Chapter 2

The Role of One- and Two-Dimensional Electrophoretic Techniques in Proteomics of the Lung

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Additional information is available at the end of the chapter

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Abstract

The current chapter was designed to keep the reader informed about the present status of pulmonary proteome. Taken together, the results documented here demonstrate that, after a decade of activity, proteomics of pulmonary diseases is catching up with its promise. The constantly growing number of reports in this area supports the view of this approach as one of the decisive methodological tools for the identification/characterization of disease-associated proteins. In terms of experimental procedures, the basic options available for proteomic investigations consist in the identification of proteins through the use of gel-based or gel-free techniques followed by MS. Obviously, the question arises of whether sophisticated technologies (such as the non-gel-based proteomic procedures) may currently be more fruitful, in terms of candidate protein marker identification, than "conventional" (read electrokinetic) approaches. In light of the versatility and high degree of reproducibility shown by these new potent strategies, a positive answer is perhaps not surprising. Nevertheless, as documented in this chapter, despite being less sophisticated than competing ones, gel-based techniques still represent a widely used procedure able to generate a reliable protein "fingerprint" and to produce qualitative and quantitative information on the protein patterns of a variety of human fluids.

Keywords: proteomics, lung diseases, 1-DE, 2-DE, 2D-DIGE, CE, MS

1. Introduction

The changes of the research strategy in the biochemical field driven by technological advancements occurred in these years allowed proteomics to progress and become one of the most captivating

branches of biochemistry. It is no exaggeration to say that, thanks to the significant step forward in the sensitivity and specificity of analytical methods, (almost) all proteins expressed by an organism can be detected/quantified even in extremely complex biological matrices. The monitoring of protein expression patterns in clinical specimens may indeed offer great opportunities to establish sets of biomarkers potentially associated with a specific disease status [1–8]. Despite these advantages, the question arises whether the application of these sophisticated proteomic procedures have profoundly affected the study of human diseases. If the number of publications in this field counts for something, the answer is undoubtedly positive. Through its ability to provide insights into both specific and system-level changes in cell, tissue and human physiology, proteomics has driven the progress observed in the last years in the elucidation of a variety of multifactorial pathological conditions, including less commonly diagnosed disorders [9–13]. In clinical proteomics several tissues and/or biological fluids are routinely analyzed for expressed proteins. Obviously, to make these analyses quantitative and reproducible, reliable profiling procedures should be established. Such procedures must rely on efficient and robust separation systems whose pivotal role is to make the complexity of mixtures simpler. Electrophoretic separations, both in-gel (1-DE and 2-DE) and in-solution (capillary electrophoresis (CE) and liquid chromatography (LC) approaches, both coupled to mass spectrometry (MS)), are currently the most attractive strategies toward the separation of hundreds/thousands of proteins. While acknowledging a remarkable success of electrophoretic approaches over the years, their intrinsic limitations cannot be hushed up. For example, the poor (if any) resolution of hydrophobic membrane proteins or of proteins that are too basic/acidic or too large/small is a well-known limit of 2-DE. The high protein amount required, the long time needed to run the samples, and the difficulty in automating the whole system are additional potential drawbacks of these techniques. The advent of LC-based procedures, characterized by unquestionable advantages, seemed to mark the fate of electrokinetic methods in the proteomic area. Being a bit of years elapsed, it can be argued that, despite the strong competition with LC, gel-based techniques do not appear to have lost this "struggle" yet [14–17]. Electrokinetic approaches, in fact, not only maintain their position but also still possess numerous characteristics currently unmatched by other proteomic methodologies. 2-DE approaches in fact remain the conventional procedure applied to the differential (control *vs.* diseased case) analysis of biological samples [18–24].

2. Outline of the chapter

The aim of this chapter is to illustrate the potential of electrokinetic procedures to the investigation of a variety of acute and chronic lung disorders. Why focus on pulmonary disorders? First, because lung diseases, which involve tens of million people, are some of the most common medical conditions in the world. Second, while few proteomic studies had been specifically designed to address this topic in the past, the depth of analysis ultimately reached by current procedures provides a new and larger context for future studies on the biology of these pathologies. This has allowed for the generation of protein profiles that are useful for exploring protein-based pathological mechanisms and/or discovering new potential therapeutic targets for a variety of pulmonary disorders.

Each paragraph of this chapter will address the proteomic data relative to a specific lung disease. Given that proteomic studies on pulmonary disorders have been carried out mostly on biofluids, the chapter will focus on the protein profiles obtained from serum/plasma, bronchoalveolar

lavage fluid, exhaled breath condensate, sputum and urine of individuals with asthma, interstitial lung disease (ILD), cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), and other "minor" pathologies. Since different fluids may serve as a rich source of information for the same disorder, specific subsections inside each paragraph have been dedicated to the presentation of the proteomic results relative to a peculiar fluid.

Given the hugeness of the subject, proteomics of lung cancer has thoroughly been investigated and greatly acknowledged elsewhere. In keeping with this large deal of literature, this topic would require a chapter dedicated, and it was intentionally left out of this chapter.

