma were added to 480µL of dilution buffer. The samples were then filtered through 0.22µm spin filters by centrifugation for 1 minute at 16,000 x g. The depletion columns were then centrifuged for 30 seconds at 400 x g to dry the beads. The end caps were attached and the diluted plasma samples were added and mixed by inverting the column. The samples were placed on a rotator (end to end) and incubated at room temperature for 30 minutes. Columns were then inverted and the tips were removed. The samples were then placed in collection tubes and centrifuged for 30 seconds at 400 x g. and the depleted flow-through was then collected for digestion.

**1.5 Protein Digestion (plasma)**

The depleted flow-through was added to a pre-rinsed Microcon YM-3 (3000 Da) molecular weight Cutoff spin cartridge (Millipore), following manufacturer's recommended protocol, and centrifuged at 14,000 x g until 100 µL of retentate remained (~30 min.). The retentate was then transferred to a clean microcentrifuge tube and proteins were reduced using 15 µL of 50 mM ammonium bicarbonate (Pierce) and 1.5µL of 100 mM DL-1,4-dithiothreitol (Acros, Geel, Belgium) and incubation at 95°C for 5 minutes. After samples cooled, they were alkylated by the addition of 3 µL of 100 mM iodoacetamide (Pierce) and incubation for 20 minutes in the dark at room temperature. 1.5 µL of 100 ng/µL porcine trypsin (Promega, Madison, WI) was then added and the samples were incubated at 37 °C for three hours. An additional 1.5 µL of 100 ng/µL trypsin was then added followed by incubation at 37 °C for approximately 16 hours. To ensure sufficient reagent mixing, all samples were vortexed (30 seconds) and centrifuged (2000 x g for one minute) following each solution addition. Samples were dried in a vacuum centrifuge at 45 °C. Samples were reconstituted with 50 µL of 3% acetonitrile with 0.1% formic acid and vortexed (30 seconds) prior to fractionation.

**1.6 Offline plasma fractionation.**

Offline fractionation of the plasma tryptic digests in each pooled sample into ten fractions was conducted using a GE healthcare MDLC Ettan (Piscataway, NJ) fitted with a GE FRAC950 fraction collector fitted with a strong cation exchange (SCX) column (Thermo Fisher Scientific Biobasic SCX, 250x2.1 mm). Ion exchange (IXE) solvent A was 20 mmol/L citric acid (Fisher) in 75% HPLC grade water and 25% acetonitrile (Fisher) (3.8g citric acid in 1 L of 25 % acetonitrile) (pH 2.65). IXE solvent B was 20 mmol/L citric acid and 1 mol/L ammonium chloride (Fisher) in 75% HPLC grade water and 25% acetonitrile (3.8g citric acid and 53g ammonium chloride dissolved in 1 L 25% acetonitrile, pH 2.65). The fraction collector was conditioned for approximately 20 minutes before each run with 100% IXE solvent A at 200 µL/minute. The tryptic digest plasma samples were reconstituted in 50 µL of IXE solvent A. Run parameters begin with a 40 µL sample injection and 0% IEX solvent B for 10 minutes, ramped to 60% IEX solvent B in 30 minutes, then to 100% IEX solvent B and held for 5 minutes. The system flow rate was 200 µL/minute and fractions were collected each minute in a 96 well plate (200 µL fractions). The fractions were lyophilized at 45°C and stored at -20°C until analysis. Samples were re-constituted with 50 µL of 3% acetonitrile with 0.1% formic acid and vortexed (30 seconds) prior to analysis.

**1.7 Liquid Chromatography.**

All nano-flow capillary liquid chromatography (ncap-LC) analyses were conducted using an Eksigent nanoLC-1D (Monmouth Junction, NJ) with a Leap technologies (Carrbaro, NC) autosampler and a Zorbax 300SB-C8 trap column (5x0.3mm). Reverse-phase separation was conducted on each of the ten fractions from each pooled sample using a New Objective Picofrit Proteopep™2 (5 cm of C18 packing and a 15 µm tip). The LC run program has a 4 minute trap wash at 10ul/min, a 10 µL injection volume and a 270 nL/minute flow rate. LC buffer A contains 0.1% formic acid in LCMS grade water (Fisher) and B contains 84% high purity acetonitrile (Fisher) with 0.1% formic acid. The LC gradient starts at 3.5% B and ramped to 9% B in 1 minute. The gradient was ramped to 70% B in 37 minutes, 97% B for 12 minutes and then returned to 3.5% B.

**1.8 Mass Spectrometry.**

All data were collected on a Thermo-Finnagan (San Jose, CA) LTQ-FTMS (a hybrid linear ion-trap with a 7 Tesla Fourier transform ion cyclotron resonance MS) with Xcalibur™ 2.0 and fitted with a New Objective Picoview 550 nanospray ionization source. Full scan data were collected at 50,000 resolution (at 400 m/z) with a mass-to-charge ratio (m/z) range of 400 to 2000. The instrument was externally calibrated no less than 5 days prior to acquisition following manufacturer recommended protocol with caffeine, NRFA and Ultramark. All data were collected using data dependent scanning with multistage MS (MS/MS) using collision-induced dissociation (CID) with a 3 m/z isolation width, normalized collision energy of 35, and 30 millisecond activation in the ion-trap MS (unit mass resolution) on the top five most abundant peptides. Charge state screening and monoisotopic precursor selection were enabled. The acquisition has a 30 second dynamic exclusion using an m/z range of 0.01 low to 1.01 high for the exclusion list with an exclusion limit of 500 *m/z* values.

**1.9 Database Searching.**

Database searching was conducted using Thermo-Finnagan Bioworks 3.3.1 SP1. The Human Refseq database was used (download November 2007 from the National Center of Biological Information) for all searches. Prior to the SEQUEST search, the Human Refseq database was indexed for Trypsin (KR), monoisotopic mass, fully enzymatic (cleavage at both sides), molecular weight range of 400-10000, 3 missed cleavage sites, and posttranslational modifications of oxidation of the methionines at 15.99492 Da and alkylation of the cysteines at 57.02146 Da. Mass accuracy was set to 20 parts per million. For all fractions of each pool, individual SEQUEST files were combined using the Bioworks Multiconsensus report function. The rigorous SEQUEST search constraints

were set with a Delta CN ≥ 0.100 and Xcorr vs. charge state of 1.9 for 1+, 2.2 for 2+, and 3.75 for 3+ as suggested by the Human Proteome Organization (HUPO) (13) and 4.0 for 4+. The number of different peptides allowed for protein identification was set to one. The total peak areas were determined using the Bioworks algorithm PepQuan with parameters set to area, mass tolerance of 0.0100, minimum threshold of 1000, number of smoothing points at 5, and including all proteins. The false discovery rate was estimated to be less than 10%. Briefly, a concatenated target-decoy database was created using the human Refseq database. Results were searched against the concatenated database and false positives were estimated as twice the number of passing decoy fragments. The false discovery rate was determined by dividing the false positives by the sum of the true positives and false positives (74).

**1.10 Statistical Analysis.**

Plasma pools vary in the distribution of peptide abundance values due to expected variability in the experimental process. To allow for comparisons across pools, the median-centered natural logarithm of peptide abundance (peak area) within each pool was calculated to standardize abundance values.. Two approaches were used to handle the large amount of missing data which is typical for MS/MS-based proteomic studies. In the first case, assuming missing data represent abundance values below the detection threshold, data were imputed to a value of one-half the minimum intensity for each pool plus a small amount of random error. In the second case, missing data were not imputed and thus no assumptions were made about the source of missing data, such as technical error or the real absence or low abundance of protein in plasma. To identify peptides correlated linearly with the presence of COPD and an increasing rate of lung function decline, the study groups were coded ordinally (control=1, SLW=2 and RPD=3) and regressed against the standardized peptide abundance values. Peptides were included if observed in at least three of the nine sample pools. The non-imputation method requires peptide presence in each of the three study groups for inclusion. Since the condition of normality of each peptide predictor in the linear regression model cannot be guaranteed, empirically derived $p$-values by a permutation test with 1,000 iterations were obtained. Multiple testing was corrected for by calculation of the false discovery rate and the corresponding $q$-values were reported (14, 15, 16, 17).

**1.11 Protein Annotation and Pathway Analysis.**

Mapping of proteins to curated molecular pathways was conducted on Kyoto Encyclopedia of Genes and Genomes (KEGG, at www.genome.jp/kegg/) (18-20). Pathway analysis was conducted with Ariadane Genomics' Pathway Studio™ software version 5.0 (Ariadne Genomics, Inc., Rockville, Maryland). The analysis was manually filtered using the expanded pathway analysis tool and limiting analysis to proteins.

**1.12 Outcome**

Offline SCX fractionation with RP-LC-ESI-MS/MS and robust database searching resulted in the observation of 1,758 unique peptides across all nine pooled samples. The filtering constraints for the imputation and non-imputation methods resulted in 1,133 and 973 peptides, respectively, for statistical analysis. At an FDR level of 10%, a total of 17 peptides were significantly associated with lung function decline for the imputation method, 20 peptides were significant for the non-imputation method, and 7 of these peptides were identified by both methods (Table 2). The regression coefficient from the linear model, along with the associated $q$-value for each method where applicable, were also presented in Table 2 for each unique peptide. A negative regression coefficient estimate indicated linearly decreasing peptide abundance levels across the 3 study groups, from controls to SLW to RPD, while a positive estimate indicated a linear increase in peptide abundance levels from controls to RPD.

**Table 2. Unique peptides differentially expressed across the 3 study groups: smokers without COPD, smokers with COPD with slow $FEV_1$ decline, and smokers with COPD with rapid $FEV_1$ decline. Reported regression coefficient estimates for each peptide were significant at the 10% false discovery rate.**

| | | | No Imputation | | Imputation | |
|---|---|---|---|---|---|---|
| **Peptide sequence** | **Gene symbol** | **Protein Name NCBI GI Number and Accession/Version Number** | **Regression coefficient[1]** | ***q*-value** | **Regression coefficient[1]** | ***q*-value** |
| **Complement System** | | | | | | |
| -.GVFVLNK.- | C3 | complement component C3 gi: 4557385, NP_000055.1 | -0.235 | <0.001 | -3.211 | <0.001 |
| K.KVFLDC*C*NYITELRR.Q | C3 | complement component C3 | 0.575 | <0.001 | na | na |
| R.VVLVAVDK.G | C3 | complement component C3 | -2.323 | <0.001 | na | na |
| K.YFKPGM#PFDLM#VFVTNPDGSPAYR.V | C3 | complement component C3 | -0.863 | 0.094 | -1.397 | <0.001 |
| R.IPIEDGSGEVVLSR.K | C3 | complement component C3 | -2.998 | 0.094 | na | na |
| K.PGFTIVGPNSVQC*YHFGLSPDLPIC*K.E | CFH | complement factor H gi: 4504375, NP_000177.1 | -0.547 | <0.001 | na | na |
| K.SSNLIILEEHLK.N | CFH | complement factor H | -0.705 | 0.059 | na | na |
| K.VKDISEVVTPR.F | CFB | complement factor B gi: 4502397, NP_001701.1 | 0.603 | <0.001 | 0.603 | 0.087 |
| R.RPASPISTIQPK.A | C8G | complement component 8, gamma polypeptide gi: 109731764, AAI13627.1 | 0.801 | <0.001 | na | na |
| R.VPANLENVGFEVQTAEDDLKTDFYK.D | C6 | complement component 6 gi: 4559406, NP_000056.1 | na | na | -2.450 | <0.001 |
| R.HLVPGAPFLLQALVR.E | C4A | complement component 4A (Rodgers blood group) gi:14577919, NP_009224.1 | na | na | -3.720 | 0.087 |
| R.LLEPHC*FPLSLVPTEFC*PSPPALK.D | C7 | complement component 7 gi: 45580688, NP_000578.2 | na | na | -2.111 | 0.087 |
| **Coagulation System** | | | | | | |
| R.LTIGEGQQHHLGGAK.Q | FGG | fibrinogen gamma chain gi: 4503715, NP_000500.1 | 1.145 | 0.059 | na | na |
| K.EKGEIQNILQK.V | KLKB1 | kallikrein B, plasma (Fletcher factor) 1 gi: 4504877, NP_000883.1 | 2.543 | <0.001 | na | na |
| K.FEVQVTVPK.I | A2M | alpha 2 macroglobulin gi: 4557225, NP_000005.1 | -0.329 | <0.001 | na | na |
| R.KAAISGENAGLVR.A | ITIH1 | inter-alpha (globulin) inhibitor H1 gi: 4504781, NP_002206.1 | 0.272 | <0.001 | na | na |

