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Review

Asthma, the ugly duckling of lung disease proteomics?

René Houtman[a,*], Edwin van den Worm[b]

[a] *Department of Pharmaceutical Proteomics, Faculty of Pharmaceutical Sciences, Utrecht University, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands*
[b] *Department of Pharmacology and Pathophysiology, Faculty of Pharmaceutical Sciences, Utrecht University, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands*

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Abstract

The human respiratory system represents a vital but vulnerable system. It is a major target for many diseases such as cancer and asthma. The incidence of these diseases has increased dramatically in the last 40–50 years. In the search for possible new therapies, many experimental tools and methods have been developed to study these diseases, ranging from animal models to in vitro studies. In the last decades, genomic and proteomic approaches have gained a lot of attention. After the major scientific breakthroughs in the field of genomics, it is now widely accepted that to understand biological processes, large-scale protein studies through proteomics techniques are required. In the battle against lung cancer, the proteomics approach has already been successfully implemented. Surprisingly, only a few proteomics studies on the ever-increasing global asthma problem have been published so far. And although proteomics also has its limitations and experimental difficulties, in our opinion, proteomics can definitely contribute to the understanding of a complex disease such as asthma. Therefore, the additional values and possibilities of proteomics in asthma research should be thoroughly investigated. A close collaboration between the different scientific disciplines may eventually lead to the development of new therapeutic strategies against asthma.

Keywords: Asthma; Lung disease proteomics; Respiratory system

Contents

* Corresponding author. Present address: Department of Pediatric Endocrinology, Academic Medical Center, Meibergdreef 9, 1105 AZ, Amsterdam, The Netherlands.
E-mail address: r.houtman@amc.uva.nl (R. Houtman).

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1. Introduction

1.1. The respiratory system

Working in combined action with the circulatory system, the main function of the respiratory system is to supply the blood with oxygen and pass out waste gases such as carbon dioxide. These tightly regulated processes, involving the CNS and vagal afferents from the lungs, are crucial for the vitality of an organism and therefore the respiratory system can be grouped among the most essential organs in the body.

The respiratory system can be divided into a conducting and a respiratory zone. In the conduction zone, the trachea, providing a means of air transport, branches into the right and left primary bronchi that enter the lungs. The total volume of this organ consists of approximately 10% solid tissue, whereas the remainder is filled with air and blood. Inside the lungs, each primary bronchus repeatedly branches into bronchi with increasingly smaller diameter. Bronchi smaller than 1 mm in diameter are called bronchioles that, via terminal bronchioles, eventually feed into the respiratory bronchioles. Here, the respiratory zone with very thin-walled air sacs or alveoli begins. The extensive branching that is achieved at this point results in an enormous enhancement of the surface area for gas exchange and the alveoli walls present minimal resistance to gas diffusion.

Lung pathology can be the result of gene defects but more often, because of the exposure to the environment, the lungs are relatively open to a range of exogenous factors that can induce pathology and impair lung function, in spite of various structural and immunological defenses. Impairment of lung function can have very serious consequences and may even cause death. Since the respiratory system plays such a vital role in everyday life, lung pathophysiology is extensively studied worldwide to obtain a better understanding of the underlying mechanisms and to eventually save lives.

1.2. Proteomics to study phenotype

If one thing can be concluded after the completion of the draft of the human genome early 2001 [1,2], it is that the number of genes in a genome is not even remotely indicative for the biological complexity of the organism. This is illustrated by humans having only twice as many genes as a fruit fly or a roundworm, and a gene count comparable to that of a mouse. The big question this raises is: ‘How do humans manage to operate at a higher level of complexity?’ The rational answer to this question is that proteins, and not genes, are responsible for the overall complexity of an organism. The interaction of proteins in an intricate network adds up to how an organism functions. This is further complicated by lipids, sugars, the dynamics of molecular shape-changes, and the intracellular compartmentalization of specific reagents and their respective concentrations. Indeed, proteins are referred to as the ‘molecular workhorses’ that determine a phenotype and it is now generally accepted that, to understand biological processes, extensive protein studies are required. This makes the proteome, the protein complement encoded by a genome, an important object for scientific research, e.g. proteomics. In various studies, lung pathology has been studied by gene expression analysis [3–5]. However, post-transcriptional mechanisms that control the rate of synthesis and half-life of proteins are responsible for a poor correlation between mRNA expression levels and protein abundance [6–8]. A typical example of this was shown in a study of parallel transcriptomic and proteomic analysis of lung tumors [9], in which a correlation between mRNA and protein expression levels was found in only 21% of the analyzed samples. In addition to this, the importance of co- and post-translational modifications for the regulation of protein function stresses the demand for techniques that directly provide both quantitative and qualitative information on proteins derived from complex mixtures. Initially, proteomics comprised only large-scale protein identification and amino acid analysis [10], but gradually it has moved beyond being a science of protein cataloguing and now includes the comprehensive and detailed analysis of proteins. Proteomics is a technology-driven science in which advances are achieved on a daily basis. Details of the techniques involved have been excellently reviewed previously [11–15]. Despite the technical advances, the vast majority of lung proteomics research is currently still largely focused on lung cancer while other lung pathologies, such as asthma, remain far less popular subjects for investigation. This may seem surprising because, looking at the incidence of lung cancer versus asthma in the world population, one would expect a more extensive proteomics effort to study the latter pathology. In this paper, innovative developments in lung cancer and asthma research using proteomics approaches are reviewed and a possible rationale for the straggling of asthma proteomics is discussed.

