

Proteomics methods and applications for the practicing clinician

Nichole A. Reisdorph, PhD*; Richard Reisdorph, PhD*; Russell Bowler, MD, PhD†; and Carolyn Broccardo, PhD*

Objective: To describe clinical proteomics from discovery techniques and their limitations, to applications in allergy, asthma, and immunology, and finally to how proteomics can be integrated into clinical practice.

Data Sources: Despite many inherent challenges, proteomics-based methods have become a powerful and popular means of profiling clinical samples for the purpose of biomarker discovery. Although several strategies exist, clinical proteomics for the purpose of biomarker discovery generally focuses on 1 of 3 basic workflows: (1) 2-dimensional gel electrophoresis to quantitate relative protein levels followed by mass spectrometry (MS) to identify proteins of interest, (2) non-gel-based methods that rely on liquid chromatography MS (LCMS) for both quantitation and identification of proteins, and (3) protein profiling methods that do not directly result in the identification of proteins but rather generate “fingerprints” that are compared among individuals or samples.

Study Selection: Regardless of the strategy being pursued, a few general experimental steps are followed that will be expounded on in the text. These proteomics techniques have been applied to discover new biomarkers in biofluids and tissues from individuals with a variety of conditions, including allergy, asthma, atopic dermatitis, inflammatory diseases, chronic obstructive pulmonary disease, and other lung diseases.

Results: After biomarker discovery, LCMS-based proteomics offers several advantages over traditional antibody-based clinical assays, including greater specificity, cost- and time-effectiveness, and the potential to multiplex up to hundreds of peptides in a single assay.

Conclusion: With many guidelines now in place and model studies on which to design future experiments, there is reason to be optimistic that candidate protein biomarkers will be discovered using proteomics and translated into clinical assays.

Ann Allergy Asthma Immunol. 2009;102:523–529.

INTRODUCTION

Proteomics, broadly defined as the study of all proteins in a biological system, is increasingly being applied to clinical samples for the purpose of biomarker discovery. Biomarkers discovered through proteomics can be used in several areas of clinical research and patient care, including disease prediction, diagnosis, staging, monitoring, and assessing drug efficacy.¹ Three major steps are required for a proteomics biomarker to be translated into a clinical assay: (1) *discovery* to identify candidate biomarkers, (2) *validation* of candidates in a large-scale clinical study, and (3) *implementation* in a clinical laboratory.² To date, most proteomics studies have focused on discovery, although progress toward validating protein biomarkers is being made in several fields. Protein biomarker discovery can entail a vast number of methods and workflows, all designed with the goal of reproducibly com-

paring relative quantities of proteins within a system; however, all consist of a few key components: sample preparation and simplification, mass spectrometry (MS), and informatics. Technological advancements in each of these areas has made large-scale proteomics clinical studies practical and enables the use of a variety of biofluids for discovery, including bronchoalveolar lavage fluid, induced sputum, blood, and potentially exhaled breath condensates.^{3–6} After a discovery phase to identify biomarkers, MS can be used as a platform for validation studies and is becoming more widely used in clinical laboratories. This review describes clinical proteomics from discovery techniques and their limitations, to applications in allergy, asthma, and immunology, and finally to how proteomics can be integrated into clinical practice.

PROTEOMICS STRATEGIES AND METHODS

Despite many inherent challenges, proteomics-based methods have become a powerful and popular means of profiling clinical samples for the purpose of biomarker discovery.⁷ Although several strategies exist, clinical proteomics for the purpose of biomarker discovery generally focuses on 1 of 3 basic workflows: (1) 2-dimensional gel electrophoresis (2DGE) to quantitate relative protein levels followed by MS

Affiliations: * Department of Immunology, National Jewish Health, Denver, Colorado; † Department of Medicine, National Jewish Health, Denver, Colorado.

Disclosures: Authors have nothing to disclose.

Received for publication October 27, 2008; Received in revised form February 19, 2009; Accepted for publication February 23, 2009.

to identify proteins of interest (Fig 1A), (2) non-gel-based methods that rely on liquid chromatography MS (LCMS) for both quantitation and identification of proteins^{8,9} (Fig 2), and (3) protein profiling methods that do not directly result in the identification of proteins but rather generate “fingerprints” that are compared between individuals or samples. The choice of strategy depends on the question being asked, sample type and availability, expertise, and other considerations. Regardless of the strategy, a few general steps are followed and will be expounded on to various degrees in the text: (1) experimental design; (2) sample collection, handling, and storage; (3) sample preparation, including 2DGE (optional, depending on workflow); (4) protein digest (optional, depending on workflow); (5) mass spectrometry; (6) data analysis, protein identification (optional, depending on workflow), and informatics; and (7) validation.