3. Principles of electrokinetic procedures

Since electrokinetic approaches play a pivotal role throughout the whole chapter, a few details about the principles of techniques cited in the following paragraphs will be given here. The term "electrokinetics" refers to the motion of charged particles by an applied electrical field. The standard laboratory technique by which charged molecules migrate through a porous matrix which

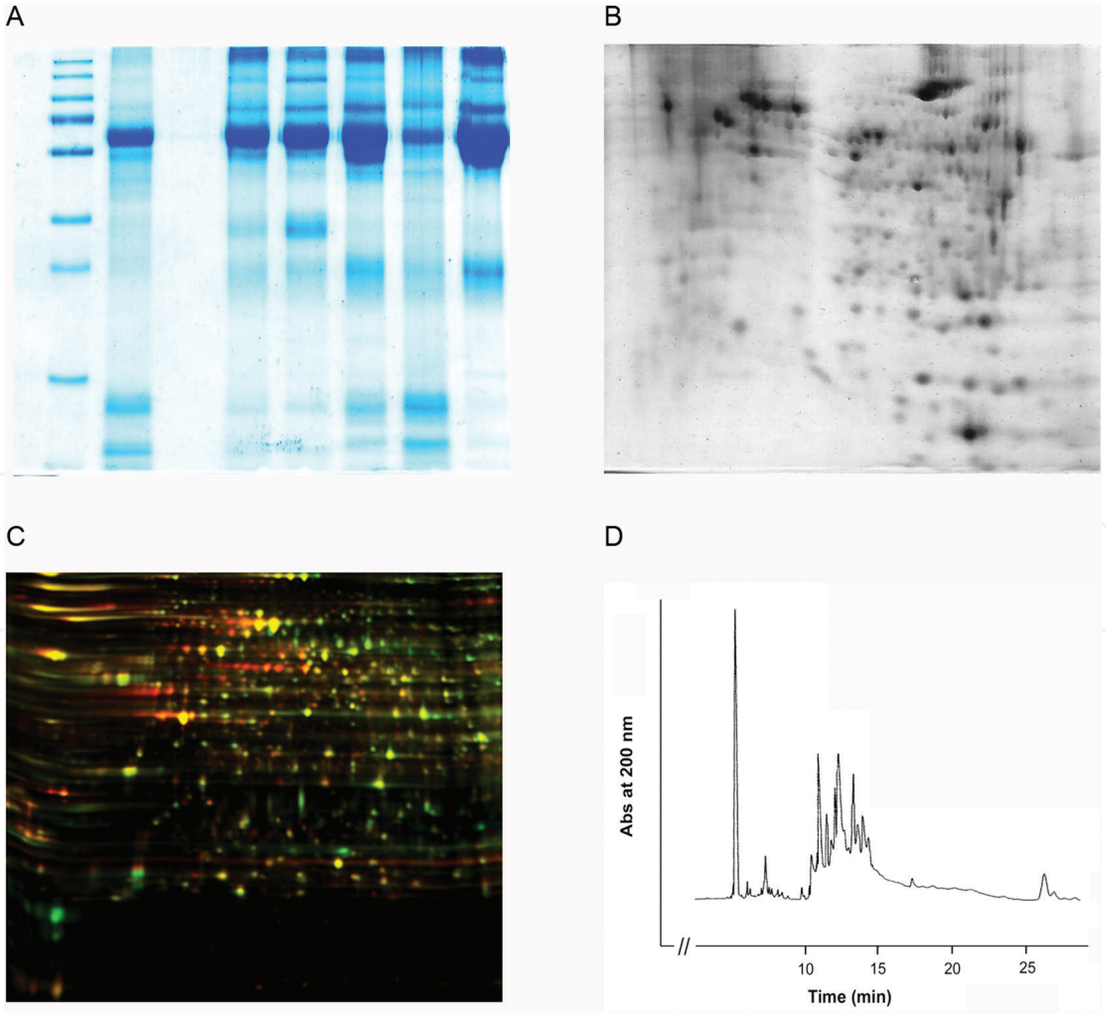

Figure 1. Representative examples of 1-DE, 2-DE, 2D-DIGE, and CE: panels A, B, C, and D, respectively (unpublished results from our laboratory).

behaves like a molecular sieve is electrophoresis. Polyacrylamide is an ideal support for separating most proteins; under denaturing/reducing conditions and with a discontinuous buffer system, one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) or 1-DE is the most widely used electrophoretic technique separating proteins primarily by mass. When the ionic detergent SDS binds to proteins, they assume a uniform negative charge. Upon application of a current, all SDS-bound proteins in the sample will migrate toward the positive electrode, and, because of the sieving effect of the gel matrix, proteins with less mass split from those with greater mass because they travel more quickly. When the mixture of proteins is very complex, higher resolution may be obtained by applying a two-dimensional (2-DE) procedure which separates proteins according to their isoelectric point in the first dimension followed by an orthogonal separation via SDS-PAGE in the second one. Similar to 2-DE is two-dimensional difference gel electrophoresis (2D-DIGE) in which, however, two or more samples are labeled with different fluorescent dyes and separated on the same gel, thus eliminating gel-to-gel variability. An instrumental evolution of the abovementioned electrophoretic techniques is capillary electrophoresis (CE) in which separation occurs in fused-silica capillaries and separation of proteins involves application of high voltages across buffer-filled capillaries. Once separated by electrophoresis, gel bands/spots are excised and proteins extracted for analysis by mass spectrometry.

Representative examples of 1-DE, 2-DE, 2D-DIGE, and CE are shown in **Figure 1** (panels A, B, C, and D, respectively).