| | | | | | | |
|---|---|---|---|---|---|---|
| K.GFPIKEDFLEQSEQLFGAKPVSLTGK.Q | SERPINF2 | serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 2 gi:260064050, NP_001159393 | na | na | -2.183 | 0.087 |
| K.TSDQIHFFFAK.L | SERPINC1 | serpin peptidase inhibitor, clade C (antithrombin), member 1 gi: 4502261, NP_000479.1 | na | na | -4.296 | 0.087 |
| **Anti-protease** | | | | | | |
| R.NLAVSQVVHK.A | SERPINA3 | serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3 gi:4501843, NP_001076.1 | -0.226 | <0.001 | na | na |
| K.VLSALQAVQGLLVAQGR.A | AGT | Angiotensinogen gi: 4557287, NP_000020.1 | -1.132 | <0.001 | -1.132 | <0.001 |
| K.DPTFIPAPIQAK.T | AGT | Angiotensinogen | -0.319 | <0.059 | na | na |
| **Metabolic** | | | | | | |
| R.EYSGTIASEANTYLNSK.S | APOB | apo-B100 precursor gi:105990532, NP_000375.2 | 0.771 | 0.059 | na | na |
| K.DKDQEVLLQTFLDDASPGDKR.L | APOB | apo-B100 precursor | na | na | -2.169 | <0.001 |
| R.ILGEELGFASLHDLQLLGK.L | APOB | apo-B100 precursor | na | na | -2.646 | 0.087 |
| K.KLVPFATELHER.L | APOA4 | apolipoprotein A-IV gi: 4502151, NP000473.1 | -0.458 | <0.001 | -0.458 | <0.001 |
| K.FLNVLSPR.G | IGFBP3 | insulin-like growth factor binding protein 3 gi:114319031, ABI63364.1 | na | na | -3.154 | <0.001 |
| R.VAGLLEDTFPGLLGLR.V | IGFALS | insulin-like growth factor binding protein, acid labile subunit isoform 1 precursor gi:225579152,NP_001139478.1 | na | na | -2.214 | 0.087 |
| **Other** | | | | | | |
| R.C*EGPIPDVTFELLR.E | A1BG | alpha-1-B glycoprotein gi: 21071030, NP_570602.2 | -1.037 | 0.059 | -2.196 | <0.001 |
| K.NGVAQEPVHLDSPAIK.H | A1BG | alpha-1-B glycoprotein gi: 21071030, NP_570602.2 | 0.238 | <0.001 | 0.238 | 0.087 |
| K.SEDC*FILDHGK.D | GSN | gelsolin (amyloidosis, Finnish type) gi:55960302, CAI14416.1 | na | na | -0.303 | 0.087 |

na, not applicable. False discovery rate > 10% for this analysis method.

[1]A negative regression coefficient estimate indicates decreasing peptide abundance levels across the 3 study groups, from controls to SLW to RPD, while a positive estimate indicates increasing peptide abundance levels across the 3 study groups.



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The 30 unique peptides identified as differentially expressed across the 3 study groups by linear regression mapped to 21 unique proteins. In Table 2 the peptides are grouped according to major function. The majority of the identified peptides (17/30), representing 12 proteins, are involved in the complement cascade which, as part of the innate immune system, promotes host defense mechanisms of bacterial lysis, phagocytosis, and immune cell recruitment and activation (21, 22). Regression analysis across the three study groups indicated a mixed pattern of over- and under-expression among the 17 complement-related peptides.

*SERPINA3*, or serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3, is relatively underexpressed in the RPD group compared with the SLW group, and highest levels are in the control group. *SERPINA3* is a protease inhibitor and lower levels of this protein in the RPD support an imbalance in proteases / anti-proteases in the RPD population. Certain allelic variants of *SERPINA3* result in reduced protease inhibitor activity and have been associated with COPD (23-25).

Fibrinogen, kallikrein B and inter-alpha (globulin) inhibitor H1, all components of the coagulation system, are relatively over expressed in the COPD groups compared with the control group, with the highest levels in the RPD group. Coagulation is a complex cascade involving plasma proteins and platelets that results in blood clot formation (26). Circulating clotting factors and their proteases and antiproteases regulate this process (27) and the coagulation system is thought to be involved in the thromboembolic complications associated with COPD and smoking (28, 29,30). Plasma levels of antithrombin (*SERPINC1*), a component of one of the principal intrinsic anticoagulant systems (31), and alpha-2 antiplasmin, a major regulator of intravascular fibrinolysis (32), are highest in the control group and lowest in the rapid $FEV_1$ decline COPD group.

Alpha-2 antiplasmin is also involved in the renin-angiotensin system (RAS) as a critical regulator of angiotensin II-mediated vascular remodeling (Huo 2008). Angiotensinogen is an inactive circulating substrate which is converted by renin to angiotensin I, the precursor peptide in the classical RAS cascade (33). The circulating and local tissue renin-angiotensin systems are involved in vascular remodeling (34) and play pivotal pathophysiological roles in hypertension (35) and diabetes (33). Experimental evidence suggests that oxidant stress-induced damage of lung microvascular endothelial cells in cigarette smokers results in endothelial cell apoptosis, capillary loss, impaired angiogenesis, and profound airspace enlargement (29).

Expression of two insulin-like growth factor binding proteins (*IGFBP3* and *IGFALS*) is lowest in the RPD COPD group and highest in the control group. This possibly reflects the lower levels of anabolic hormones, such as insulin-like growth factors and testosterone, found in chronic inflammatory muscle-wasting conditions such as COPD, chronic heart failure, acquired immunodeficiency syndrome, and cancer (36, 37).

Three peptides mapping to apolipoprotein B100 had a mixed pattern of differential expression across the study groups. Apolipoprotein B is the major structural protein of very low- and low-density lipoproteins (VLDL, LDL), and apoB-containing lipoproteins transport cholesterol from the liver and gut to peripheral tissues (38). On the other hand, apolipoprotein A-IV is the major protein component of high-density lipoproteins (HDL) which reverse transport cholesterol from the periphery to the liver for excretion (38) and constitutes a potent endogenous inhibitor of lipid oxidation (39). Apolipoprotein A-IV is relatively underexpressed in the COPD-RPD group compared with the COPD-SLW and control groups.

Gelsolin (*GSN*) is an actin-binding protein involved in regulating host response to cellular damage in bacterial sepsis (40). Two peptides with opposite directions of differential expression across the study groups mapped to alpha-1B-glycoprotein, a plasma protein of unknown function.

Ariadne Genomics' Pathway Studio™ identified nine other proteins with multiple connections to the 21 proteins identified as differentially expressed across the three groups in this study (Figure 1). These included insulin (*INS*, gi:307072, AAA59179.1), plasminogen (*PLG*, gi:387026, AAA60113.1), fibrinogen alpha (*FGA*, gi 11761629, NP_068657.1), coagulation factor 2 (*F2*, gi4503635 NP_000497.1), interleukin 6 (*IL6*, gi:10834984, NP_000591.1) and interleukin 1 beta (*IL1B*, gi:386816, AAA74137.1), signal transducer and activator of transcription 3 (*STAT3*, gi:21618340, NP_644805.1), cyclin dependent kinase 2 (*CDK2*, gi:30582481, AAP35467.1) and FAM3A (family with sequence similarity 3, member A, gi:57284179, CAI43239.1). Changes in the regulation of plasminogen and its role in coagulation have been associated with smoking and COPD (28). *IL6* and *IL1B* promote the inflammatory response and both have been observed to be increased in the sputum (and *IL6* in serum) of smokers and persons with COPD (41, 42, 43, 30).

Furthermore, in a large genome-wide association study, a specific small nucleotide polymorphism found in the *IL6* receptor gene is identified as associated with COPD (44). This suggests that *IL6* signaling may be an important pathway in COPD. An *IL1B* gene polymorphism has also been linked with COPD in a Korean population (45). In addition to the human data linking *ILB* to COPD, a recent mouse model overexpressing *IL1B* in the lung demonstrated similar tissue changes with inflammation, tissue remodeling and distal airway enlargement (46). Of the 9 additional proteins found by pathway analysis, insulin, plasminogen, interleukin 6 and interleukin 1 beta had the greatest number of interactions with the 21 differentially expressed proteins observed, suggesting that the these additional proteins may represent common mechanistic pathways for COPD in cigarette smokers and for rate of lung function decline in COPD.

**1.13 Summary**

Using high-resolution MS proteomics and two rigorous statistical methods, multiple peptides were identified whose expression is linearly correlated across three groups of cigarette smokers classified spirometrically as having COPD with slow or no lung function decline, COPD with rapid decline and an unaffected control group. Thirty unique peptides, representing 21 proteins, differentiated the three groups. The majority of the peptides observed are components of the complement or coagulation cascades, consistent with the chronic and abnormal inflammatory response that is the hallmark of COPD and which is often associated with a prothrombotic state (28). Ariadne Genomics' Pathway Studio™ analysis identified nine additional proteins that had multiple interactions with the 21 observed proteins. Interestingly, the four proteins with the greatest number of interactions with the 21 differentially expressed proteins were insulin, plasminogen, interleukin 6, and interleukin 1 beta, all of which have been previously associated with COPD or its complications. Insulin resistance, metabolic syndrome and diabetes have been shown to be associated with COPD (47, 48,37). Both COPD and metabolic syndrome/insulin resistance appear to be systemic proinflammatory, prothrombotic disorders with significant associated, and often common, comorbidities (47,36, 48).

There is increasing evidence that the clinical features of COPD correlate poorly with airflow limitation as measured by spirometry (8) and, therefore, that spirometric parameters alone were inadequate as diagnostic and prognostic biomarkers for this complex disease (73). A more comprehensive evaluation using a multidimensional index (BODE) that incorporates *b*ody mass index, airflow *o*bstruction, *d*yspnea, and *e*xercise capacity, has been shown to be more predictive of mortality than $FEV_1$ alone (49).

Although offline peptide fractionation enables the identification of a greater number of low-abundance plasma proteins, offline fractionation adds to the instrument time required for the data collection from each sample. In this study, each pooled sample was fractionated offline into 10 well-separated fractions, thus increasing the data collection time by a factor of 10. In the interest of reasonable data collection times, the 18 plasma samples in each study group

were grouped into 3 pools of 6 samples each, for a total of 90 RP-LC-MS/MS samples in the study, each requiring approximately 2 hours per data collection (not including blanks and quality controls collected every 10 samples). A disadvantage of sample pooling is the inability to collect information on individual variation. However, a benefit is the dilution of undesired individual variation (noise), and the amplification of any signal, by the factor of dilution (*i.e.*, 6 in this study).

**Example 2: Differential Protein Expression Among Two Groups Of Adult Cigarette Smokers With Mild To Moderate COPD But Different Rates Of Lung Function Decline**

The plasma proteomes of 40 adult cigarette smokers with mild to moderate COPD were analyzed. Subjects were clinically characterized as having either rapid decline (RPD, n = 20) or slow to no decline (SLW, n = 20) in $FEV_1$ over a five-year interval. The accurate mass and time (AMT) tag technology utilized is a comprehensive high-throughput proteomic approach based upon a putative time and mass tag database (PMT), high resolution LC separations and high mass accuracy measurements using FT-ICR MS with a 9.4-tesla magnetic field (50-53). Proteins identified as differentially abundant between the two clinical COPD categories (RPD vs. SLW) are exemplary biomarkers of rate of lung function decline in COPD and are useful for monitoring and/or determining disease progression in a subject

**2.1 Subjects**

Subjects were selected from the 624 participants in the Lung Health Study (LHS) at the University of Utah study center. LHS was a prospective, randomized, multicenter clinical study sponsored by the National Heart, Lung, and Blood Institute (NHLBI) which enrolled male and female otherwise healthy cigarette smokers, aged 35–60 years, with mild or moderate COPD during 1986–1989 (11). Lung function was measured by spirometry at baseline, annually for 5 years, and once during 1998–2001 (12). A subset of 244 participated in the Genetics of Nicotine Addiction Project (GAP) during 2003–2004 in which lung spirometry and smoking status were assessed and a plasma sample for proteomic analysis was obtained.

Lung function was assessed as $FEV_1$ and $FEV_1$%predicted (*e.g.*, adjusted for age, sex, and height) (1). The annualized rate of lung function decline during the 5 years of LHS was calculated for each participant as the slope of the linear regression of $FEV_1$% predicted. The 20 subjects with the steepest rate of decline in $FEV_1$ (rapid decliners, RPD) and the 20 subjects with the least steep or no annualized rate of decline in $FEV_1$ (slow decliners, SLW) were selected for proteomic analysis. Characteristics of the study groups are shown in Table 3. Over the first 5 years of LHS, the rapid decliners had an average annual decrease in $FEV_1$ of 1.52%predicted/year, while the slow decliners had an average increase of 0.73%predicted/year. At the end of LHS, 5/20 (25%) of the RPD and 9/18 (50%) of the SLW participants no longer smoked. At the GAP assessment approximately 12 years later, 8/20 (40%) of the RPD and 11/18 (61%) of the SLW participants no longer smoked. Two SLW subjects had unacceptably low plasma peptide levels and were excluded from this proteomic analysis.