Experimental Design and Sample Preparation

The importance of sample choice and experimental design in proteomics has been explored in several recent reviews.^{10,11}

Important considerations include sample availability, sample integrity and storage requirements, sample volume, protein concentration and dynamic range, time of collection, time of processing, and the inclusion of protease inhibitors. For example, although arguably a rich source of biomarkers, plasma proteomics holds several challenges, including the presence of highly abundant proteins, relatively rapid protein degradation, and protein concentrations spanning 10^{12} orders of magnitude,¹² which decreases the likelihood of identifying low abundant protein biomarkers. Simplification of the sample is therefore generally required, and many proteomics studies now focus on a greatly simplified subproteome, such as a specific organelle (mitochondria) or a specific class of protein (glycoproteins). Several commercial products are available for fractionation or simplification; for example, antibody-based columns are available that deplete the most highly abundant proteins, such as albumin, from plasma and bronchoalveolar lavage fluid, thus enabling detection of lower abundance proteins.^{13–15} Similarly, enrichment or depletion of

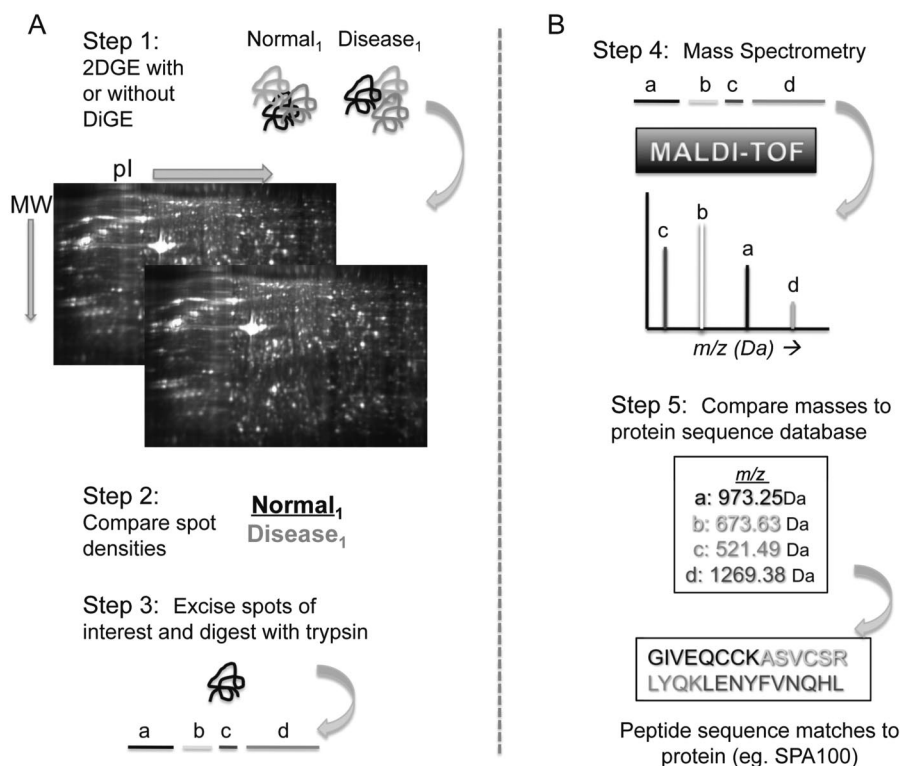


Figure 1. Two-dimensional gel electrophoresis (2DGE) (A) followed by mass spectrometry (B) to quantitate and identify candidate protein biomarkers. In step 1, samples are solubilized and proteins separated based on their isoelectric point (pI) followed by separation according to molecular weight (MW). When difference gel electrophoresis (DiGE) is used, 2 samples are individually labeled with dyes, combined, and analyzed together. In non-DiGE experiments, samples are analyzed separately. In both DiGE and non-DiGE, sophisticated software is used to determine and compare spot densities (step 2). Proteins exhibiting differential expression are excised and digested with a protease such as trypsin, which reproducibly cleaves after the amino acids lysine and arginine (step 4). In step 5, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) is used to measure the masses of peptides generated by trypsin. These masses are compared with sequences in a protein sequence database to determine if the measured masses match a predicted protein sequence. When liquid chromatography tandem mass spectrometry is used, sequence information is generated for individual proteins, often resulting in greater overall confidence in identification. m/z indicates mass-to-charge ratio.