4. Asthma

Asthma is an airway disease originating from complex interactions between genetic factors and environmental agents. It affects over 300 million people around the world and is characterized by airflow limitation resulting in shortness of breath. Bronchial obstruction is extremely variable and often reversible in asthma. Being a global health problem, tools which allow its early diagnosis, monitoring, and follow-up would offer great advantages for a better insight into its physiopathology. The articles discussed in the following sections prove that the proteomic content of sputum/induced sputum and BALf may allow for the identification of specific biomarkers in asthma.

4.1. Sputum/induced sputum

2-DE coupled to MALDI/TOF allowed Lee et al. [25] to compare the proteomic profiles of sputum from patients with neutrophilic-type uncontrolled asthma (UA) and from patients with neutrophilic controlled asthma (CA). It could be observed that, while a few proteins (including calgranulin S100A9) were overexpressed in sputum of UA patients, others, associated with inflammation, anti-inflammation, enzymatic activity, and immunity signaling, were downregulated. Differences in protein abundance and composition between asthma and rhinitis were revealed by the proteomic approach (2D-DIGE and MS) applied by Suojalehto et al. [26] to induced sputum (IS) and nasal lavage fluid (NLF) of patients. Their findings showed that (i) fatty acid-binding protein 5 (FABP5) was upregulated in IS of asthmatics, (ii) vascular endothelial growth factor (VEGF) was increased in NLF of asthmatics, and (iii) in NLF of these subjects,

FABP5 and VEGF were positively correlated with cysteinyl leukotriene (CysLT). Based on their results, the authors hypothesized that, in asthma, FABP5 may contribute to the airway remodeling and inflammation by regulating the levels of CysLTs which, in turn, induce VEGF production.

4.2. Bronchoalveolar lavage fluid

A differential study on BALf of asthmatic individuals was performed by Wu et al. [27] who produced the first comprehensive database of BALf proteins. Abundant proteins were depleted by immunoaffinity chromatography, and all others were separated by 1-DE and identified by nano-LC-MS/MS. Chemokines and cytokines and a variety of matrix metalloproteases (MMPs) were upregulated in subjects after segmental allergen challenge. Other highly overexpressed proteins included pulmonary surfactants and LPLUNC1.

5. Interstitial lung disease

Interstitial lung disease (ILD) describes a collection of more than 200 lung disorders that involve inflammation and fibrosis of the alveoli, distal airways, and septal interstitium of the lungs. Typically, ILD presents with progressive breathlessness, lung crackles, and a diffusely abnormal chest radiograph. This disorder can be caused by long-term exposure to hazardous materials or by some types of autoimmune diseases, such as rheumatoid arthritis. In some cases, however, the etiology remains unknown. The following sections summarize the latest proteomic studies aimed at clarifying the pathophysiological mechanisms involved in the development and progression of ILD. All recent proteomic studies in this area based on electrokinetic approaches have been performed on BALf.

5.1. Idiopathic pulmonary fibrosis

Idiopathic pulmonary fibrosis (IPF) is a progressive fibroproliferative disorder characterized by continuous production of extracellular matrix that alters parenchymal lung structure and effective gas exchange. Cigarette smoking, exposure to agriculture and farming, livestock, wood and metal dust, stone, and silica have been associated with significantly increased risk of IPF. The etiopathogenesis of the disease is not completely understood, and the prognosis is very poor. In light of this, proteomic studies aimed at the discovery of molecules involved in triggering and progression of this disorder could help to a better understanding of its physiopathology. As expected, all proteomic approaches to study this disorder which involved the use of an electrokinetic procedure have been performed on BALf.

Kim et al. [28] compared by 2-DE and nano-LC-MS/MS the proteomes of BALf from patients affected by IPF and healthy subjects. An increase of haptoglobin and a decrease of α1-antitrypsin, α1-antichymotrypsin, macrophage capping protein, angiotensinogen, hemoglobin chain B, apolipoprotein (Apo A-I), clusterin, protein disulfide isomerase A3, immunoglobulin, and complement C4A were observed in IPF subjects. That a local treatment with Apo A-I could be effective against the development of experimental lung fibrosis was shown in mice. The main goal of the study performed by Ishikawa and colleagues [29] was to discover