**Table 3. Characteristics of study subjects**

| | RPD | | | SLW | | |
|---|---|---|---|---|---|---|
| **Characteristic** | **Baseline** | **Year 5** | **GAP** | **Baseline** | **Year 5** | **GAP** |
| No. of subjects | | 20 | | | 18 | |
| Male, % | | 70 | | | 67 | |
| Age, mean (SD) | | | 64.9 (5.1) | | | 64.4 (7.4) |
| Cigarettes per day, mean (SD) | 36.4 (16.6) | 26.5 (13.5) | 15.9 (16.8) | 30.2 (10.7) | 10.6 (13.4) | 7.2 (11.0) |
| Years smoked, mean (SD) | 30.5 (5.2) | - | 43.0 (6.9) | 29.1 | - | 35.8 (9.3) |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | (6.5) | | |
| $FEV_1$(L), mean (SD) | 2.7 (0.57) | 2.33 (0.63) | 1.68 (0.59) | 2.73 (0.63) | 2.73 (0.69) | 2.4 (0.65) |
| $\Delta FEV_1$(mL/year), mean (SD)[a] | | -75.0 (47.3) | | | 4.9 (43.7) | |
| $FEV_1$ %predicted, mean (SD) | 75.3 (10.28) | 67.7 (12.89) | 54.6 (16.95) | 75.6 (10.0) | 78.7 (12.18) | 76.6 (14.6) |
| $\Delta FEV_1$ %predicted/year, mean (SD)[b] | | -1.52 | | | 0.73 | |

$FEV_1$, forced expiratory volume in one second; RPD, $FEV_1$ rapid decliner group; SLW, $FEV_1$ slow decliner group; GAP, Genetics of Nicotine Addiction Project, an average of 17 years after baseline
a Difference in $FEV_1$ (mL/year) at Year 5 from baseline; for RPD *vs.* SLW, $p<0.001$
b Difference in $FEV_1$ (%predicted) at Year 5 from baseline; for RPD *vs.* SLW, $p<0.001$

**2.2 Plasma Sampling, Processing and MS analysis**

Plasma samples were obtained from each subject at least 2 h after eating by venipuncture using a sodium citrated Vacutainer tube (BD, Franklin Lakes, NJ). Within 10 min of collection, blood was centrifuged at 1500 × g for 15 min at 2–6°C. The top-most plasma was removed and centrifuged at 1500 × g for an additional 15 min. Plasma samples were shipped on dry ice, stored at -80°C and thawed just before analysis. The plasma samples were analyzed using a comprehensive high-throughput proteomic approach, the accurate mass and time (AMT) tag technology, to facilitate comprehensive high-throughput proteomic measurements. This technology is based upon a putative mass and time (PMT) tag database, high resolution LC separations and high mass accuracy measurements using FT-ICR MS with a 9.4-tesla magnetic field (50-53). This approach involved pooling a subset of randomly selected plasma samples after depletion of abundant proteins and digestion with trypsin. A standard shotgun proteomic analysis was performed where the pool was then separated by strong cation exchange and analyzed by reversed phase capillary LC (rp-LC) coupled directly with an electrospray IT mass spectrometer using a data-dependent MS/MS mode. The results were then used to populate the PMT database. All samples were then analyzed using a high resolution FT-ICR MS system. The data analysis incorporated both the FT-ICR MS accurate mass measurements of intact proteins and the PMT database. This two-stage approach utilized FT-ICR MS to validate peptide AMTs from the PMTs identified using the conventional MS/MS method. This approach provided greater confidence in peptide identifications as well as the foundation for later measurements without the need for MS/MS resulted in greater sensitivity and increased throughput (50-53). Details of each step were discussed below.

**2.2.1 Depletion of abundant proteins from plasma**

The 12 most abundant proteins were depleted using GenWay Seppro 12 spin-columns (GenWay Biotech, Inc., San Diego, CA, now ProteomeLab-IgY-12, Beckman Coulter, Inc., Fullerton, CA) following the manufacturer's protocol. The removal of abundant proteins was monitored by SDS-PAGE.

**2.2.2 In-solution tryptic digestion of plasma**

TCA-precipitable protein from the depleted plasma samples was denatured by the addition of urea to 8 M, thiourea to 2 M, DTT to 5mM, and heating to 60°C for 30 min. The sample was then diluted fourfold with 100 mM ammonium bicarbonate, and calcium chloride was added to 1 mM. Methylated, sequencing-grade trypsin (Promega, Madison, WI) was added at a substrate-to-enzyme ratio of 50:1 (mass:mass) and incubated at 37°C for 15 h. Sample cleanup was achieved using a 1-mL SPE $C_{18}$ column (Supelco, Bellefonte, PA). The peptides were eluted from each

column with 1 mL methanol and concentrated via SpeedVac. The samples were reconstituted to 10 μg/μL with 25 mM ammonium bicarbonate and frozen at -20°C until analyzed.

**2.2.3 Strong cation exchange separation**

From all 40 samples, six randomly selected plasma samples were depleted of abundant proteins, digested with trypsin as described above, and pooled. Strong cation exchange chromatography was performed on the pooled peptide sample utilizing a Synchropak S 300, 100 × 2 mm chromatographic column (Thermo Hypersil-Keystone, Bellefonte, PA). A one-hour gradient was utilized at a flow rate of 200 μL/min with fractions collected every 2 min. The beginning solvent system was 25% acetonitrile and 75% water containing 10 mM ammonium formate at pH 3.0, adjusted with formic acid; the ending solvent system was 25% acetonitrile and 75% water containing 200 mM ammonium formate at pH 8.0. The peptide mixture was resuspended in 25% acetonitrile and 75% water containing 10 mM ammonium formate at pH 3.0 with formic acid prior to injection. Fractions were lyophilized and stored at -20°C until LC MS/MS analysis.

**2.2.4 MS/MS analysis of peptides**

Peptide samples were analyzed by reversed phase capillary LC (rp-LC) coupled directly with electrospray tandem mass spectrometers (Thermo Finnigan, models LCQ Duo and DecaXP, San Jose, CA). Chromatography was performed on a 60-cm, 150-μm id × 360-μm od capillary column (Polymicro Technologies, Phoenix, AZ) packed with Jupiter $C_{18}$ 5-μm-diameter particles (Phenomenex, Torrence, CA). A solvent gradient was used to elute the peptides using 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The gradient was linear from 0–5% solvent B in 20 min, followed by 5–70% solvent B in 80 min, and then 70–85% solvent B in 45 min. Solvent flow rate was 1.8 μL/min. The capillary LC system was coupled to a LCQ IT mass spectrometer (Thermo Finnigan, San Jose, CA) using an in-house manufactured ESI interface, in which no sheath gas or makeup liquid was used. The temperature of the heated capillary and the electrospray voltage was 200°C and 3.0 kV, respectively. Samples were analyzed using the data-dependent MS/MS mode over the *m/z* range of 300–2000. The three most-abundant ions detected in each MS scan were selected for collision-induced dissociation.

**2.2.5 Putative mass and time (PMT) tag database from plasma results**

The raw LC-IT data from the pooled sample described above and data from previous multidimensional analysis (54) were reanalyzed to populate the PMT database using a PMT quality score of 1.0 [requires a minimum cross-correlation score (Xcorr) of 2] and a discriminate score of 0.5 (52). This database was used to generate the AMT tag results.

**2.2.6 FT-ICR Mass Spectrometry**

A modified and enhanced Bruker Daltonics 9.4-tesla FT-ICR MS (Bruker Daltonics Inc., Billerica, MA) was employed for the high-throughput proteomics, as described by Belov *et al.* (55). Briefly, the FT-ICR mass spectrometer is combined with the capillary LC system and modified for concurrent internal mass calibration and auto-sampling. Tryptic peptides for each individual sample were resuspended in mobile phase A (0.1% TFA) and analyzed separately using RP capillary LC coupled to an ESI interface with a FT-ICR MS, as previously described (52). Analysis of the LC FT-ICR data was performed using in-house software tools that included ICR-2LS (Pacific Northwest National Laboratory, http://omics.pnl.gov/software/ICR2LS.php). The initial analysis of raw LC FT-ICR data involved a mass transformation or de-isotoping step using ICR-2LS. To generate relative abundances for the peptides, each sample was analyzed by FT-ICR in duplicate.

**2.3 Database searching**

The SEQUEST algorithm (56) was run on each of the datasets against the human protein database from the National Center for Biotechnology Information (RefSeq release 10, March 2005). All data were collected using the multidimensional protein identification technology (MudPIT) approach developed by Yates and coworkers (57, 58). Briefly, all accepted SEQUEST results have a delta Cn of 0.1 or greater. Peptides with a +1 charge state were accepted if they were fully tryptic and have a Xcorr of at least 1.9. Peptides with a +2 charge state were accepted if they were fully tryptic or partially tryptic and have an Xcorr of at least 2.2. Peptides with +2 or +3 charge states with an Xcorr of at least 3.0 or 3.75, respectively, were accepted regardless of their tryptic state (58).

**2.4 Data analysis**

When peptides were detected in some samples but not others, the undetected peptides were considered to be missing. On average, 60% of data (e.g., potential peptides) were missing across all quantitative MS runs. Missing values could be due, in part or in combination, to several sources, including true absence of the peptide in blood plasma, an abundance of peptides at a level below the detection limit of MS, and failure to correctly identify a peptide. Since the source of the missing data was unclear, a single method of handling missing data would not be appropriate for all peptides. Therefore, differences in peptide abundance between RPD and SLW were assessed using three separate statistical methods. In Method 1, missing values represent peptides not observed. In Method 2, missing values were imputed to a value below the detection threshold to account for low abundance peptides. In Method 3, a conservative proxy measure of peptide abundance was calculated that avoids the imputation of data.

Replicates were averaged for Methods 1 and 2. Each run was standardized by the respective median log base 2 intensity value to allow for direct comparisons across all samples. For Method 2, missing data was imputed to a value of one-half the minimum intensity for each run plus a small amount of random error (SD = 0.01). For Method 3, peptide abundance was coded as an ordinal variable corresponding to the number of times the peptide was observed in each replicate (*e.g.*, 0, 1, or 2). This proxy coding correlates significantly with the observed quantitative outcome ($r = 0.45$, $p$-value <0.0001).

Analysis was restricted to peptides present in at least 20% of the samples. Tests for association of peptide abundance using the ordinal variable with RPD-SLW group were carried out using exact logistic regression implemented in the R statistical software program (59). The exact option was used to correct for small cell counts, and parameter estimates were obtained by Markov chain Monte Carlo 100,000 simulations following 1,000 burn-in iterations. For Methods 1 and 2, a two-sided t-test was performed and empirically derived p-values were obtained by 1,000 permutations of the data. The set of empirical p-values were corrected for multiple testing by reporting the false discovery rate (14-17). All statistical analyses were performed using R version 2.5.1 software (http://www.r-project.org).

**2.5 Protein annotation/function**

Mapping of proteins to curated molecular pathways was conducted on the Kyoto Encyclopedia of Genes and Genomes (KEGG, on the world wide web at www.genome.jp/kegg/) (18-20).

**2.6 Outcome**

**2.6.1 Peptide/protein identification and data processing**

A total of 3,549 non-redundant peptides were identified from 80 independent MS runs (2 technical replicates per sample), representing 533 proteins. Overall, the peptide abundance levels from technical replicates were very similar (mean $R^2 = 0.964$, SD = 0.054). The average number of unique peptides detected in all samples was 1,362.46 (SD =

414.17). Two samples (4 MS runs) displayed lower-than-acceptable numbers of peptides, and hence were omitted from further statistical analysis.

**2.6.2 Peptide-level results**

Peptide analysis revealed that 12, 49, and 10 peptides were significant at a false discovery rate of 5% for statistical Methods 1, 2, and 3, respectively. Figure 2 illustrates the overlap on the peptide level among the three statistical methods.

**2.6.3 Protein-level results**

Since each of the statistical methods has its own strengths and weaknesses, the proteomic analysis was conducted on the proteins associated with the non-redundant peptides (Table 4) from all three methods. The 33 proteins associated with the 55 peptides were found by at least one of the three statistical methods and hence were used for subsequent network/pathway analysis. Figure 2 also illustrates the overlap among the statistical methods on the protein level.