2. Lung cancer proteomics

Lung cancer is one of the most aggressive and lethal pathologies of the lungs and can be caused both by environmental factors such as smoking and inhalation of asbestos

or radon gas [16–18], as well as genetic factors [4,19,20]. During the past 50 years, the incidence of women in the US diagnosed with lung cancer has increased by approximately 600%, compared to a slight decrease among males. This results in an annual death of approximately 68,000 American women, a number equal to all deaths for breast and gynaecologic cancers combined. The increase in the number of female smokers is an obvious cause for this dramatic increase, but a variety of data suggest that genetic, metabolic and hormonal factors may also be responsible for the increase in lung cancer in women [21]. Most lung cancers start in the lining of the bronchi, but occasionally they may start in other areas such as the trachea, the bronchioles, or the alveoli. Two main types of lung cancer can be distinguished: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) [22]. Approximately 25% of all lung cancers are SCLC. Although the cancer cells initially are small, they can multiply quickly and form large tumors that can spread to the lymph nodes, the brain, the liver, or the bones. SCLC is only rarely found in non-smoking individuals and therefore it is generally believed that smoking is the major cause of this pathology. The fact that this cancer frequently spreads and forms metastases has triggered proteomics initiatives to search for useful tumor markers in patient serum [23]. The other 75% of lung cancer cases are NSCLC. Non-small cell lung cancers are categorized into three types: squamous cell carcinoma, adenocarcinoma, and large cell carcinoma [24]. Squamous cell carcinoma makes up 25–40% of all lung cancers and in many cases smoking has been has been pinpointed as the major cause [25]. All types of NSCLC, as well as various environmental factors that can induce lung cancer, have been subjected to proteomic research and lung proteomic databases have been constructed [26–39].

2.1. Two-dimensional electrophoresis

The approach employed in most studies is generally fairly straightforward. The effect of a single variable on expression of proteins is assessed and aims for finding disease-correlated proteins. A typical approach, as depicted in Fig. 1, involves protein extraction from control and disease samples, followed by separation of the protein mixture by two-dimensional electrophoresis (2DE). After visualization