new markers for both IPF and chronic obstructive pulmonary disease (COPD). By comparing the 1-DE and 2-DE profiles of BALf and IS of IPF and COPD patients with those of controls, they showed a reduction in the levels of hemoglobin α (Hbα) and hemoglobin β (Hbβ) monomers and complexes only in IPF patients. Furthermore, MS analyses revealed that a modification at Cys 105 (most likely the site involved in complex formation) of Hbα was responsible for the lack of the Hbα complexes formation in IPF patients. 2-DE, MS, Western blotting, and ELISA were applied by Ohlmeier et al. [30] to lung tissues and BALf of IPF, COPD, and alpha-1 antitrypsin-deficient (AATD) patients with the aim to investigate the different receptors for advanced glycation end product (RAGE) isoforms. Their analyses revealed a decrease of the full-length RAGE (FL-RAGE) and its C-terminal processed variant (cRAGE) in the lung tissues of IPF and COPD patients but not in AATD. The endogenous secretory RAGE (esRAGE) level was reduced in IPF but remained unchanged in COPD. The 2-DE proteomic study by Hara et al. [31] aimed at searching for BALf biomarkers allowing to distinguish IPF from other fibrotic interstitial pneumonias. Their study showed that S100A9 protein (or calgranulin B, a member of the calcium-binding S100 protein family) was upregulated in IPF patients compared to patients with other fibrotic interstitial diseases and healthy controls. Based on these findings, S100A9 was thought to be a good candidate biomarker to discriminate IPF from other fibrotic interstitial pneumonias. The research group by Landi et al. [32] used the electrokinetic approach to compare the proteome of BALf from patients affected by four interstitial lung diseases (sarcoidosis, idiopathic pulmonary fibrosis, pulmonary Langerhans cell histiocytosis, fibrosis associated to systemic sclerosis) with that of controls (smokers and nonsmokers). The proteins they identified (i.e., plastin 2, annexin A3, 14-3-3ε, and S10A6) could be directly or indirectly related to the pathophysiology of the different interstitial lung diseases. The same research group compared the profile of BALf proteins from IPF patients, never-smokers, and smokers to understand which protein(s) could be potentially related to disease progression and pathogenesis. Their findings showed that angiotensin system maturation, renin angiotensin-aldosterone system, heme metabolism, coagulation system, response to hypoxia, oxidative stress, and iron transport were the metabolic pathways involved in disorder [33]. The same group also investigated the molecular patterns and their variability in familial and sporadic IPF patients through a differential proteomic analysis based on 2-DE and MS [34]. It was observed that, while in familial IPF, the upregulated proteins were those involved in wounding and immune responses, coagulation system, and ion homeostasis, and those involved in the oxidative stress response were upregulated in sporadic IPF.

5.2. Sarcoidosis

Sarcoidosis is a multisystem disorder characterized by the formation of epithelioid cell granulomas. Even though lungs, lymph nodes, and eyes are most commonly involved, any organ or system of the body can be affected. Patients with genetic susceptibility undergoing persistent exposure to unknown inhaled antigens may develop an excessive immune response mediated by antigen-presenting cells (APC) that can trigger the pathological mechanisms of the disease. However, its etiology still remains an enigma and a challenge for researchers and clinicians due to its unknown, variegated, and unpredictable presentation. The recent state of the art relative to the application of proteomic studies aimed at gaining insights into the mechanisms of the disease is reported in this section.

Silva et al. [35] have used a 2D-based proteomic approach to analyze BALf of patients affected by sarcoidosis and chronic beryllium disease (CBD) with the aim of comparing the protein profiles at the site of active inflammation. Briefly, the differentially expressed proteins were highlighted by 2D-DIGE and identified by MALDI-TOF. In accordance with the ongoing chronic inflammation response in lungs of these patients, most of these proteins were immune-related proteins.

While being a well-known risk factor for the development of various interstitial lung diseases, smoking is generally not related to the occurrence of sarcoidosis. To evaluate better the influence of cigarette smoking on the proteome of BALf from sarcoidosis patients and to study the pathogenetic mechanism of the disease, Landi et al. [36] investigated by 2-DE the protein profile and network of BALf from sarcoidosis patients, smokers (SC), and nonsmokers (NSC) used as controls. MALDI-TOF MS revealed that 34 spots contained unique proteins involved in lipid, mineral, and vitamin D metabolism and immune regulation of macrophage function. These findings confirmed that, differently from other ILDs, the expression profile of proteins from sarcoidosis patients was more comparable to that of NSC than of SC.

5.3. Lung fibrosis associated with systemic sclerosis

Systemic sclerosis (SSc) is a disease of unknown origin characterized by increased deposition of collagen and other extracellular matrix proteins in skin and multiple internal organs. About 70% of SSc patients develop a severe and progressive lung fibrosis, ILD being the main cause of mortality. Nevertheless, the mechanisms involved in the onset and progression of fibrosis remains unknown. To clarify the causative role of ILD in SSc, Shirahama et al. [37] analyzed the protein profiles of BALf from patients affected by SSc with and without pulmonary fibrosis (SSc-fib+ and SSc-fib−). 2-DE combined with MALDI-TOF MS was the method of choice. The authors showed that, among other proteins identified, α2-macroglobulin, α1-antitrypsin, and pulmonary surfactant protein A were upregulated in SSc-fib+ patients, while α2 heat shock protein (HSP) and glutathione S-transferase (GST) were downregulated in the same patients compared to SSc-fib ones. These results suggested that these proteins could be potentially involved in the development of ILD in SSc patients.

5.4. Rheumatoid arthritis-associated lung disease

Rheumatoid arthritis (RA) is a systemic autoimmune disease affecting 0.5–1.0% of the adult population worldwide. Rheumatoid arthritis-associated interstitial lung disease (RA-ILD) occurs in 10–30% of RA patients and is associated with increased mortality in up to 10% of RA patients. The pathogenetic mechanisms of RA-ILD are still unknown. The most common pulmonary patterns in RA-ILD are usual interstitial pneumonia (UIP) and nonspecific interstitial pneumonia (NSIP). Nevertheless, the less common organizing pneumonia (OP) and lymphocytic interstitial pneumonia are not as rare as hypothesized. The proteomic profiles of UIP and OP were compared by Suhara et al. [38] by analyzing BALf of patients with 2-DE and nano-LC-ESI-MS/MS. The levels of the fragmented proteins gelsolin and Ig kappa chain C region were found to be significantly higher in UIP patients compared to those with an OP pattern. Conversely, the levels of α1-antitrypsin, C-reactive protein (CRP), haptoglobin β, and surfactant protein A (isoform 5) were significantly higher in the OP than in the UIP patterns.