**Table 4. Fifty-five unique peptides identified from statistical Methods 1, 2, or 3 as differentially expressed between $FEV_1$ rapid (RPD) and slow (SLW) decliner subjects with chronic obstructive pulmonary disease**

| | | | | Model coefficients[a] | | |
|---|---|---|---|---|---|---|
| Peptide | NCBI Genbank Accession | Gene symbol | Gene name | Method 1 | Method 2 | Method 3 |
| GAAANLELIFVGPQHAGNYR | NP_570602.2 | A1BG | alpha 1B-glycoprotein precursor | 0 | -1.50 | 0 |
| LHDNQNGWSGDSAPVELILSD ETLPAPEFSPEPESGR | NP_570602.2 | A1BG | alpha 1B-glycoprotein precursor | 0 | 2.67 | 0 |
| VTLTCVAPLSGVDFQLR | NP_570602.2 | A1BG | alpha 1B-glycoprotein precursor | 0 | -2.48 | -2.77 |
| ASVSVLGDILGSAMQNTQN | NP_000005.1 | A2M | alpha-2-macroglobulin precursor | 0 | -2.24 | 0 |
| DSFHLDEQFTVPVEMMQAR | NP_000925.1 | SERPIN F2 | alpha-2-plasmin inhibitor | 1.14 | 1.39 | 0 |
| EATEGKIQEFLSGLPEDTVLLL LNAIHFQGFWR | NP_000925.1 | SERPIN F2 | alpha-2-plasmin inhibitor | 0 | -3.32 | -1.99 |
| GFPRGDKLFGPDLK | NP_000925.1 | SERPIN F2 | alpha-2-plasmin inhibitor | 0 | -0.88 | 0 |
| AGANVLAKNK | NP_055757.1 | ANKRD 6 | ankyrin repeat domain 6 | 0 | 2.37 | 1.81 |
| PYADEFK | NP_000473.1 | APOA4 | apolipoprotein A-IV precursor | 0 | 1.19 | 0 |
| ALYWVNGQVPDGVSK | NP_000375.1 | APOB | apolipoprotein B precursor | 0 | 1.80 | 0 |
| IAELSATAQEIIK | NP_000375.1 | APOB | apolipoprotein B precursor | 0.94 | 0 | 0 |
| LLLQMDSSATAYGSTVSKR | NP_000375.1 | APOB | apolipoprotein B precursor | -1.82 | 0 | 0 |
| KLISVDTEHSNIYLQNGPDR | NP_000087.1 | CP | ceruloplasmin precursor | 0 | 1.80 | 0 |
| MFGNLQGLTMHVGDEVNWYL MGMGNEIDLHTVHFHGHSFQY K | NP_000087.1 | CP | ceruloplasmin precursor | 0 | 2.00 | 0 |
| TLLSNLEEAK | NP_001822.2 | CLU | clusterin isoform 1 | 0 | -0.86 | 0 |
| ETYDFDIAVLR | NP_000495.1 | F10 | coagulation factor X preproprotein | 0 | -1.17 | 0 |
| SSNNPHSPIVEEFQVPYNK | NP_001725.1 | C1S | complement component 1, s subcomponent | 0 | 2.65 | 2.02 |
| AGDFLEANYMNLQR | NP_000055.1 | C3 | complement component 3 | 0.84 | 0.84 | 0 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | precursor | | | |
| PLSWDIPELVNMGQWK | NP_000055.1 | C3 | complement component 3 precursor | 0 | 1.89 | 0 |
| QKPDGVFQEDAPVIHQEMIGG LR | NP_000055.1 | C3 | complement component 3 precursor | 0 | 2.19 | 0 |
| QLYNVEATSYALLALLQLKDF DFVPPVVR | NP_000055.1 | C3 | complement component 3 precursor | 0 | 2.78 | 0 |
| RQGALELIK | NP_000055.1 | C3 | complement component 3 precursor | -0.63 | 0 | 0 |
| SPYQIHFTK | NP_000055.1 | C3 | complement component 3 precursor | 0 | -1.22 | 0 |
| SYTVAIAGYALAQMGR | NP_000055.1 | C3 | complement component 3 precursor | 0 | 2.41 | 0 |
| NPSDPMPQAPALWIETTAYAL LHLLLHEGKAEMADQAAAWL TR | NP_00100202 9 | C4B | complement component 4B preproprotein | 0 | -2.23 | 0 |
| PGNSDPNMIPDGDFNSYVR | NP_00100202 9.1 | C4B | complement component 4B preproprotein | 1.13 | 0 | 0 |
| EKFSDASYQSINIPVTQNMVPS SR | NP_001726.1 | C5 | complement component 5 | 0 | 1.41 | 0 |
| FSDASYQSINIPVTQNMVPSSR | NP_001726.1 | C5 | complement component 5 | 0 | 1.55 | 0 |
| AKDLHLSDVFLK | NP_000056.1 | C6 | complement component 6 precursor | 0 | -0.89 | 0 |
| DISEVVTPR | NP_001701.1 | CFB | complement factor B preproprotein | 0 | 3.53 | 2.63 |
| HVIILMTDGLHNMGGDPITVID EIRDLLYIGK | NP_001701.1 | CFB | complement factor B preproprotein | 0 | 3.73 | 2.39 |
| AVPHPDSQPDTIDHDLLLLQLS EK | NP_001919 | CFD | complement factor D preproprotein | 0 | 1.24 | 0 |
| NDFTWFK | NP_000177.1 | CFH | complement factor H isoform a precursor | 0 | -1.46 | 0 |
| YQIWTTVVDWIHPDLKR | NP_000195.1 | CFI | complement factor I preproprotein | 0 | -1.60 | 0 |
| PNMIDAATLK | NP_000500.1 | FGG | fibrinogen, gamma chain isoform gamma-A | -1.42 | 0 | 0 |
| TYNPDESSKPNMIDAATLK | NP_000500.1 | FGG | fibrinogen, gamma chain isoform gamma-A | 0 | -2.39 | 0 |
| GATYNIIVEALKDQQR | NP_002017 | FN1 | fibronectin 1 isoform 3 preproprotein | 0 | 3.00 | 2.00 |
| TPFVTHPGYDTGNGIQLPGTSG QQPSVGQQMIFEEHGFRR | NP_002017 | FN1 | fibronectin 1 isoform 3 preproprotein | 0 | 2.19 | 1.84 |
| AGALNSNDAFVLK | NP_000168.1 | GSN | gelsolin isoform a precursor | 1.18 | 1.49 | 0 |
| IEGSNKVPVDPATYGQFYGGD SYIILYNYR | NP_000168.1 | GSN | gelsolin isoform a precursor | 1.27 | 1.57 | 0 |
| AAISGENAGLVR | NP_002206.1 | ITIH1 | inter-alpha (globulin) inhibitor H1 | 0 | -3.74 | 0 |
| AHNVVTMR | NP_002208.1 | ITIH3 | inter-alpha (globulin) inhibitor H3 | 0 | -1.53 | 0 |
| ANTVQEATFQMELPK | NP_002209.2 | ITIH4 | inter-alpha (globulin) inhibitor H4 | 0 | 1.99 | 0 |
| RLGVYELLLK | NP_002209.2 | ITIH4 | inter-alpha (globulin) inhibitor H4 | 0 | -2.75 | 0 |
| LWAYLTINQLLAER | NP_002207.1 | ITIH2 | inter-alpha globulin | 0 | -1.80 | 0 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | inhibitor H2 polypeptide | | | |
| SLPLLMDSVIQALAELEQKVPA AK | NP_443122.2 | PGLYR P2 | peptidoglycan recognition protein 2 precursor | 0 | -3.53 | -3.41 |
| EIIIHQNYK | NP_000883.1 | KLKB1 | plasma kallikrein B1 precursor | 0 | 3.01 | 0 |
| NNEEYLALIFEKGGSYLGR | NP_002817.2 | QSOX1 | quiescin Q6 sulfhydryl oxidase 1 isoform a | 0 | -1.75 | 0 |
| ELTPEVLQEWLDELEEMMLVV HMPR | NP_000479.1 | SERPIN C1 | serine (or cysteine) proteinase inhibitor, clade C *(antithrombin), member 1; antithrombin* | 0 | 2.56 | 0 |
| FATTFYQHLADSKNDNDNIFLS PLSISTAFAMTK | NP_000479.1 | SERPIN C1 | serine (or cysteine) proteinase inhibitor, clade C *(antithrombin), member 1; antithrombin* | 0 | 3.29 | 0 |
| LYGSEAFATDFQDSAAAK | NP_001076.1 | SERPIN A3 | serpin peptidase inhibitor, clade A, member 3 | 0.88 | 0.88 | 0 |
| NLAVSQVVHK | NP_001076.1 | SERPIN A3 | serpin peptidase inhibitor, clade A, member 3 | -0.59 | 0 | 0 |
| KALQTEMAR | NP_000233.1 | MBL2 | soluble mannose-binding lectin precursor | 0 | -1.51 | 0 |
| GILGKDVSGFSDPYCLLG | NP_954712.1 | UNC13 D | unc-13 homolog D | 0 | 0.79 | 2.58 |
| DWHGVPGQVDAAMAGR | NP_000629.2 | VTN | vitronectin precursor | 0.79 | 0.79 | 0 |

a) Positive coefficient indicates more peptide in RPD *vs.* SLW; negative coefficient indicates less peptide in RPD *vs.* SLW; non-zero coefficients were significant at a false discovery rate <5%

This example used an untargeted global proteomic approach to investigate novel plasma proteins associated with the rate of $FEV_1$ decline in cigarette smokers with COPD. Using three rigorous statistical methods, multiple peptides were found that discriminated between COPD subjects with rapid and slow decline in lung function. The combined analysis identified 55 peptides that putatively correspond to 33 proteins. The majority of these proteins reside in the coagulation and complement cascades, as identified by KEGG (18-20) (Fig. 3). Of these proteins, only serum fibrinogen has been previously linked to an accelerated decline of lung function in COPD subjects (60-62). These proteins represent biomarkers for lung function decline in COPD.

**2.6.4 Complement.**

The complement system, a complex, multi-protein cascade, is part of the innate immune system and plays an important role in host defenses by promoting bacterial lysis, phagocytosis, and immune cell recruitment and activation (21, 22). In the present example, 12 of the identified proteins are known to be involved in the complement system (Fig. 3). Chronic inflammation and an abnormal inflammatory response to noxious inhaled particles or gases were considered to be key pathogenic mechanisms in COPD (1, 60, 63, 64). Exacerbations of COPD and decline in lung function have been linked to bacterial and viral pulmonary infections (33-35). It is therefore possible that the differential complement activity suggested between the RPD and SLW in this study is the result of an ongoing or recurrent microbial stimulus in the lungs of the rapid decliners, leading to direct complement activation as well as activation secondary to the antibody response to these microbes by the adaptive immune system. A boosted adaptive immune response to viral infections and bacterial colonization of the airways can also result in some subjects with COPD, even after smoking cessation. Molecular mimicry between foreign microbial antigens and self-determinants can result in self-reactivity with autoantibody formation to autoantigens and subsequent complement activation (6, 65). Chronic inflammation in COPD is associated with increased levels of interleukin (IL)-6, which subsequently increases hepatic synthesis of

proinflammatory and prothrombotic proteins such as C-reactive protein, fibrinogen and other coagulation factors (4, 66). A recent genetic association study found that the IL-6R locus is associated with COPD (67). IL-6 expression, which is also increased in cigarette smokers (30, 43), has been reported to influence complement 3 and 4 gene expression (68).

In addition to the complement pathway, other proteins were identified that have been shown to play a role in bacterial defenses. Peptidoglycan recognition protein 2 is a bacterial binding protein that is produced by the liver and is part of the non-complement-related innate immune system (69). It may reflect a greater bacterial load in COPD subjects with accelerated lung function decline. Gelsolin is an actin-binding plasma protein that has been reported to be important in regulating the host response to cellular damage that occurs during bacterial sepsis (40). Another identified protein, inter-alpha-inhibitor, plays a role in coagulation during endotoxic shock (70). The identification of these proteins and components of the complement pathway as differentially expressed between rapid and slow $FEV_1$ decliners in COPD suggests that bacterial host defenses may be playing a role in the progression and severity of COPD.

**2.6.5 Coagulation**

Coagulation involves a complex cascade of plasma protein and platelet activation that results in blood clot formation (26). This process is regulated by circulating clotting factors and their proteases and antiproteases (27). In this study, 12 interleukinoteins that were involved in coagulation were identified as differentially expressed between RPD and SLW (Fig. 3). COPD is associated with higher levels of procoagulant proteins in the blood (71,72). Coagulation may play an important role in COPD progression and has been implicated in the thromboembolic complications associated with COPD and smoking (30, 28, 29). Except for fibrinogen, described above, the proteins identified in the present study differed from those previously reported.

**THE USE OF ADDITIONAL PROTEIN BIOMARKERS**

The proteins identified in Examples 1 and 2 may be employed with other protein biomarkers of lung function for use in the methods described herein, such as methods of diagnosing lung disease, providing a prognosis to a subject having lung disease, and/or distinguishing individuals with rapid or slow decline in lung function. The proteins identified in Examples 1 and 2 may also be employed with other protein biomarkers of lung function in forming compositions for use conducting the methods described herein. A number of such protein biomarkers have been described. In one embodiment such protein biomarkers include those protein biomarkers capable of use in distinguishing between subjects with rapidly declining pulmonary function and slowly declining pulmonary function. One group of such markers is listed in Table 5a and was described in WO 20081003066 A2, which is hereby incorporated by reference. Those proteins described in WO 20081003066 A2 as having a two-fold or greater difference in abundance between slow decline conditions and rapid decline conditions are listed in Table 5b. In one embodiment, any number of the proteins of either Table 5a or 5b can be employed in the methods described herein with proteins identified in the present study.