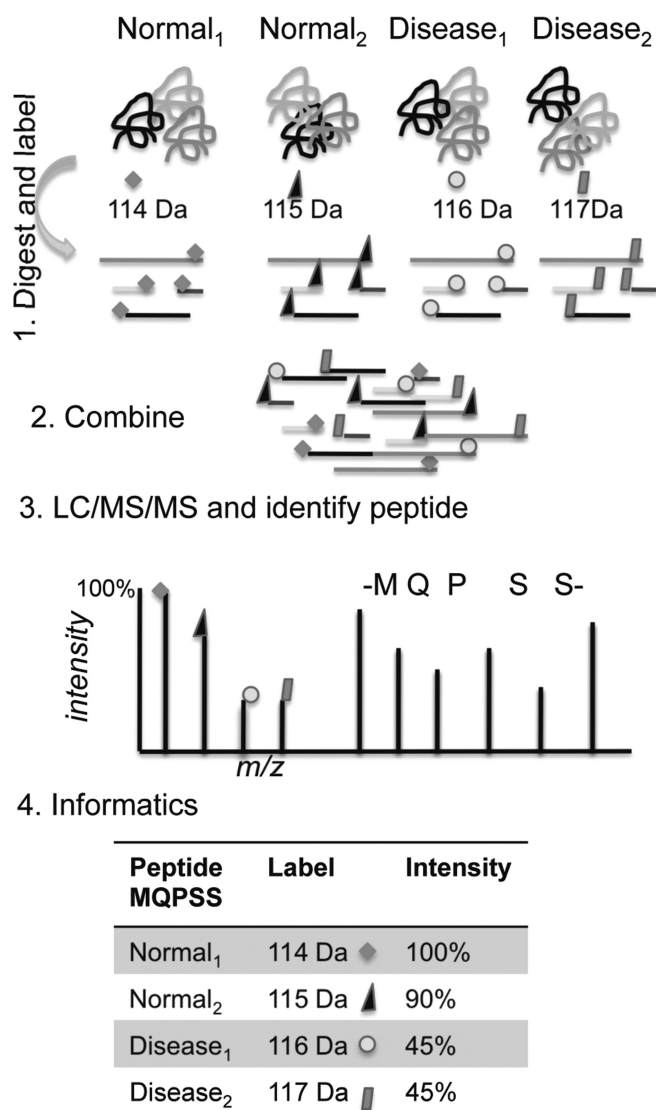


Figure 2. Quantitative proteomics using a labeling strategy. In this example, proteins are extracted from a biofluid, digested, and labeled with tags. Although not shown in the figure, the tags are connected to linker molecules that vary in mass; the mass of the entire label (tag mass plus the linker mass) is identical for each of the labels (ie, the labels are isobaric). Samples from 2 healthy individuals are labeled with tags 114 and 115 Da, whereas samples from 2 individuals with disease are labeled with tags 116 and 117Da (step 1). After labeling, samples are combined (step 2) and can be fractionated to reduce complexity. Peptides are resolved using high-performance liquid chromatography before entry into a mass spectrometer. An example of a resulting mass spectrum for peptide MQPSS is shown in step 3. In this example, there is a higher intensity of the tags 114 and 115, indicating more peptide and protein present in the normal vs disease samples. LC/MS/MS indicates liquid chromatography tandem MS; m/z, mass-to-charge ratio.

proteins that contain distinct functional groups, such as phosphoproteins, glycoproteins, or otherwise modified proteins, can be accomplished using antibodies.¹⁶ For example, Benson

et al¹⁷ used extensive fractionation of nasal lavage combined with MS to distinguish between patients presenting with allergic rhinitis (AR) and asthmatic chronic rhinosinusitis (CR). Criticisms of depletion or enrichment techniques include potential loss of proteins and poor reproducibility.¹³

Several methods also exist for separating proteins on columns based on their chemical properties, many of which can be automated. Appropriate column chemistries for proteins include strong cation exchange, anion exchange, carbon-based resins, and size exclusion. These techniques can be used at various points of sample preparation.^{17,18} Proteins can also be electrophoretically resolved based on their isoelectric points (pI), the pH at which the protein has a net neutral charge. For example, off-gel and free flow electrophoresis systems separate proteins based on their pI while maintaining proteins in a liquid state.¹⁹ In addition, 2DGE can be used to effectively resolve proteins, where pI is used for separation in the first dimension and molecular weight in the second dimension (Fig 1). 2DGE strategies have been used extensively in allergy, asthma, and immunology studies^{3,4,20} (Table 1).

Mass Spectrometry

MS is an essential component of any proteomics study⁸ and is used for the identification, and often quantitation, of proteins. With the exception of some protein profiling studies, after sample preparation or simplification, proteins are digested with a protease such as trypsin,²¹ which cleaves after the amino acids arginine and lysine and results in the formation of peptides of predictable and reproducible masses. After digestion, peptides are analyzed in mass spectrometers, which measure the mass or, more precisely, the mass-to-charge ratio (m/z) of molecules. Identification occurs through matching the predicted masses and sequences of peptides or amino acids in a protein database to the measured masses from the mass spectrometer (Fig 1B). Although several types of MS systems exist, the 2 most common MS configurations used in proteomics are (1) matrix-assisted laser-desorption/ionization time of flight (MALDI-TOF) and (2) liquid chromatography tandem MS (LC/MS/MS). In both cases peptides are ionized through the addition of a proton and travel through a mass analyzer to a detector, generating a mass spectrum. During MALDI, peptides are combined on a plate with an energy-absorbing matrix, a laser transfers energy to the matrix, and the energy is transferred to the peptides, which become ionized. The peptides then enter a mass analyzer such as a TOF mass analyzer. In TOF MS, the times that it takes the peptides to reach the detector are recorded and converted to m/z and a mass spectrum is generated. The peptide mass information is then compared with a protein database.