Based on these considerations, the hypotheses were made that (i) identified proteins may play a role in the onset and progression of UIP and OP and (ii) fragmented gelsolins may contribute to the development of pulmonary fibrosis.

5.5. Pulmonary Langerhans cell histiocytosis

Pulmonary Langerhans cell histiocytosis (PLCH) is a rare interstitial lung disease associated with the exposure to tobacco smoke. To provide new insights into its pathogenesis and to compare this disorder with COPD, Ghafouri et al. [39] analyzed BALf from a biopsy-proven case of PLCH, a COPD patient, and a healthy control. Proteins were separated by 2-DE, and their patterns were analyzed with a computerized 2-DE imaging system. If compared with the profiles of the COPD patient and the healthy control, that of PLHC patient lacked significant amounts of protective and anti-inflammatory proteins. These findings opened a new route for a better understanding of the pathophysiology of PLCH.

6. Cystic fibrosis

Cystic fibrosis (CF) is an autosomal recessive monogenic disease caused by mutations in the gene coding for the CF transmembrane conductance regulator (CFTR) protein. The main clinical features of this disorder are progressive bronchiectasis and pancreatic exocrine insufficiency. Since airway inflammation and recurrent infections leading to respiratory failure represent the major causes of morbidity and mortality among CF patients, clinical research is mainly focused on these topics. The characterization of proteins from different sources and the study of their interactions within the lung microenvironment may be useful tools toward the identification of biomarkers for the diagnosis/prognosis of this disorder. The following subsections show recent reports on proteomic research in this field.

6.1. Induced sputum

The proteomic approach based on 1-DE, MALDI-TOF-MS, and LC-ESI-MS/MS allowed Schulz et al. [40] to analyze high molecular mass proteins in induced sputum from (i) CF adults with and without acute exacerbation, (ii) CF children with stable disease and preserved lung function, and (iii) healthy adults and children (controls). The main high molecular mass proteins in sputum from all subjects investigated were MUC5B and MUC5AC, two mucins that, in exacerbated CF adults, seemed degraded and also showed increased sialylation and reduced sulfation/fucosylation. In addition, while two CF children showed mucin profiles similar to those of exacerbated CF adults, the remaining CF children had profiles comparable to those of healthy controls. Based on these observations, the authors suggested that the processes of mucin glycosylation and degradation may be considered as predictive biomarkers of lung condition in CF patients.

6.2. Serum

Charro et al. [41] worked on immunodepleted sera of CF patients and of healthy CF and non-CF carriers. The combination of 2D-PAGE and shotgun LC-MS/MS showed that members of the apolipoproteins family were deregulated in CF patients, while heat shock 70 kDa protein 5

and the multifunctional enzyme NDKB (an ion sensor in epithelial cells, pancreatic secretion, neutrophil-mediated inflammation, and energy production) were identified exclusively in the CF group. This interesting comparative study allowed the authors to identify deregulated proteins involved in tissue remodeling, complement system dysfunction with consequent impairment on defense mechanisms and chronic inflammation, nutritional imbalance, and colonization by *P. aeruginosa*.

7. Chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is an "umbrella" term that combines different pathological conditions such as emphysema, chronic bronchitis, nonreversible asthma, and some types of bronchiectasis characterized by irreversible airflow limitations. Its clinical features are a general progressive airflow limitation, destruction of the lung parenchyma, and/or local fibrosis. Even though the main cause of the disorder is cigarette smoking, continuous inhalation of toxic gases and particles which promotes chronic airway inflammation may contribute to its development. COPD can also be determined by genetic factors, i.e., the deficiency of α-1 antitrypsin (AATD).

The following subsections describe electrokinetic-based proteomic studies aimed at elucidating the mechanism underlying the pathobiology of the disease.

7.1. Plasma

To shed new light on the molecular mechanisms of COPD, Merali et al. [42] investigated the plasma proteome of 10 COPD patients and 10 healthy controls. Briefly, abundant proteins in pooled plasma from each group were immunodepleted; samples were then fractionated by 1-DE prior to ESI-MS/MS. A few proteins including acute-phase response proteins, CRP, fibrinogen, coagulation factors, and adhesion molecules were upregulated in COPD patients compared to controls. By contrast, molecules involved in protease-induced lung tissue injury and repair were downregulated.

7.2. Induced sputum

In a proteomic study aimed at identifying proteins involved in COPD pathogenesis, Ohlmeier et al. [43] analyzed induced sputum of nonsmokers, smokers, and smokers with moderate COPD by cysteine-specific 2D-DIGE coupled with MS. Among other candidates, polymeric immunoglobulin receptor (PIGR), a protein involved in specific immune defense and inflammation, appeared upregulated in smokers and subjects with COPD, thus suggesting its possible role in the regulation of inflammation during COPD pathogenesis.

7.3. Bronchoalveolar lavage fluid

The application of 2-DE followed by LC-MS/MS allowed Plymoth et al. [44] to investigate the global proteome of BALf from 29 light and heavy smokers and 18 never-smokers in 6–7 years of follow-up study. During this study 7 of the 29 smokers developed moderate COPD.