**Table 5a Proteins Identified in WO20081003066 A2**

| NCBI GI and Accession Number/Verison R | Protein Description |
|---|---|
| gi\|4501987\|ref\|NP_001124.1\| | afamin precursor; alpha-albumin [Homo sapiens] |
| gi\|4502027\|ref\|NP_000468.1\| | albumin precursor; PRO0883 protein [Homo sapiens] |
| gi\|21071030\|ref\|NP_570602.2\| | alpha 1B-glycoprotein [Homo sapiens] |
| gi\|4501843\|ref\|NP_001076.1\| | alpha-1-antichymotrypsin, precursor; alpha-1-antichymotrypsin; antichymotrypsin [Homo sapiens] |
| gi\|4557225\|ref\|NP_000005.1\| | alpha-2-macroglobulin precursor [Homo sapiens] |
| gi\|11386143\|ref\|NP_000925.1\| | alpha-2-plasmin inhibitor; alpha-2-antiplasmin [Homo sapiens] |
| gi\|4557287\|ref\|NP_000020.1\| | angiotensinogen precursor; angiotensin II precursor; pre-angiotensinogen; angiotensin I [Homo sapiens] |
| gi\|4557321\|ref\|NP000030.1\| | apolipoprotein A-I precursor [Homo sapiens] |
| gi\|4502149\|ref\|NP001634.1\| | apolipoprotein A-II precursor [Homo sapiens] |
| gi\|4502151\|ref\|NP000473.1\| | apolipoprotein A-IV precursor [Homo sapiens] |
| gi\|4502153\|ref\|NP_000375.1\| | apolipoprotein B precursor; apoB-100; apoB-48 [Homo sapiens] |
| gi\|4502157\|ref\|NP_001636.1\| | apolipoprotein C-I precursor [Homo sapiens] |
| gi\|4557325\|ref\|NP_000032.1\| | apolipoprotein E precursor; apolipoprotein E3 [Homo sapiens] |
| gi\|4557327\|ref\|NP_000033.1\| | beta-2-glycoprotein 1 precursor [Homo sapiens] |
| gi\|4557373\|ref\|NP_000051.1\| | biotinidase precursor [Homo sapiens] |
| gi\|4502517\|ref\|NP_001729.1\| | carbonic anhydrase I; carbonic dehydratase [Homo sapiens] |
| gi\|4503011\|ref\|NP_001299.1\| | carboxypeptidase N, polypeptide 1, 50kD precursor [Homo sapiens] |
| gi\|4557485\|ref\|NP_000087.1\| | ceruloplasmin (ferroxidase); Ceruloplasmin [Homo sapiens] |
| gi\|42716297\|ref\|NP_001822.2\| | clusterin isoform 1; complement-associated protein SP-40 [Homo sapiens] |
| gi\|4503635\|ref\|NP_000497.1\| | coagulation factor II precursor; prothrombin [Homo sapiens] |
| gi\|4503625\|ref\|NP_000495.1\| | coagulation factor X precursor; prothrombinase; factor Xa [Homo sapiens] |
| gi\|4557379\|ref\|NP_000053.1\| | complement component 1 inhibitor precursor [Homo sapiens] |
| gi\|4502493\|ref\|NP_001724.1\| | complement component 1, r subcomponent [Homo sapiens] |
| gi\|7706083\|ref\|NP_057630.1\| | complement component 1, r subcomponent-like precursor; complement C1r-like proteinase; C1r-like serine protease analog [Homo sapiens] |
| gi\|4502495\|ref\|NP_001725.1\| | complement component 1, s subcomponent [Homo sapiens] |
| gi\|14550407\|ref\|NP_000054.2\| | complement component 2 precursor; C3/C5 convertase [Homo sapiens] |
| gi\|4557385\|ref\|NP_000055.1\| | complement component 3 precursor; acylation-stimulating protein cleavage product [Homo sapiens] |
| gi\|4502503\|ref\|NP_000706.1\| | complement component 4 binding protein, alpha; Complement component 4-binding protein, alpha polypeptide; complement component 4-binding protein, alpha [Homo sapiens] |
| gi\|50345296\|ref\|NP_00100202 9.1\| | complement component 4B preproprotein; Chido form of C4; basic C4; C4A anaphylatoxin [Homo sapiens] |
| gi\|38016947\|ref\|NP_001726.2\| | complement component 5 [Homo sapiens] |
| gi\|4559406\|ref\|NP_000056.1\| | Complement component 6 precursor [Homo sapiens] |
| gi\|45580688\|ref\|NP_000578.2\| | complement component 7 precursor [Homo sapiens] |
| gi\|4557389\|ref\|NP_000553.1\| | complement component 8, alpha polypeptide precursor [Homo sapiens] |
| gi\|4502511\|ref\|NP_001728.1\| | complement component 9 [Homo sapiens] |
| gi\|4502397\|ref\|NP_001701.1\| | complement factor B preproprotein; C3 proactivator; C3 proaccelerator; glycine-rich beta-glycoprotein; C3/C5 convertase [Homo sapiens] |
| gi\|4504375\|ref\|NP_000177.1\| | complement factor H; H factor-1 (complement); factor H-like 1; H factor 2 (complement); H factor 1 (complement) [Homo sapiens] |
| gi\|11761629\|ref\|NP_068657.1\| | fibrinogen, alpha chain isoform alpha preproprotein [Homo sapiens] |
| gi\|11761631\|ref\|NP_005132.1\| | fibrinogen, beta chain preproprotein [Homo sapiens] |
| gi\|4503715\|ref\|NP_000500.1\| | fibrinogen, gamma chain isoform gamma-A precursor [Homo sapiens] |
| gi\|47132557\|ref\|NP_997647.1\| | fibronectin 1 isoform 1 preproprotein; cold-insoluble globulin; migration-stimulating factor [Homo sapiens] |
| gi\|4504165\|ref\|NP_000168.1\| | gelsolin isoform a [Homo sapiens] |
| gi\|11321561\|ref\|NP_000604.1\| | hemopexin [Homo sapiens] |
| gi\|4504355\|ref\|NP_000176.1\| | heparin cofactor II [Homo sapiens] |
| gi\|4504579\|ref\|NP_000195.1\| | I factor (complement) [Homo sapiens] |

| NCBI GI and Accession Number/Verison R | Protein Description |
|---|---|
| gi\|21489959\|ref\|NP_653247.1\| | immunoglobulin J chain [Homo sapiens] |
| gi\|4504781\|ref\|NP_002206.1\| | inter-alpha (globulin) inhibitor H1; inter-alpha (globulin) inhibitor, H1 polypeptide [Homo sapiens] |
| gi\|4504783\|ref\|NP_002207.1\| | inter-alpha (globulin) inhibitor H2; inter-alpha (globulin) inhibitor, H2 polypeptide [Homo sapiens] |
| gi\|10092579\|ref\|NP_002208.1\| | inter-alpha (globulin) inhibitor H3; Inter-alpha (globulin) inhibitor, H3 polypeptide; pre-alpha (globulin) inhibitor, H3 polypeptide [Homo sapiens] |
| gi\|31542984\|ref\|NP_002209.2\| | inter-alpha (globulin) inhibitor H4 (plasma Kallikrein-sensitive glycoprotein); Inter-alpha (globulin) inhibitor, H4 polypeptide; inter-alpha (globulin) inhibitor, H polypeptide-like 1 [Homo sapiens] |
| gi\|10835141\|ref\|NP_000563.1\| | interleukin 10 precursor; cytokine synthesis inhibitory factor [Homo sapiens] |
| gi\|4504893\|ref\|NP_000884.1\| | kininogen 1; alpha-2-thiol proteinase inhibitor; bradykinin [Homo sapiens] |
| gi\|4505047\|ref\|NP_002336.1\| | lumican [Homo sapiens] |
| gi\|33188445\|ref\|NP_036222.3\| | microfilament and actin filament cross-linker protein isoform a; actin cross-linking factor; 620 kDa actin binding protein; macrophin 1; trabeculin-alpha; actin cross-linking family protein 7 [Homo sapiens] |
| gi\|19923106\|ref\|NP_000437.3\| | paraoxonase 1; Paraoxonase [Homo sapiens] |
| gi\|21361845\|ref\|NP_443122.2\| | peptidoglycan recognition protein L precursor [Homo sapiens] |
| gi\|4504877\|ref\|NP_000883.1\| | plasma kallikrein B1 precursor; kallikrein 3, plasma; Kallikrein, plasma; kallikrein B plasma; Fletcher factor [Homo sapiens] |
| gi\|4505881\|ref\|NP_000292.1\| | plasminogen [Homo sapiens] |
| gi\|151465432\|ref\|XP_376519.2\| | PREDICTED: ankyrin repeat domain 6 [Homo sapiens] |
| gi\|42662334\|ref\|XP_375941.1\| | PREDICTED: FLJ45139 protein [Homo sapiens] |
| gi\|42656986\|ref\|XP_098238.8\| | PREDICTED: SH3 domain protein D19 [Homo sapiens] |
| gi\|51464068\|ref\|XP_209550.4\| | PREDICTED: similar to Carboxypeptidase N 83 kDa chain (Carboxypeptidase N regulatory subunit) [Homo sapiens] |
| gi\|51458647\|ref\|XP_497680.1\| | PREDICTED: similar to prohibitin [Homo sapiens] |
| gi\|51460685\|ref\|XP_497833.1\| | PREDICTED: similar to SULT6B1 [Homo sapiens] |
| gi\|4506117\|ref\|NP_000304.1\| | protein S (alpha); Protein S, alpha [Homo sapiens] |
| gi\|13325075\|ref\|NP_002817.2\| | quiescin Q6 [Homo sapiens] |
| gi\|5803139\|ref\|NP_006735.1\| | RBP4 gene product [Homo sapiens] |
| gi\|21361198\|ref\|NP_000286.2\| | serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1; protease inhibitor 1 (anti-elastase), alpha-1- antitrypsin [Homo sapiens] |
| gi\|4507377\|ref\|NP_000345.1\| | serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 7; thyroxine-binding globulin; thyroxin-binding globulin [Homo sapiens] |
| gi\|4502261\|ref\|NP_000479.1\| | serine (or cysteine) proteinase inhibitor, clade C (antithrombin), member 1; antithrombin III [Homo sapiens] |
| gi\|39725934\|ref\|NP_002606.3\| | serine (or cysteine) proteinase inhibitor, Glade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1; pigment epithelium-derived factor [Homo sapiens] |
| gi\|4502133\|ref\|NP_001630.1\| | serum amyloid P component precursor; pentaxin-related; 9.5S alpha-1-glycoprotein [Homo sapiens] |
| gi\|7382460\|ref\|NP_001031.2\| | sex hormone-binding globulin; Sex hormone-binding globulin (androgen binding protein) [Homo sapiens] |
| gi\|4557739\|ref\|NP_000233.1\| | soluble mannose-binding lectin precursor; Mannose-binding lectin 2, soluble (opsonic defect); mannose binding protein [Homo sapiens] |
| gi\|4507659\|ref\|NP_003283.1\| | translocated promoter region (to activated MET oncogene); Tumor potentiating region (translocated promoter region) [Homo sapiens] |
| gi\|46195765\|ref NP_954712.1\| | unc-13 homolog D [Homo sapiens] |
| gi\|32483410\|ref\|NP_000574.2\| | vitamin D-binding protein precursor; vitamin D-binding alpha-globulin [Homo sapiens] |
| gi\|18201911\|ref\|NP_000629.2\| | vitronectin precursor; serum spreading factor; somatomedin B; complement S-protein; epibolin [Homo sapiens] |

**Table 5b Proteins Identified in WO20081003066 A2 having twofold or greater difference in abundance between slow and rapid decline in lung function**

| NCBI GI Reference Number | Protein Description | Anti-Log (Rapid decline conditions vs. Slow decline conditions | Average Rapid decline conditions to Slow decline conditions Ratio | Standard Deviation | Number of Significant Peptides |
|---|---|---|---|---|---|
| 4501843 | Antichymotrypsin | 0.87 | 0.83 | 0.05 | 2 |
| 4557225 | Alpha-2-macroglobulin | 0.88 | 1.05 | 0.48 | 4 |
| 4502153 | Apolipoprotein B | 1.48 | 1.22 | 0.21 | 17 |
| 4557485 | Ceruloplasmin | 0.97 | 0.82 | 0.34 | 5 |
| 4557385 | Complement component 3 | 0.64 | 0.71 | 0.22 | 15 |
| 11761629 | Fibrogen, alpha chain isoform | 1.24 | 1.29 | 0.37 | 6 |
| 11761631 | Fibrogen, beta chain | 1.41 | 1.23 | 0.30 | 5 |
| 4503715 | Fibrogen, gamma chain isoform | 1.21 | 1.24 | 0.50 | 5 |
| 47132557 | Fibronectin 1 isoform 1 | 0.75 | 0.63 | 0.17 | 2 |
| 4504165 | Gelsolin isoform a | 0.76 | 0.85 | 0.30 | 4 |
| 4504893 | Kininogen 1; bradykinin | 1.20 | 1.27 | 0.10 | 2 |
| 4504877 | Plasma kallikrein B1; kallikrein 3, plasma | 0.46 | | | 1 |
| 21361198 | Serine (or cysteine) proteinase inhibitor; alpha-1-antitrypsin | 1.60 | 1.37 | 0.15 | 4 |
| 4502133 | Serum amyloid P component | 1.26 | 1.23 | 0.04 | 2 |
| 32483410 | Vitamin D-binding protein | 0.86 | | | 1 |

Another group of protein biomarkers that are capable of distinguishing between subjects with rapidly declining pulmonary function and slowly declining pulmonary function, are described in WO 20081003066 A2, which is hereby incorporated by reference, and listed in Table 6 parts a - d. In some embodiments, any number of the proteins identified in Table 6, or in any of its separate subsections (6a, 6b, 6c or 6d), may be employed in the methods described herein with any number of the proteins identified in the present study and/or in combination with any number of the proteins identified in Tables 5a or 5b.