In proteomics experiments, LC/MS/MS is generally coupled to nano-electrospray ionization. During nano-electrospray ionization, the sample is introduced via a high-performance liquid chromatography (HPLC) system through a fine needle. High voltage, drying gas, and heat are applied to achieve ionization. The sample is retained in a liquid state during LC and peptides can be further separated on a column,

Table 1. Proteomics Strategies in Various Respiratory Conditions^a

Disease	Sample preparation strategy	Quantitative strategy	Protein candidates	Biofluid	Reference(s)
Allergens	Various	2DGE	Various depending on allergen	Serum	41–44
Allergy	Antibody enrichment	LC/MS/MS	Quantitation of phosphorylation events	Mast cells	45
Asthma (mouse)	HPLC/None	MALDI-TOF/2DGE	Complement 3 fragment f, leukotriene receptor, cytoskeletal, antioxidant, calcium binding	Various	18, 33, 35, 36
Asthma (human)	None	DiGE	MMP, cytokines, C-reactive proteins, S100 proteins, Ym1, calgranulin, PDE4C, thioredoxin 2, GST-M3	Various	30, 32, 14
COPD	Metal affinity	SELDI	Profiling study, no candidates generated	Plasma	37
Inflammatory diseases	None	SELDI	Preliminary identification of calgranulins, Clara cell secretory protein	Sputum	5
Interstitial lung disease	Dialysis to remove salts	2DGE	MIF, calgranulin, antioxidant peroxysomal enzyme, thioredoxin peroxidase 2	BAL	20
Lung cancer	Various	2DGE, nonlabeling LC/MS/MS, MALDI imaging	CK7 cleavage products, cathepsin D, AOE372, ATP5D, GSTM4, UCHL1, galactosyltransferase, cytosolic inorganic pyrophosphatase, triosephosphate isomerase	Various	9, 27, 29
Rhinitis or rhinosinusitis	Multiple separations	Nonlabeling LC/MS/MS	α_2 -Macroglobulin, long palate protein, lung or nasal epithelium carcinoma-associated protein	Nasal lavage	17

Abbreviations: 2DGE, 2-dimensional gel electrophoresis; COPD, chronic obstructive pulmonary disease; DiGE, difference gel electrophoresis; HPLC, high-performance liquid chromatography; LC/MS/MS, liquid chromatography tandem mass spectrometry; MALDI-TOF, matrix-assisted laser-desorption ionization time of flight; MMP, matrix metalloproteinase; SELDI, surface-enhanced laser desorption ionization.

^a A variety of proteomics strategies have been used to discover candidate biomarkers in respiratory diseases. Many of those listed are expounded on in the text.

resulting in a higher number of protein identifications compared with MALDI-TOF when complex mixtures are analyzed. After HPLC and ionization, peptides enter a mass analyzer and often tandem MS is used to obtain sequence information. In MS/MS using an ion trap, peptides of a single mass are “trapped” in an electromagnetic field and fragmented, and the resulting fragments are detected and a second mass spectrum is formed. Because fragmentation of a peptide often results in sequence information, a single, high-quality peptide can often be used to identify a protein. In addition, this information can be used to localize posttranslational modifications, such as phosphorylation, glycosylation, and ubiquitination. Additional mass analyzers include TOF, ion traps, Fourier transform, and quadrupoles; combinations of these form several tandem mass spectrometers such as quadrupole TOF (Q-TOF) instruments.

In summary, in MALDI-TOF a laser desorbs samples and matrix from a solid surface and ionized molecules travel through a flight tube at a rate proportional to their mass (smaller peptides travel faster than larger peptides). In LC/MS/MS, peptides are first separated on a column and then ionized using electrospray ionization, their mass is detected, and then a peptide is fragmented in a mass analyzer, generating amino acid sequence information. LC/MS/MS is generally considered more sensitive than MALDI-TOF (ie, less protein is required for identification). Conversely, MALDI-TOF is generally more of a high throughput method compared with LC/MS/MS.