The authors observed that, although protein components were variably expressed in individuals, inflammatory and redox proteins were unambiguously upregulated in smokers compared to never-smokers.

To elucidate putative molecular mechanisms underlying gender-based differences in the pathophysiology of COPD, Kohler et al. [45] applied 2D-DIGE coupled to nano-LC-MS to investigate the proteome of non-symptomatic smokers and smokers with COPD. The authors observed significant gender differences with a subset of 19 proteins providing a highly predictive model for classification of female non-symptomatic smokers from female smokers with COPD. Subsequent pathway analyses correlated the observed alterations to downregulation of the lysosomal pathway and upregulation of the oxidative phosphorylation pathway. No altered proteins were found in the corresponding male classification model.

An interesting research (based on 2-DE and MALDI-TOF/TOF) by Pastor et al. [46, 47] evidenced a distinct proteomic reactive oxygen species (ROS) protein signature in BALf from COPD and lung cancer patients. Their findings highlight the role of ROS proteins in the pathogenic pathways of both diseases and provide new candidate biomarkers and predictive tools for lung cancer and COPD diagnosis.

8. Other pulmonary disorders

8.1. Idiopathic pulmonary arterial hypertension

Idiopathic pulmonary arterial hypertension (IPAH) is a rare, life-threatening disorder clinically characterized by sustained elevations of pulmonary artery pressure (PAP). Together with intimal proliferation, in situ thrombi, deposition of extracellular matrix, and inflammation, the remodeling of the pulmonary arterial vasculature with medial and adventitial thickening of the arteries and arterioles is the main feature of IPAH. This leads to increased pulmonary vascular resistance, right heart failure, and death. The latest proteomic studies aimed at providing insights into the molecular mechanisms underlying IPAH are presented in this section. All recent proteomic studies based on the use of an electrokinetic approach have been performed on serum/plasma.

The first proteomic approach aimed at identifying protein changes in serum from IPAH patients was performed by Yu et al. [48] by using a combination of 2-DE and MS. The attention of the authors was focused on α-1 antitrypsin and vitronectin that, being downregulated in IPAH patients, were indicated as proteins involved in the development of the disorder. In fact, while the decrease of plasma vitronectin might favor platelet aggregation and thrombus formation, the decrease of α-1 antitrypsin might induce apoptosis of endothelial cells resulting in proliferation of apoptosis-resistant endothelial cell arteriolar occlusion. 2-DE and immunoblotting were the combination applied by Terrier et al. [49] to identify the target antigens of antifibroblast antibodies (AFAs) in patients affected by IPAH. Among the 21 protein spots specifically recognized by serum IgG antibodies from IPAH patients, 16 proteins involved in (i) regulation of cytoskeletal function, (ii) cell contraction, (iii) oxidative stress, (iv) cell energy metabolism, and (v) other key cellular pathways were identified.

The comparative proteomic analysis based on 2-DE and MALDI-TOF performed by Zhang et al. [50] on serum of IPAH patients and controls identified, among other proteins showing significant changes, leucine-rich α-2-glycoprotein (LRG) as a possible specific prognostic biomarker of IPAH. 2D-DIGE coupled to MALDI-TOF-MS was used by Yeager et al. [51] to investigate the plasma proteome of IPAH children with good and poor outcome to long-term vasodilator therapy. Before and after therapy, serum amyloid A (SAA-4) was found to be fourfold lower in patients with good outcome compared to those with poor outcome, while serum amyloid P (SAP) was 1.3-fold lower prior to therapy in patients with good outcome. Since these proteins regulate circulating mononuclear phagocytes, they may contribute to the differential response to chronic vasodilator therapy.

8.2. Pulmonary embolism

Pulmonary emboli usually arise from thrombi originating in the deep venous system of the lower extremities that travel to the lung and can lodge at the bifurcation of the main pulmonary artery or the lobar causing hemodynamic compromise. Mortality rates in patients with acute pulmonary embolism (PE) vary from 1.4 to 17.4% during the first 3 months of treatment. The causes of most early deaths are complications such as acute pulmonary arterial hypertension and right heart failure, whereas medical problems underlying PE are responsible for most late deaths. The subsections reported below describe the recent advances in PE proteomics.

8.2.1. Plasma

To identify biomarkers useful for the risk stratification in patients with acute symptomatic PE, Insenser et al. [52] studied the plasma proteome of PE patients with low, intermediate, and high risk. 2D-DIGE was used to compare the abundance of plasma proteins in the three cohorts, and candidate protein markers were identified by MALDI-TOF. Differences among patients with different PE severities were observed in a panel of four biomarkers (haptoglobin, hemopexin, α2-macroglobulin, and Ig α-1-chain C region) involved in iron metabolism pathways and acute-phase response. Among them, the reduced concentrations of haptoglobin could be considered an accurate signal for the biochemical detection of high-risk PE.

8.2.2. Urine

A proteomic approach based on capillary electrophoresis coupled to mass spectrometry (CE-MS) was applied by von zur Mühlen et al. [53] for noninvasive identification of PE specific urinary markers of pathophysiological relevance. The analysis of urine from patients with symptoms associated with deep vein thrombosis and pulmonary embolism (DVT + PE) allowed the authors to identify 62 urinary peptides, i.e., fragments of type I collagen and a fragment of fibrinogen β-chain, whose presence in organized and acute thrombus has been demonstrated by the authors using immunohistochemistry.