**Table 6(a): Proteins identified in Signatures as disclosed in WO 20081003066 A2. The listed accession numbers correspond to entries within the National Center for Biotechnology Information (NCBI) database maintained by the National Institutes of Health.**

| Protein | Other names | Human polynucleotide accession no. (NCBI) | Human protein accession no. (NCBI) |
|---|---|---|---|
| Apolipoprotein H | Beta-2 glycoprotein I | NM_000042 | NP_000033 |
| CD40 | CD40L receptor | NM_001250 | NP_001241 |
| Haptoglobin | Hp2-alpha | NM_005143 | NP_005134 |
| IL-8 | Interleukin-8 | NM_000584 | NP_000575 |
| MCP-1 | CCL2 | NM_002982 | NP_002973 |
| TNF-RII | TNFRSF1B | NM_001066 | NP_001057 |
| Apolipoprotein CIII | Apoc3 | NM_000040 | NP_000031 |
| GM-CSF | Colony stimulating factor 2 | NM_000758 | NP_000749 |
| IgA | Immunoglobulin type A | BC087841 | AAH87841 |
| MIP-1α | CCL3 | NM_002983 | NP_002974 |
| Tissue factor | Coagulation factor III | NM_001993 | NP_001984 |
| TNF-α | TNF superfamily member 2 | NM_000594 | NP_000585 |
| α1-antitrypsin | Serpin A1 | NM_000295 | NP_000286 |
| CRP | C-reactive protein | NM_000567 | NP_000558 |
| Fibrinogen | FGA | NM_000508 | NP_000499 |
| MDC | CCL22 | NM_002990 | NP_002981 |
| sVCAM-1 | Soluble VCAM-1 | NM_001078 | NP_001069 |
| IL-4 | Interleukin-4 | AF395008 | AAK71324 |

**Table 6b: Differences in plasma markers between COPD rapid decliners and individuals without lung disease**

| Marker | Fold change | p value (FPR) | q value (FDR) |
|---|---|---|---|
| Alpha-1-antitrypsin | 1.11 | 0.0238 | 0.062 |
| Alpha fetoprotein | 1.38 | 0.0498 | 0.094 |
| Apolipoprotein A1 | 1.38 | 0.0020 | 0.017 |
| Apolipoprotein H | 1.15 | 0.0029 | 0.019 |
| Carcinoembryonic antigen | 1.75 | 0.0022 | 0.017 |
| Eotaxin | 2.64 | 0.0007 | 0.008 |
| Factor VII | 1.16 | 0.0448 | 0.091 |
| Fibrinogen | 1.18 | 0.0231 | 0.062 |
| GM-CSF | 1.51 | 0.0061 | 0.026 |
| Haptoglobin | 2.02 | 0.0115 | 0.034 |
| IL-10 | 1.54 | 0.0116 | 0.034 |
| IL-13 | 1.69 | 0.0086 | 0.031 |
| IL-1 alpha | 1.16 | 0.0336 | 0.079 |
| IL-3 | 1.46 | 0.0496 | 0.094 |
| IL-4 | 4.21 | <0.0001 | 0.000 |
| IL-5 | 1.59 | 0.0041 | 0.023 |
| IL-7 | 2.16 | 0.0044 | 0.023 |
| IL-8 | 1.20 | 0.0398 | 0.088 |
| MCP-1 | 1.51 | <0.0001 | 0.001 |
| Serum amyloid P | 1.28 | 0.0049 | 0.023 |
| Tissue factor | 1.19 | 0.0410 | 0.088 |
| TNF-RII | -1.19 | 0.0071 | 0.028 |
| Thrombopoietin | 7.55 | 0.0117 | 0.034 |
| sVCAM-1 | -1.20 | 0.0002 | 0.003 |
| VEGF | 1.18 | 0.0301 | 0.075 |

**NCBI Accession Numbers and NCBI GI Numbers for the markers appearing in table 6b are as follows.**

| Marker | Accession number and GI | | Marker | Accession number and GI |
|---|---|---|---|---|
| alpha-1-antitrypsin | AAB59495.1 GI:177831 | | IL-3 | AAC08706.1 GI:3002475 |
| alpha fetoprotein | AAB58754.1 GI:178236 | | IL-4 | AAH70123.1 GI:47123367 |
| apolipoprotein A1 | AAD34604.1 GI:4960066 | | IL-5 | NP_000870.1 GI:4504671 |
| apolipoprotein H | AAA51766.1 GI:178857 | | IL-7 | AAH47698.1 GI:29126905 |
| Carinoembryonic antigen | CAA44076.1 GI:1877203 | | IL-8 | AAH13615.1 GI:15488984 |
| Eotaxin | CAB07027.1 GI:2462478 | | MCP-1 | AAB29926.1 GI:545465 |
| Factor VII | AAA51983.1 GI:180334 | | Serum amyloid P | BAA00060.1 GI:220068 |
| Fibrinogen | CAA50740.1 GI:394794 | | Tissue factor | AAA61152.1 GI:339506 |
| GM-CSF | AAA52578.1 GI:183364 | | TNF-RII | NP_001057.1 GI:4507577 |
| Haptoglobin | AAA88080.1 GI:386783 | | Thrombopoietin | AAB33390.1 GI:914226 |
| IL-10 | NP_000563.1 GI:10835141 | | sVCAM-1 | |
| IL-13 | NP_002179.2 GI:26787978 | | VGEF | CAC19513.2 I:220732299 |
| IL-1 alpha | CAA27448.1 GI:33786 | | | |

**Table 6c: Differences in plasma markers between COPD slow decliners and individuals without lung disease**

| Marker | Fold change | p value (FPR) | q value (FDR) |
|---|---|---|---|
| Apolipoprotein H | 1.16 | 0.0230 | 0.696 |
| Cancer antigen 19.9 | 2.29 | 0.0078 | 0.563 |
| Eotaxin | 1.68 | 0.0358 | 0.696 |
| VEGF | 1.11 | 0.0545 | 0.696 |

**NCBI Accession Numbers and NCBI GI Numbers for the markers appearing in table 6c are as follows.**

| Marker | Accession number and GI |
|---|---|
| apolipoprotein H | AAA51766.1 GI:178857 |
| Cancer antigen 19.9 | none found |
| Eotaxin | CAB07027.1 GI:2462478 |
| VEGF | CAC19513.2 GI:220732299 |
| | |

**Table 6d: Differences in plasma markers between COPD rapid and slow decliners.**

| Marker | Fold change | p value (FPR) | q value (FDR) |
|---|---|---|---|
| Cancer antigen 19.9 | -1.43 | 0.0355 | 0.300 |
| IgA | -1.57 | 0.0120 | 0.157 |
| IL-4 | 1.27 | 0.0008 | 0.054 |
| IL-5 | 1.48 | 0.0139 | 0.157 |
| Insulin | -5.86 | 0.0165 | 0.160 |
| MCP-1 | 1.44 | 0.0026 | 0.089 |
| MDC | 1.28 | 0.0422 | 0.300 |
| MIP-1 alpha | -1.26 | 0.0468 | 0.300 |
| Tissue factor | 1.32 | 0.0125 | 0.157 |
| sVCAM-1 | -1.31 | 0.0043 | 0.098 |

**NCBI Accession Numbers and NCBI GI Numbers for the markers appearing in table 6d are as follows**

| Marker | Accession number and GI |
|---|---|
| Cancer antigen 19.9 | |
| IgA | CAA10818.1 GI:2632187 |
| IL-4 | AAH70123.1 GI:47123367 |
| IL-5 | NP_000870.1 GI:4504671 |
| Insulin | AAA59179.1 GI:307072 |
| MCP-1 | AAB29926.1 GI:545465 |
| MDC | AAB29191.1 GI:455835 |
| MIP-1 alpha | P10147.1 GI:127078 |
| Tissue factor | AAA61152.1 GI:339506 |
| sVCAM-1 | |

Among the protein biomarkers identified herein, a group of protein biomarkers not described in either WO20081003066 A2 or WO 20081003066 A2 have been identified. Those protein biomarkers, which include insulin, plasminogen, interleukin 6, interleukin 1 beta (*IL1B*), signal transducer and activator of transcription 3 (*STAT3*), cyclin dependent kinase 2 (*CDK2*) and FAM3A (family with sequence similarity 3, member A) which were identified using Ariadne Genomics' Pathway Studio™, are listed in Table 7.

**Table 7**

| **Protein** | **GI Number and NCBI Accession/Version** | **SEQ ID NO:** |
|---|---|---|
| complement component 4A (Rodgers blood group) | GI:14577919, NP_009224 (Precursor) | 1 |
| serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3 | GI:4501843, NP_001076.1 | 2 |
| complement component 8, gamma polypeptide | GI: 109731764, AAI13627.1 | 3 |
| serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium ), | GI:260064050, NP_001159393 | 4 |
| insulin-like growth factor binding protein 3 | GI:114319033, DQ884398.1 | 5 |
| insulin-like growth factor binding protein, acid labile subunit | GI:4826772, NP_004961.1 | 6 |
| gelsolin (amyloidosis, Finnish type) | GI:119607896, EAW87490.1 | 7 |
| complement factor D | GI:42544239, NP_001919.2 | 8 |
| fibronectin 1 isoform 3 preproprotein | GI:16933542, NP_002017.1 | 9 |
| Insulin | GI:307072, AAA59179.1 | 10 |
| plasminogen | GI:387026, AAA60113.1 | 11 |
| Interleukin 6 | GI:10834984, NP_000591.1 | 12 |
| interleukin 1 beta | GI:386816, AAA74137.1 | 13 |
| signal transducer and activator of transcription 3 | GI:21618340, NP_644805.1 | 14 |
| cyclin dependent kinase 2 | GI:30582481, AAP35467.1 | 15 |
| family with sequence similarity 3, member A (*FAM3A*) | GI:57284179, CAI43239.1 | 16 |

Other substitutions, modifications, changes and omissions may be made in the design, operating conditions and arrangement of the aspects and embodiments described herein without departing from the spirit of the invention as expressed in the appended claims.

Additional advantages, features and modifications will readily occur to those skilled in the art. Therefore, the invention in its broader aspects is not limited to the specific details, and representative devices, shown and described herein. Accordingly, various modifications may be made without departing from the spirit or scope of the general inventive concept as defined by the appended claims and their equivalents.

All of the references cited herein, including patents, patent applications, and publications, are hereby incorporated in their entireties by reference.

The scope of the claims below is not restricted to the particular embodiments described above. The examples are described for illustrative purposes and are not intended to limit the methods and compositions of the present disclosure in any manner. Those of skill in the art will recognize a variety of parameters that can be changed or modified to yield the same results.