Data Analysis: Protein Identification

Assignment of protein identification to peptides of interest is accomplished by querying databases with MS or MS/MS

spectral data. Databases are based on nucleic acid sequences; therefore, prior protein characterization is not required for identification. A variety of curated databases, containing sequences for many species, are available. Databases are queried using 1 or more software programs called search engines. Additional “back-end” informatics programs are used for quantitation, and separate software can be used to determine the functional and biological significance of identified proteins.

QUANTITATIVE PROTEOMICS WORKFLOWS

As mentioned, generally 3 strategies are used in clinical proteomics: (1) 2DGE, (2) LCMS, and (3) protein profiling. In 2DGE proteins are first separated based on their pI and then by molecular weight in a gel matrix (Fig 1A). Proteins are stained and their densities compared using software to determine differences among groups or samples. When difference gel electrophoresis (DiGE) is combined with 2DGE, proteins from 2 samples are labeled with fluorescent dyes before analysis. After 2-dimensional separation the gel is scanned such that distinct images for each sample are generated. Measuring signal intensity for individual protein spots can result in statistically relevant information when an internal standard is used and/or when a sufficient number of replicates are analyzed. One advantage to DiGE is that 2 samples are resolved on a single gel, eliminating some of the variability inherent in 2DGE studies. A major disadvantage to all 2DGE studies lies in the fact that significant sample loss can occur, especially for hydrophobic, high-molecular-weight, and insoluble proteins.

Quantitative LCMS-based experiments can be performed with or without labeling, and several labeling strategies exist^{8,22} for the purpose of biomarker discovery. In these studies, the mass spectrometer is used to measure the relative amount of peptides among samples, either directly by comparing peptide intensity or indirectly via detection of molecular tags that are attached to peptides. Protein identification occurs either simultaneously or in a subsequent step. In 1 label-dependent method, peptides are labeled with isobaric tags before mass spectral analysis (Fig 2). During analysis the tags are released and their relative abundance among samples is determined. An advantage of label-dependent approaches is that 2 or more samples can be analyzed for quantitative differences in a single MS run. In label-independent methods peptides are analyzed directly and samples must be analyzed individually. Because peptide intensity and/or the number of peptides detected are directly related to their abundance in a sample, relative peptide abundance can be compared among samples for quantitative purposes. The advantages of LC/MS/MS techniques include the ability to analyze large numbers of proteins without relying on gel-based methods and the potential for high-throughput analysis. A comparison of 3 common quantitative techniques, including the 2DGE- and LC/MS-based techniques, showed reasonable comparison when simple mixtures were used but saw variability in the sensitivity and accuracy of the methods when whole cell

lysates from HCT116 and HCT116 p53 knockout cells were compared.²³

Importantly, after discovery of candidate biomarkers, regardless of the methods used, extensive validation must occur. Although the techniques described herein are not suitable for large-scale clinical studies, high-throughput, multiplex assays can be developed using mass spectrometers for the purpose of validation. These assays can then be translated directly into clinical assays.

Quantitative MS methods also exist for intact proteins; these have more immediate potential to become clinical assays, although they are also used for discovery of biomarkers. Termed *molecular imaging* and *protein profiling*^{24,25} in these experiments, a nonsimplified sample is subjected to MALDI-TOF and the resulting mass spectra are analyzed for common patterns (ie, “fingerprints”). For example, Groseclose and colleagues²⁶ extracted protein from formalin-fixed paraffin-embedded lung tissue and subjected tissue microarrays to MALDI imaging MS to assist in the diagnosis of adenocarcinoma and squamous cell carcinoma. These strategies are also being used in other applications of lung cancer and to track drug metabolism in animals.²⁷

BIOINFORMATICS

After quantitation and identification of candidate proteins, a proteomics workflow begins to resemble a genomics workflow in terms of bioinformatics; at this point programs capable of generating molecular pathway and biological network information are required. In addition, determining the statistical and biological significance of up to hundreds of proteins requires sophisticated software. Several software packages specifically designed for proteomics data are available for this purpose. Both commercial and open-source platforms are available. Workflows and available software have been reviewed elsewhere.^{24,28}

APPLICATION OF PROTEOMICS TO IMMUNOLOGY, ASTHMA, AND ALLERGY

Although proteomics is still generally being applied at the discovery phase, there are some cases in which useful biomarkers have been discovered in both biofluids and tissues^{9,29} (Table 1). For example, as mentioned, Benson et al¹⁷ were able to distinguish between patients presenting with AR and those with asthmatic CR using a LC/MS/MS approach. Three chromatographic techniques, including depletion of high abundant proteins using antibodies, were used followed by MS to identify and quantitate proteins. Proteins found predominantly in AR included α_2 -macroglobulin, long palate protein, and lung or nasal epithelium carcinoma-associated protein. Proteins found to be associated with CR included lipocalin and lactotransferrin.