Compared to asthma, interstitial lung disease, cystic fibrosis, and COPD, fewer articles have been published for "minor" diseases including acute respiratory distress syndrome (ARDS), high-altitude pulmonary edema (HAPE), and invasive pulmonary aspergillosis (IPA). This is, in large part, due to the lower prevalence of these disorders which results in difficulties

recruiting cohorts of patients whose size allows detection of statistically significant data. Nevertheless, Chang et al. [54] and Sixt et al. [55] investigated the proteomics of ARDS; proteomic data on HAPE have been published by Ahmad et al. [56, 57] and by Yang et al. [58], and preliminary data on IPA have been produced by Brasier et al. [59].

9. Tuberculosis

With more than 10.4 million new cases and about 1.8 million deaths each year, tuberculosis (TB) still remains an urgent global health problem. Being all deaths mainly due to the increasing spread of *Mycobacterium tuberculosis* drug-resistant strains, early diagnosis and treatment of infection would be essential for the prevention. However, routine laboratory tests for drug-resistant TB with sufficient sensitivity and specificity are still not available. This is the rationale for the development of electrophoretic-based proteomic approaches aimed at identifying host TB-associated proteins or antigens useful for the serodiagnosis of drug-resistant *M. tuberculosis* strains. To better understand and monitor the disease process, Tanaka et al. [60] analyzed whole blood supernatants from TB patients by 2D-DIGE followed by MS. Among others, the authors observed that retinol-binding protein 4 (RBP4) and fetuin-A were significantly lowered in patients with active TB compared to controls, thus suggesting that they could be considered potential biomarkers for monitoring the course of the disease during clinical treatment.

Method	Advantages/disadvantages	Lung disease	Matrix	Ref. no.
1-DE	—Allows separation of all types of proteins, even those insoluble in water	Asthma	BALf	[27]
	—Overlapping of closely spaced bands leading to limited resolution	IPF	BALf/IS	[29]
	—Often needs the coupling of another detection technique, i.e., immunoblotting or MS	CF	IS	[40]
		COPD	Plasma	[29, 42]
2-DE	—Good resolution of protein mixtures	Asthma	IS	[25]
	—Allows discernment of posttranslational modifications	IPF	BALf	[29–34]
	—Comparison of multiple gels facilitated by image analysis software	Sarcoidosis	BALf	[32, 35–36]
	—Unable to resolve low molecular weight proteins (<10 kDa)	Lung fibrosis associated to SSc	BALf	[32, 37]
	—Presence of high-abundance protein (i.e., albumin, immunoglobulin) hiding low-abundance proteins	RA-ILD	BALf	[38]
		PLCH	BALf	[32, 39]
	—Final identification requires spot removal from gels, digestion, and peptide analysis by MS	CF	Serum	[41]
	—Low throughput	COPD	BALf	[29, 30, 44, 46, 47]
		IPAH	Serum	[48–50]
		TB	Serum	[61]

Method	Advantages/disadvantages	Lung disease	Matrix	Ref. no.
2D-DIGE	—Very sensitive	Asthma	Sputum/NLF	[26]
	—Ratio of protein expression can be obtained in a single gel	COPD	IS	[43]
			BALf	[45]
	—An internal standard can be introduced in each gel reducing gel-to-gel variation	IPAH	Serum	[51]
	—Presence of high-abundance protein (i.e., albumin, immunoglobulin) hiding low-abundance proteins	PE	Plasma	[52]
	—Unable to resolve low molecular weight proteins (<10 kDa)	TB	Serum	[60]
	—Low throughput			
	—Final identification requires spot removal from gels, digestion, and peptide analysis by MS			
CE	—Less expensive than LC-MS	PE	Urine	[53]
	—Very small volumes of samples injected (nL)			
	—Detection of basic and hydrophilic peptides			
	—Fast separation			
	—Limited loading capacity			
	—Unavailability of an integrated system as a marketed solution			

Table 1. List of proteomic approaches considered in this report together with their advantages/disadvantages, the type of pulmonary disorder, the matrix considered, and the original article of reference.

Using an immune-proteomic approach based on 2-DE coupled to MALDI-TOF/TOF, Zhang et al. [61] analyzed serum from healthy controls and patients infected with drug-resistant or drug-susceptible *M. tuberculosis* strains. By comparing the immune-reactive proteins, the authors identified antigens present only in drug-resistant strains. Among the identified immune-reactive proteins, Rv2031c, Rv3692, and Rv0444c had the greater antigenic activity. This feature made them possible candidate biomarkers for the serum diagnosis of drug-resistant TB.

The list of proteomic approaches considered in this report, together with their advantages/disadvantages, the type of pulmonary disorder, the matrix considered, and the original article of reference, is shown in **Table 1**.