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**Claims**

1. A method of providing a diagnosis or prognosis of a subject having, or suspected of having a lung disease, comprising making a determination of one or more proteins in Table 7, or one or more peptides of a protein in Table 7 in a biological sample from a subject; said method optionally comprising making a determination of one or more proteins in Tables 2, 4, 5 and/or 6, or one or more peptides of one or more proteins in Tables 2, 4, 5 and/or 6.
2. The method of claim 1, comprising making a determination of two or more, three or more, four or more, five or more, six or more, eight or more, ten or more, fifteen or more, twenty or more, twenty five or more, thirty or more, or forty or more different proteins or peptides from different proteins in Tables 2, 4, 5 6 and/or 7.
3. The method of claim 1 or claim 2, wherein the disease is selected from the group consisting of obstructive pulmonary disease, chronic systemic inflammation, emphysema, asthma, pulmonary fibrosis, cystic fibrosis, obstructive lung disease, COPD, lung cancer, and pulmonary inflammatory disorder.
4. The method of any of the preceding claims, wherein the biological sample is a biological fluid.
5. The method of any of the preceding claims wherein the biological fluid is blood, plasma, serum, lymphatic fluid, saliva, sputum and/or urine.
6. The method of any of the preceding claims wherein said determination is conducted by or more of: FT-ICR MS, LC FT-ICR, accurate mass and time (AMT) technology, putative mass and time (PMT) technology, multistage mass spectrometry (MS/MS), liquid chromatography separation with mass spectroscopic analysis (LC-MS) using techniques such as MS/MS, data dependant scanning, product ion scans, single ion monitoring, single reaction monitoring, multiple reaction monitoring, MALDI-MS or MS/MS, ESI-MS or MS/MS, offline SCX fractionation with RP-LC-ESI-MS/MS, two-dimensional gel electrophoresis, protein array analysis, polymerase chain reaction, an array, quantitative RT-PCR array, multiplex PCR, quantitative DNA array, autoradiograph analysis, quantitative hybridization, immunohistochemistry, antibodies, immunoelectrophoresis, SDS-PAGE electrophoresis, chromatography, quantitative ligand-binding, quantitative rRNA-based amplification, fluorescent probe hybridization, fluorescent nucleic acid sequence specific amplification, loop-mediated isothermal amplification and/or ligase amplification (*e.g.*, ligase chain reaction).
7. The method of any of claims 1 – 5, wherein said determination is conducted by an immunoaffinity assay.
8. The method of claim 7, wherein said immunoaffinity assay is an ELISA or Western blot.
9. The method of any of claims 1 – 5, wherein said determination is conducted by measuring or observing the quantity or concentration of one or more nucleic acids expressed from a gene encoding one or more different proteins in Tables 2, 4, 5 6 and/or 7.
10. The method of claim 9, wherein said measuring or observing is conducted by a method employing one or more of: nucleic acid amplification, polymerase chain reaction, nucleic acid array analysis, RT-PCT, quantitative RT-PCR. real time PCR, multiplex PCR, quantitative hybridization assay, chromatography, quantitative rRNA-based amplification, fluorescent probe hybridization, loop-mediated isothermal amplification and/or ligase amplification (*e.g.*, ligase chain reaction).
11. The method of any of the preceding claims wherein the diagnosis or prognosis is a diagnosis or prognosis of COPD.
12. The method of any of claims 1 – 11, wherein said diagnosis or prognosis is:

(a) a diagnosis or prognosis of rapid decline in lung function relative to individuals not having lung disease, said method optionally comprising making a determination of one or more proteins in any of Tables 2, 4, and/or 6(b), or one or more peptides of the protein in any of Tables 2, 4, and/or 6(b) in said sample; or

(b) a diagnosis or prognosis of slow decline in lung function relative to individuals not having lung disease, said method optionally comprising making a determination of one or more proteins in any of Tables 2, 4, and/or 6(c), or one or more peptides of the protein in any of Tables 2, 4, and/or 6(c) in said sample; or

(c) a diagnosis or prognosis of rapid decline in lung function relative to individuals having a slow decline in lung function, said method optionally comprising making a determination of one or more proteins in any of Tables 2, 4, 5 and/or 6(d), or one or more peptides of the protein in any of Tables 2, 4, 5 and/or 6(d) in said sample.

13. The method of claim 12, comprising making a determination of two or more, three or more, four or more, five or more, six or more, eight or more, ten or more, fifteen or more, twenty or more, twenty five or more, thirty or more, or forty or more different proteins or peptides from different proteins in any of Tables 2, 4, 5, 6 and/or 7.

14. The method of any of the preceding claims comprising making a determination of two, three, four, five, six, seven or more proteins or peptide fragments of the proteins in Table 2 that have negative coefficients.

15. The method of any of the preceding claims comprising making a determination of two, three, four, five, six, seven or more proteins or peptide fragments of the proteins in Table 2 that have positive coefficients.

16. The method of any of claims 1 – 15, wherein the diagnosis or prognosis is COPD with a rapid or slow decline in $FEV_1$.

17. The method of claim 16, comprising making a determination of two, three, four, five, six, seven or more proteins or peptide fragments of the proteins in Table 4 that have positive coefficients.

18. The method of any of the preceding claims further comprising making a determination of one or more proteins, or peptide fragments thereof, selected from the group consisting of: Insulin 2, proinsulin, plasminogen, fibrinogen, alpha polypeptide, interleukin 6, coagulation factor II, interleukein 1 beta, signal transducer and activator of transcription 3, cyclin dependent kinase 2, family with sequence similarity 3, member A (FAM3A), and peptide fragment of any of the foregoing.

19. The method of any of the preceding claims, wherein said peptides are greater than seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen, seventeen, eighteen, twenty, twenty two, twenty five, thirty, or thirty five amino acids in length.

20. The method of any of the foregoing claims comprising making a determination of two or more, three or more, four or more, five or more, six or more, eight or more, ten or more, or fifteen or more proteins in Table 7

21. A composition comprising two or more proteins in Table 7, or one or more peptides of two or more protein in Table 7, said composition optionally comprising two or more proteins in any of Tables 2, 4, 5 and/or 6, or two or more peptides of two or more different proteins in any of Tables 2, 4, 5 and/or 6; provided that said two or more proteins are different.

22. The composition of claim 21, comprising three or more, four or more, five or more, six or more, eight or more, ten or more, fifteen or more, twenty or more, twenty five or more, thirty or more, or forty or more different proteins or peptides from different proteins in any of Tables 2, 4, 5 6 and/or 7.

23. The composition of claim 22, wherein the proteins are selected from the proteins in table 2 and/or Table 4.

24. The composition of claim 22, wherein the proteins are selected from the proteins in table 7.

25. The composition of claim 22, wherein the proteins or peptide fragments are selected from the proteins found in both table 2 and table 4.
26. The composition of any of claims 22 – 25, further comprising one, two, three, four, five, six, seven, eight or more of proteins, or peptide fragments thereof, selected from the group consisting of: Insulin 2, proinsulin, plasminogen, fibrinogen, alpha polypeptide, interleukin 6, coagulation factor II, interleukein 1 beta, signal transducer and activator of transcription 3, cyclin dependent kinase 2, family with sequence similarity 3, member A (FAM3A), and peptide fragment of any of the foregoing.
27. The composition of any of claims 22 - 26, wherein the peptides are greater than seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen, seventeen, eighteen, twenty, twenty two, twenty five, thirty, or thirty five amino acids in length.
28. A composition comprising two, three, four, five, six, seven or more nucleic acid molecules comprising a nucleotide sequence encoding the proteins and/or peptides of any of claims 21 - 27 or a fragment of said nucleotide sequences, or a complementary nucleotide sequence of either; said nucleic acids molecules optionally comprising a promoter operatively coupled to said nucleotide sequence, fragment of said nucleotide sequence, or complementary nucleotide sequence of either.
29. The composition of claim 28, wherein said nucleotide sequence has at least 80-90 percent, 80-95 percent, 85-95 percent, or 95-100 percent sequence identity to a contiguous sequence of 21 or more, 24 or more, 27 or more or 30 or more, nucleotides of a nucleic acid sequence encoding the proteins and/or peptides of any of claims 21 to 27 or a fragment of said nucleotide sequences, or a complementary nucleotide sequence of either.
29. A composition comprising two or more different antibodies or fragments thereof, wherein said different antibodies or antigen binding fragments thereof are specific to two or more different proteins or peptides present in the compositions of claims 21 - 27.
30. The composition of claim 29, wherein said two or more different antibodies or antigen binding fragments thereof, are three, four, five, six seven or more different antibodies, or antigen binding fragments thereof, each specific for a different protein or peptide fragment found in Table 7.
31. The composition of any of claims 22 - 30, wherein said composition is in the form of:
    a) an array having two or more proteins or peptide fragments attached to two or more different spatially addressable locations;
    b) an array having two or more antibodies or antigen binding fragment thereof attached to two or more different spatially addressable locations; or
    c) an array having two or more nucleic acids attached to two or more spatially addressable locations.
32. The composition of claim 31, wherein said attachment is a covalent attachment.
33. The composition of claim 31, wherein said attachment is a non-covalent attachment.
32. The array of claims 31 - 33, wherein in the array has at least 20, 30, 40, 50, 100, 200, 500, 1,000, or 10,000 spatially addressable locations per centimeter square.
33. A method of treating a subject having or suspected of having a lung disease or of following the course of lung disease in a subject having or suspected of having a lung disease comprising:
    (a) making a first determination at a first time, of at least one protein or peptide fragment of a protein identified in Table 7, and/or in Tables 2, and/or Table 4, in a first sample from the subject; and

(b) making a second determination of at least the same protein or peptide fragment from a second sample obtained from the subject at a second time; and comparing the first measurement to the second measurement to determine the progression or regression of the lung disease.

34. The method of claim 33, further comprising making a determination of one or more proteins, or peptides of proteins, appearing in either or both of tables 5 or 6 in either said first or said second sample.
35. The method of any of claims 33 – 34, wherein at least one determination conducted by measuring or observing the quantity or concentration of one or more nucleic acids expressed from a gene encoding one or more different proteins in Table 7, and/or in Tables 2, and/or Table 4.
36. The method of any of claims 33-35, wherein at least one therapeutic agent was administered to said subject.
37. The method of claim 36, wherein said first sample was obtained from said subject before said second sample, and wherein said therapeutic agent is administered after said first sample was obtained from said subject, and before said second sample was obtained from said subject.
38. The method of any of claims 33-37, wherein said therapeutic agent is selected from the group consisting of: immunosuppressants, corticosteroids, β2(beta 2)-adrenergic receptor agonists, anticholinergics, and oxygen.
39. The method of any of claims 1 - 20 or 33 -38, further comprising changing the treatment of a subject based upon said diagnosis or prognosis or said progression, regression, or stability of said lung disease.