An LC/MS approach was also used to profile plasma peptides from control and asthmatic mice.¹⁸ In these studies, a combination of HPLC and MALDI-TOF was used to show an increase in complement 3 fragment f in control compared with asthmatic mice. Other proteomic strategies have also

been used to discover biomarkers for asthma, including matrix metalloproteinases (MMP),³⁰ cytokines,^{14,31–33} and C-reactive protein. Several members of the MMP family are up-regulated in the lungs of humans and mice that exhibit the asthma phenotype. A 2DGE DiGE study of MMP2/9^{–/–} mice vs wild type resulted in the identification of several proteins as potential substrates, including Ym1, S100A8, S100A9, and calgranulin. The S100 proteins have also been implicated in atopic dermatitis, where 2DGE was used to determine a role for S100A11 in regulating skin barrier integrity.³² Additional experimental studies have established that up-regulation of MMPs is at least partly coordinated by cytokines. MMPs have also been implicated in the progression of other chronic lung disorders, such as chronic obstructive pulmonary disease (COPD) and bronchiectasis.³⁴

2DGE has also been used in a chronic asthmatic mouse model, where protein targets of cysteinyl leukotriene receptor type 1 were discovered, including chitinases, lungkine, γ -actin, and surfactant protein D.³⁵ Generating chronic inflammation of the lungs in same mouse model, Wong and Zhao³⁶ discovered 100 proteins differentially expressed in chronically inflamed lungs, including cytoskeletal, calcium-binding, and antioxidant proteins.

Conversely, surface-enhanced laser desorption ionization (SELDI) uses protein profiles or “fingerprints” to differentiate among clinical phenotypes, and protein identifications are not made during the analysis. Nonetheless, proteomic profiling using SELDI has been used as a screening method to distinguish among COPD phenotypes³⁷ or chronic rhinosinusitis subtypes³⁸ using serum. SELDI has also been applied to sputum samples to distinguish patients with asthma, COPD, cystic fibrosis, and bronchiectasis from healthy individuals.⁵ SELDI, as well as other proteomics methods, have also been applied to saliva, for example, in understanding Sjögren syndrome.³⁹ In no case was protein identification confirmed, and perhaps for this reason the feasibility of using SELDI in a clinical laboratory setting has yet to be proven.⁴⁰ Additional criticisms of the technique include poor reproducibility, especially between laboratories, and low sensitivity and specificity compared with traditional MALDI-TOF.

Proteomics has also been applied extensively to the analysis of allergens for wheat,⁴¹ soy,⁴² peanut,⁴³ and mold.⁴⁴ In general, protein extracts from allergens are separated using 2DGE and then blotted with sera from allergic patients. Candidate allergens are extracted from the gels and identified using MS.

In addition to global studies, proteomics can also be used to elucidate molecular pathways or functions, potentially leading to drug targets and better understanding of disease mechanism. For example, Cao and colleagues⁴⁵ used MS to quantitatively characterize phosphorylation events in mast cells, whose activation leads to the release of mediators in the early-phase allergic response.

TRANSLATION INTO CLINICAL SETTING

After biomarker discovery, a targeted LCMS analysis can be developed that is appropriate for application in a clinical laboratory. A technique commonly used in small molecule analysis, for example, in newborn screening,⁴⁶ is now being applied to targeted protein analysis.⁴⁷ Termed *multiple reaction monitoring*, this directed approach uses MS and stable isotope-labeled internal standards to accurately and specifically detect and quantitate up to hundreds of molecules in a single multiplex assay. This method is more precise than antibody-based assays (eg, enzyme immunoassay), which are often not able to distinguish among similar molecules, such as protein isoforms. Enzyme immunoassays may also require large-scale production of antibodies and are not as readily multiplexed compared with LCMS. Thus, multiple reaction monitoring assays can be a cost- and time-effective means of conducting large-scale validation studies.

CHALLENGES AND REALITIES

Although there is immense potential for generating valuable biomarkers using clinical proteomics studies, many challenges remain at every step of a typical protein biomarker discovery workflow. Despite improvements in sample preparation, MS technology, and bioinformatics, current methods allow analysis of less than 20% of typical proteomes. Although fractionation techniques are somewhat effective at improving our ability to analyze larger portions of proteomes, this can significantly reduce throughput and increase costs associated with profiling studies. Despite these challenges, proteomics can be successfully applied to clinical science when sound experimental design and techniques are used.¹¹ LCMS can then offer several advantages over traditional antibody-based clinical assays, including greater specificity, cost- and time-effectiveness, and the ability to multiplex up to hundreds of peptides in a single assay. With many guidelines now in place and model studies on which to design future experiments, there is reason to be optimistic that candidate protein biomarkers will be discovered using proteomics and translated into clinical assays.

ACKNOWLEDGMENTS

We thank Michael Armstrong and Roger Powell for their helpful suggestions and critical review of the article.