10. General considerations

Although the characterization of the full proteome is still challenging, the recent technological innovations have improved our ability to obtain cross-sectional time and space snapshots of protein levels that reflect observed phenotypes more closely than those of genomic techniques. The current successes in the use of proteomic approaches to understand disease and enable drug development resulted in optimism that many more effective diagnostic tests

and treatments tailored to genetic, environmental, and lifestyle factors of individuals will be developed. Showing a great ability in providing reference data for the identification of groups of individuals who share various attributes, these approaches have opened new opportunities for the discovery of potential diagnostic/prognostic protein biomarkers in several pulmonary disorders. By helping researchers in understanding the pathogenesis of respiratory diseases and improve patient care, the recent findings have indeed progressively increased the interest for the application of proteomics in clinical practice. The current chapter was designed to keep the reader informed about the present status of pulmonary proteome. Taken together, the results documented here demonstrate that, after a decade of activity, proteomics of pulmonary diseases is catching up with its promise. The constantly growing number of reports in this area supports the view of this approach as one of the decisive methodological tools for the identification/characterization of disease-associated proteins. In terms of experimental procedures, the basic options available for proteomic investigations consist in the identification of proteins through the use of gel-based or gel-free techniques followed by MS. Undoubtedly, the striking improvement in technologies related to accuracy, when coupled to quantitative approaches, has a great impact on the quality of the results. Obviously, the question arises of whether sophisticated technologies (such as the non-gel-based proteomic procedures) may actually be more fruitful, in terms of candidate protein marker identification, than "conventional" (read electrokinetic) approaches. In light of the versatility and high degree of reproducibility shown by these new potent strategies, a positive answer is perhaps not surprising, at least for one reason. The very high number of peptides identified and quantified results in a higher accuracy, which translates into improved alignment and quantification across spectra. Nevertheless, as documented in this chapter, despite being less sophisticated than competing ones, gel-based techniques still represent a widely used procedure able to generate a reliable protein "fingerprint." Though it may seem nonsense, it is precisely the "limited" amount of information produced by electrokinetic approaches that may result in an easy interpretation of data. The possibility to compare a sample in physiological and pathological conditions allows, in fact, immediate detection of possible relevant changes in protein expression which differentiate the two conditions. These changes are essential in demonstrating progression from health to disease and understanding the relationship between function and modification. The wide spectrum of examples presented in this chapter confirms that the application of 1-DE/2-DE/2-DIGE/CE (followed by MS) to a variety of biological fluids from individuals with different respiratory diseases may result in the production of data with clinical relevance which allow a better understanding of the molecular basis of the disorder investigated. However, as it can be observed from the data presented in this chapter, while peculiar proteins are pointed out as potential biomarkers of specific disorders, a good number of proteins is implicated across a variety of different diseases. This makes the notion of a single biomarker to indicate a specific disease more difficult. For example, while α2-macroglobulin and surfactant protein A have been indicated as candidate biomarkers of both lung fibrosis associated with systemic sclerosis and asthma [27, 37], the former protein (together with other proteins) was suggested to be also a potential biomarker of pulmonary embolism [52]. Indeed, for greater confidence in disease diagnosis or prognosis, a suite of biomarkers would provide more specificity than a single one. In other words, should the identification of hundreds of candidate biomarkers come at the

price of sacrificing their specificity? This discrepancy, however, is only apparent and may be reconciled with a harmonization of results. In fact, given that a single molecule can hardly discriminate complex processes without context, the finding of common signatures for several pulmonary disorders contributes to drawing intriguing parallels among them. This obviously results in a better understanding of the disease mechanism. On the other hand, the fact that several proteins found (e.g., in COPD) have not been reported in other chronic lung diseases suggests that the merits of proteomics in hunting down biomarkers with high specificity cannot be under-evaluated. In this context, the finding of significantly altered levels of cathepsin B, ATP synthase, and chaperonin in the BALf of female COPD patients, but not in that of males, while supporting the hypothesis that these proteins were the most prominent marker candidates for this gender only, confirmed the abovementioned merits of proteomics [45]. Proteomic studies have also discovered panels/clusters of oxidant/antioxidant enzymes that may be utilized for the assessment of disease severity [44]. Overproduction of ROS can also cause oxidative modifications of several important antioxidant/defense enzymes, which may be associated with alterations in enzyme conformation, and thus they can function as markers of the degree of oxidative stress present in the airways [46, 47]. The huge amount of experimental data generated in some cases (i.e., COPD and asthma) represent the first attempts to identify the principal pathways involved in the pathogenesis and already allow interesting candidate biomarkers to emerge [26, 43].

As the goal of the authors is to offer a broad picture of the subject, readers interested in learning more about specific techniques or their application to pulmonary diseases are encouraged to refer to excellent review articles in this field which show the merits of proteomic techniques in producing qualitative and quantitative information on the protein patterns of a variety of human fluids/tissues [62–65]. Taken together, these articles represent a good resource which describes in depth the status of electrokinetic (and chromatographic) proteomic methods and provide a comprehensive picture of proteomics of pulmonary disorders to date.

11. Conclusions

Aside from the interest in deciphering the function of individual proteins, the set of data produced by proteomic methods represent the starting point for studying large-scale interactions that serve to discover general important properties for interaction participation. The fact that highly interactive proteins are often well conserved and/or essential or that homologous proteins, and, in particular, proteins with domains from the same family, tend to interact more frequently than others will likely improve the knowledge of their intrinsic properties. Thus, the understanding of the role these proteins play in the pathogenesis of respiratory diseases, while opening the door to much more powerful protein diagnostics, reinforces the linkage between basic medical research and clinical laboratory medicine. Addressing these concerns is obviously a top priority for the field, the ultimate goal of researchers being to understand the biology of disease and to translate this knowledge into the clinic.

There is no doubt that this branch of respiratory proteomics will have substantial improvement in the future.

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Conflict of interest

The authors declare no conflict of interest.

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