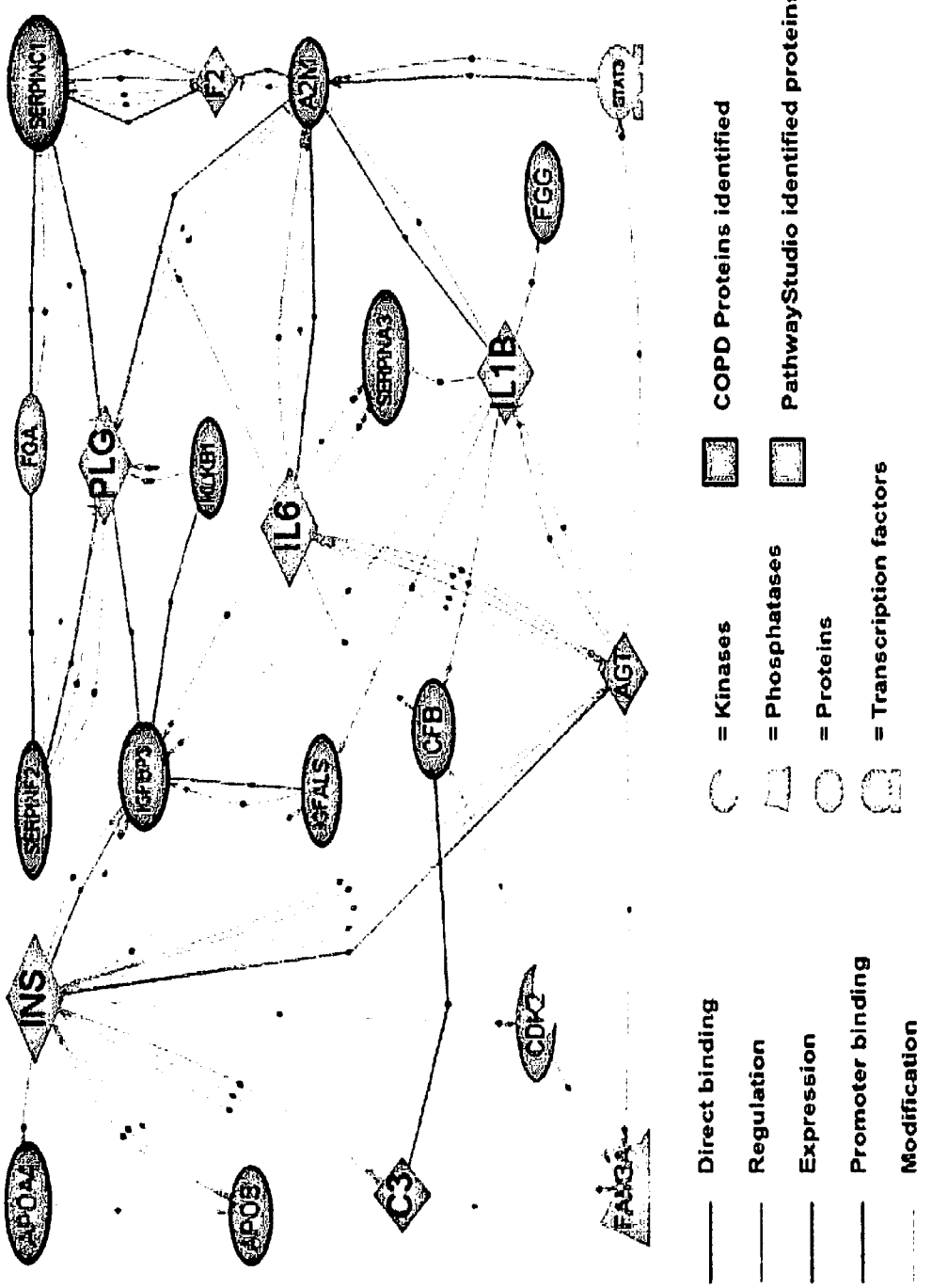
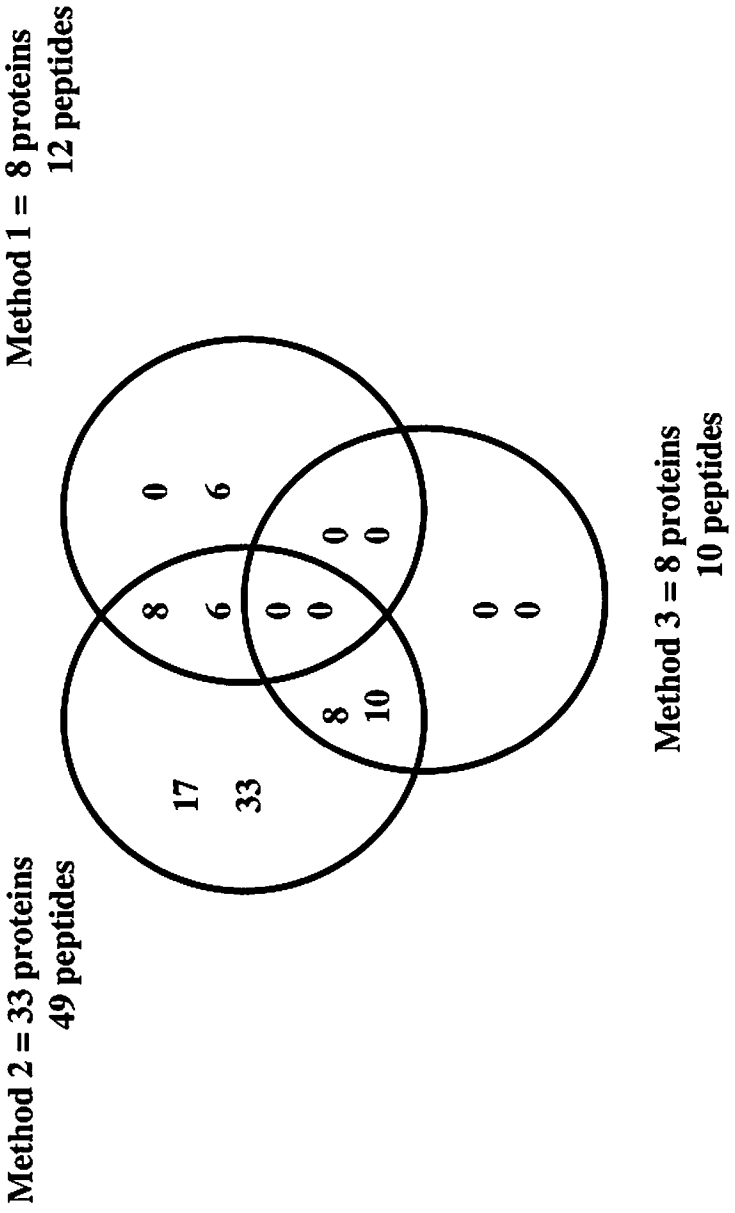

SERPINC1
F2
A2M
STAT3
FGA
PLG
KLKB1
IL6
SERPINA3
IL1B
FGG
SERPINF2
IGFBP3
IGFALS
CFB
AG1
INS
CDK2
APOA4
APOB
C3
FABP4
= Kinases
= Phosphatases
= Proteins
= Transcription factors
COPD Proteins identified
PathwayStudio identified proteins
Direct binding
Regulation
Expression
Promoter binding
Modification

Figure 1

FIG. 2


Method 1 = 8 proteins
12 peptides
Method 2 = 33 proteins
49 peptides
Method 3 = 8 proteins
10 peptides
0
6
8
6
0
0
0
0
0
0
8
10
17
33

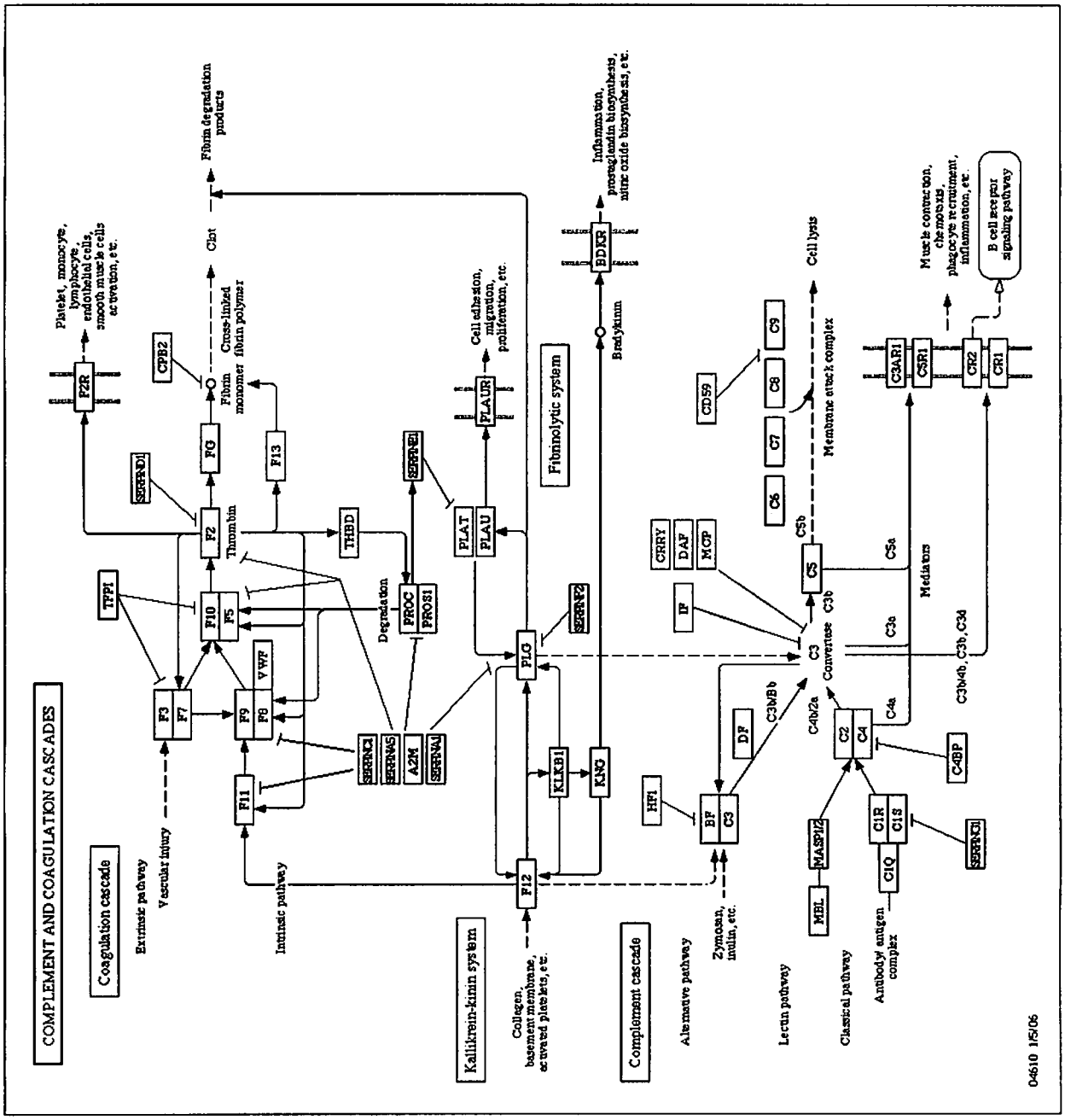

COMPLEMENT AND COAGULATION CASCADES
Coagulation cascade
Extrinsic pathway
Vascular injury
Intrinsic pathway
Kallikrein-kinin system
Collagen, basement membrane, activated platelets, etc.
Complement cascade
Alternative pathway
Zymosan, inulin, etc.
Lectin pathway
Classical pathway
Antibody antigen complex
Fibrinolytic system
Degradation
Bradykinin
Inflammation, prostaglandin biosynthesis, nitric oxide biosynthesis, etc.
Platelet, monocyte, lymphocyte, endothelial cells, smooth muscle cells activation, etc.
Fibrin degradation products
Clot
Cross-linked monomer fibrin polymer
Fibrin
Thrombin
Cell adhesion, migration, proliferation, etc.
Membrane attack complex
Cell lysis
Converase C3b
C3a
C5a
C5b
Mediators
C3b4b, C3b, C3d
C4a
C4b2a
C3bBb
Muscle contraction, chemotaxis, phagocyte recruitment, inflammation, etc.
B cell receptor signaling pathway
04610 1/5/06

Figure 3.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 11/20151

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G01N 33/53, G01N 33/48 (2011.01)
USPC - 436/501, 435/86
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8)-G01N 33/53, G01N 33/48 (2011.01)
USPC-436/501, 435/86

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST(PGPB,USPT,USOC,EPAB,JPAB); Google Patents; Google Scholar
lung disease, biomarker, protein, peptide, nucleic acid, complement component, insulin, igfbp, fibrinogen, interleukin 6, IL-6, stat3, cyclin dependent kinase 2, cdk-2, serpin peptidase inhibitor, gene expression array,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | US 2008/0044843 A1 (PERLEE et al.) 21 February 2008 (21.02.2008) (para [0002], [0007], [0008], [0075], [0111], [0115], Table 12) | 1-3, and 33b-35 |
| X | US 2008/0227117 A1 (FEHNIGER et al.) 18 September 2008 (18.09.2008) (para [0010]-[0020], [0027]-[0034]) | 1-3, and 33b-35 |

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 19 April 2011 (19.04.2011) | 02 MAY 2011 |
| Name and mailing address of the ISA/US<br>Mail Stop PCT, Attn: ISA/US, Commissioner for Patents<br>P.O. Box 1450, Alexandria, Virginia 22313-1450<br>Facsimile No. 571-273-3201 | Authorized officer:<br>Lee W. Young<br>PCT Helpdesk: 571-272-4300<br>PCT OSP: 571-272-7774 |

Form PCT/ISA/210 (second sheet) (July 2009)

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 11/20151

**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-20, 27-32a, 32b, 33a, 36-39
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: claims 1-3 and 33b-36, directed to a method of diagnosing a lung disease comprising making a determination of one or more proteins in Table 7 in a sample from a subject.

Group II: claims 21-26, directed to a composition comprising two or more proteins or peptides in Table 7.

- Please see extra sheet for continuation -

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-3 and 33b-36

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**

International application No.
PCT/US 11/20151

Continuation of Box III: Lack of Unity of Invention

The inventions listed as Groups I - II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The special technical feature of the Group I claims is a method of diagnosing a lung disease comprising making a determination of one or more proteins in Table 7 in a sample from a subject - not required by the claims of Group II. The special technical feature of the Group II claims is a composition comprising two or more proteins or peptides in Table 7 - not required by the claims of Group I. Neither of these special technical features is common to the other group, nor do they correspond to a special technical feature in the other group.

There is not a definitive common technical element shared by the above groups, because there are more than three proteins or proteins or peptides in Table 7, it is possible that the proteins or peptides of Group I may not share a single common technical element with the proteins or peptides of Group II. However, there is also the potential for the peptides to be shared in common. The technical elements of Group I, however, do not represent an improvement over the prior art of US 2008/0044843 A1 to Perlee et al. (see para [0008], diagnosis of COPD in a human subject, para [0028], use of more than one marker, and Table 12, which discloses the diagnosis of a lung disease (COPD) in a subject comprising making a determination of one or more markers, and Table 12, which discloses several markers (IL-6, IGFBP-3, insulin) listed in Applicants' Table 7). Therefore, the inventions of Group I and Group II lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.

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摘要(译)

已经使用全面的高通量蛋白质组学方法鉴定了与COPD相关的独特蛋白质，其能够区分可能经历肺功能快速（RPO）或缓慢（SLW）下降的受试者。映射到21种独特蛋白质的30种肽与COPD吸烟者的年度肺功能下降速率线性相关，其特征在于具有快速或缓慢下降的吸烟者和没有COPD的吸烟者。使用三种不同的统计方法来评估数据，RPO和SLW组由55种肽区分，这些肽映射到33种独特的蛋白质。许多鉴定的肽是蛋白质的蛋白水解片段，其参与补体和/或凝血系统或具有抗蛋白酶或代谢功能。