REFERENCES

1. Hanash S. Disease proteomics. *Nature*. 2003;422:226–232.
2. Granger CB, Van Eyk JE, Mockrin SC, Anderson NL. National heart, lung, and blood institute clinical proteomics working group report. *Circulation*. 2004;109:1697–1703.
3. Wattiez R, Falmagne P. Proteomics of bronchoalveolar lavage fluid. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2005;815:169–178.
4. Magi B, Bargagli E, Bini L, Rottoli P. Proteome analysis of bronchoalveolar lavage in lung diseases. *Proteomics*. 2006;6:6354–6369.
5. Gray RD, MacGregor G, Noble D, et al. Sputum proteomics in inflammatory and suppurative respiratory diseases. *Am J Respir Crit Care Med*. 2008;178:444–452.
6. Nicholas B, Skipp P, Mould R, et al. Shotgun proteomic analysis of human-induced sputum. *Proteomics*. 2006;6:4390–4401.

7. Mischak HA, Banks RE, Conaway M, et al. Clinical proteomics: a need to define the field and to begin to set adequate standards. *Proteomics Clin Appl*. 2007;1:148–156.
8. Aebersold R, Mann M. Mass spectrometry-based proteomics. *Nature*. 2003;422:198–207.
9. Bowler RP, Ellison MC, Reisdorph N. Proteomics in pulmonary medicine. *Chest*. 2006;130:567–574.
10. Lescuyer P, Hochstrasser D, Rabilloud T. How shall we use the proteomics toolbox for biomarker discovery? *J Proteome Res*. 2007;6:3371–3376.
11. Good DM, Thongboonkerd V, Novak J, et al. Body fluid proteomics for biomarker discovery: Lessons from the past hold the key to success in the future. *J Proteome Res*. 2007;6:4549–4555.
12. Anderson NL, Anderson NG. The human plasma proteome: history, character, and diagnostic prospects. *Mol Cell Proteomics*. 2002;1:845–867.
13. Hoffman SA, Joo WA, Echan LA, Speicher DW. Higher dimensional (hi-d) separation strategies dramatically improve the potential for cancer biomarker detection in serum and plasma. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2007;849:43–52.
14. Wu J, Kobayashi M, Sousa EA, et al. Differential proteomic analysis of bronchoalveolar lavage fluid in asthmatics following segmental antigen challenge. *Mol Cell Proteomics*. 2005;4:1251–1264.
15. Ogata Y, Charlesworth MC, Higgins L, et al. Differential protein expression in male and female human lumbar cerebrospinal fluid using ITRAQ reagents after abundant protein depletion. *Proteomics*. 2007;7:3726–3734.
16. Drake RR, Schwegler EE, Malik G, et al. Lectin capture strategies combined with mass spectrometry for the discovery of serum glycoprotein biomarkers. *Mol Cell Proteomics*. 2006;5:1957–1967.
17. Benson LM, Mason CJ, Friedman O, et al. Extensive fractionation and identification of proteins within nasal lavage fluids from allergic rhinitis and asthmatic chronic rhinosinusitis patients. *J Sep Sci*. 2009;32:44–56.
18. Yeo S, Roh GS, Kim DH, et al. Quantitative profiling of plasma peptides in asthmatic mice using liquid chromatography and mass spectrometry. *Proteomics*. 2004;4:3308–3317.
19. Righetti PG, Castagna A, Herbert B, Candiano G. How to bring the “unseen” proteome to the limelight via electrophoretic pre-fractionation techniques. *Biosci Rep*. 2005;25:3–17.
20. Magi B, Bini L, Perari MG, et al. Bronchoalveolar lavage fluid protein composition in patients with sarcoidosis and idiopathic pulmonary fibrosis: a two-dimensional electrophoretic study. *Electrophoresis*. 2002;23:3434–3444.
21. *Current Protocols in Protein Science*. Maltham, MA: John Wiley & Sons Inc; 2008.
22. Old WM, Meyer-Arendt K, Aveline-Wolf L, et al. Comparison of label-free methods for quantifying human proteins by shotgun proteomics. *Mol Cell Proteomics*. 2005;4:1487–1502.
23. Wu WW, Wang G, Baek SJ, Shen RF. Comparative study of three proteomic quantitative methods, DIGE, CICAT, and ITRAQ, using 2D gel- or LC-MALDI TOF/TOF. *J Proteome Res*. 2006;5:651–658.
24. Gulcicek EE, Colangelo CM, McMurray W, et al. Proteomics and the analysis of proteomic data: an overview of current protein-profiling technologies. *Curr Protoc Bioinformatics*. 2005;chap 13, unit 13.11.
25. Cornett DS, Reyzer ML, Chaurand P, Caprioli RM. MALDI imaging mass spectrometry: molecular snapshots of biochemical systems. *Nat Methods*. 2007;4:828–833.
26. Groseclose MR, Massion PP, Chaurand P, Caprioli RM. High-throughput proteomic analysis of formalin-fixed paraffin-embedded tissue microarrays using MALDI imaging mass spectrometry. *Proteomics*. 2008;8:3715–3724.
27. Massion PP, Caprioli RM. Proteomic strategies for the characterization and the early detection of lung cancer. *J Thorac Oncol*. 2006;1:1027–1039.
28. Deutsch EW, Lam H, Aebersold R. Data analysis and bioinformatics tools for tandem mass spectrometry in proteomics. *Physiol Genomics*. 2008;33:18–25.
29. Hirsch J, Hansen KC, Burlingame AL, Matthay MA. Proteomics: current techniques and potential applications to lung disease. *Am J Physiol Lung Cell Mol Physiol*. 2004;287:L1–23.
30. Greenlee KJ, Corry DB, Engler DA, et al. Proteomic identification of in vivo substrates for matrix metalloproteinases 2 and 9 reveals a mechanism for resolution of inflammation. *J Immunol*. 2006;177:7312–7321.
31. Halapi E, Hakonarson H. Recent development in genomic and proteomic research for asthma. *Curr Opin Pulm Med*. 2004;10:22–30.
32. Howell MD, Fairchild HR, Kim BE, et al. Th2 cytokines act on s100/a11 to downregulate keratinocyte differentiation. *J Invest Dermatol*. 2008;128:2248–2258.
33. Roh GS, Shin Y, Seo SW, et al. Proteome analysis of differential protein expression in allergen-induced asthmatic mice lung after dexamethasone treatment. *Proteomics*. 2004;4:3318–3327.
34. Sepper R, Prikk K. Proteomics: is it an approach to understand the progression of chronic lung disorders? *J Proteome Res*. 2004;3:277–281.
35. Wong WS, Zhu H, Liao W. Cysteinyl leukotriene receptor antagonist mk-571 alters bronchoalveolar lavage fluid proteome in a mouse asthma model. *Eur J Pharmacol*. 2007;575:134–141.
36. Wong WS, Zhao J. Proteome analysis of chronically inflamed lungs in a mouse chronic asthma model. *Int Arch Allergy Immunol*. 2008;147:179–189.
37. Bowler RP, Canham ME, Ellison MC. Surface enhanced laser desorption/ionization (SELDI) time-of-flight mass spectrometry to identify patients with chronic obstructive pulmonary disease. *COPD*. 2006;3:41–50.
38. Das S, Maeso PA, Becker AM, et al. Proteomics blood testing to distinguish chronic rhinosinusitis subtypes. *Laryngoscope*. 2008;118:2231–2234.
39. Baldini C, Giusti L, Bazzichi L, Lucacchini A, Bombardieri S. Proteomic analysis of the saliva: a clue for understanding primary from secondary Sjogren’s syndrome? *Autoimmun Rev*. 2008;7:185–191.
40. Kiehnopf M, Siegmund R, Deufel T. Use of SELDI-TOF mass spectrometry for identification of new biomarkers: potential and limitations. *Clin Chem Lab Med*. 2007;45:1435–1449.
41. Akagawa M, Handoyo T, Ishii T, et al. Proteomic analysis of wheat flour allergens. *J Agric Food Chem*. 2007;55:6863–6870.
42. Batista R, Martins I, Jeno P, Ricardo CP, Oliveira MM. A proteomic study to identify soya allergens: the human response to transgenic versus non-transgenic soya samples. *Int Arch Allergy Immunol*. 2007;144:29–38.
43. Chassaigne H, Norgaard J, Hengel AJ. Proteomics-based approach to detect and identify major allergens in processed peanuts by capillary LC-Q-TOF (MS/MS). *J Agric Food Chem*. 2007;55:4461–4473.
44. Benndorf D, Muller A, Bock K, et al. Identification of spore allergens from the indoor mould *aspergillus versicolor*. *Allergy*. 2008;63:454–460.
45. Cao L, Yu K, Banh C, et al. Quantitative time-resolved phosphoproteomic analysis of mast cell signaling. *J Immunol*. 2007;179:5864–5876.
46. Wilcken B, Wiley V. Newborn screening. *Pathology*. 2008;40:104–115.
47. Serkova N, Reisdorph N, Tissot van Patot M. Metabolic markers of hypoxia: systems biology application in biomedicine. *Toxicol Mechanisms Methods*. 2007;18:81–95.

Requests for reprints should be addressed to:
 Nichole Reisdorph, PhD
 National Jewish Health
 1400 Jackson St, K924b
 Denver, CO 80206
 E-mail: ReisdorphN@njc.org