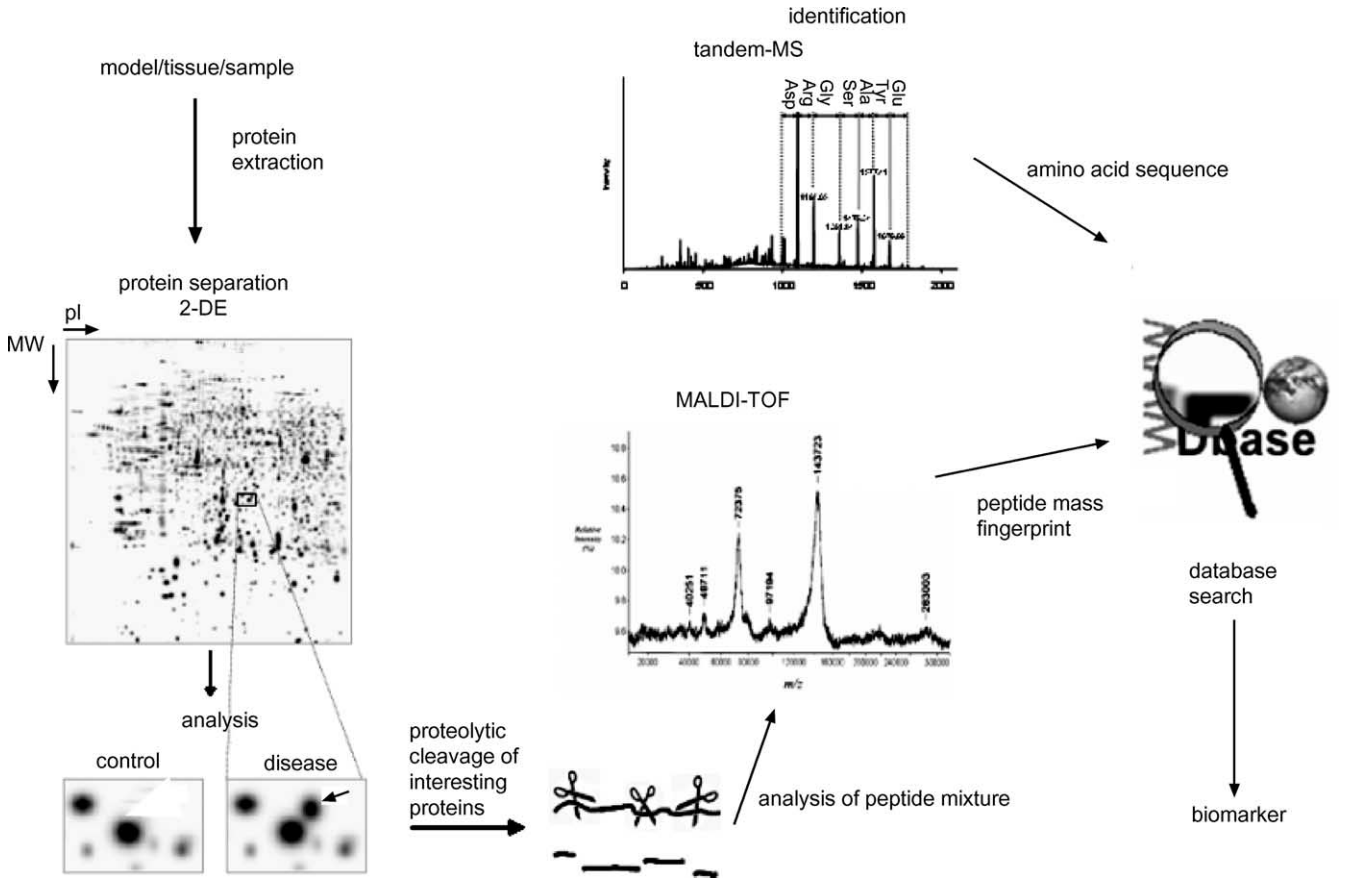

Fig. 1. General strategy for proteome analysis by 2DE. Protein samples are separated by 2DE. After visualization of the separated proteins, expression patterns are analyzed. Proteins of interest are excised from the gel and subjected to proteolytic cleavage. The resulting peptides diffuse out of the gel and are analyzed using MS. Peptide mass fingerprints and/or and peptide sequence data, obtained by MALDI-TOF or tandem-MS, respectively, are submitted to database searching and eventually protein identification.

of the separated proteins, protein patterns are analyzed and compared. For identification of proteins with altered expression, protein spots are excised from the 2DE gels and subjected to in-gel digestion by an endopeptidase with known specificity. Proteolytic fragments diffuse out of the gel and are analyzed by mass spectrometry (MS), either through peptide mass fingerprinting or protein sequencing combined with database searching [40]. The samples subjected to analysis via this approach range from human or animal cell lines to patient material. Besides protein expression analysis of lung tumor cell lines or tissue, alteration of expression due to treatment with anti-cancer drugs such as boheme [41] and paclitaxel [27] has been investigated to study the underlying molecular mechanisms of cancer. Although 2DE is currently still unsurpassed in its resolving power for separation of complex protein mixtures, it is not without difficulties and shortcomings. One of the major difficulties is the enormous dynamic range in protein expression which is illustrated by the fact that 90% of the proteome of a typical cell is made up off only 10% of the 10,000–20,000 different protein species. An additional factor that adds to this complexity is the large variety of biochemical characteristics of proteins (hydrophobicity, charge, p*I*, etc.), which makes solubilization of all proteins during sample preparation virtually impossible. To reduce complexity, samples can be pre-fractionated for specific subsets of proteins by affinity enrichment and application of specific extraction buffers and methods. An example hereof is biotinylation of membrane proteins on living A549 cells, a lung adenocarcinoma cell line [42]. After enrichment for membrane proteins using monomer avidin columns, simplified samples were subjected to 2DE for protein profiling.

Besides its limitations due to the high dynamic range of proteins, 2DE also suffers from high variation that is introduced during sample preparation, protein loading and by the complex nature of the staining procedures employed. In order to obtain statistically significant quantitative data from which valid conclusions regarding the in vivo situation can be drawn, it is necessary to produce a number of replicate gels from the same sample [43–45]. This may become a problem when the amount of sample, for example patient material that often can only be obtained by unpleasant procedures, is limited. Although the analysis of human patient material will directly deliver cancer-related biomarkers, the limitation of the amount of sample may enforce the choice for alternative models, such as cell lines and animal systems. On the other hand, studying cancer in (immortalized) cell lines may be tricky since these cell lines may not provide a proper representation of the real in vivo situation.

2.2. MS-based proteomics

Although most proteomics studies aim at the identification of cancer markers, alternative approaches use MS to find cancer-related spectra. Matrix-assisted laser desorption/ionisation time of flight MS (MALDI-TOF) has been used directly from 1-mm regions of single frozen surgically resected tissue sections for profiling of protein expression in tumors. Obtained MS spectra were aligned, and the class-prediction model that was built from these spectra proved to be very accurate in the classification of lung tumors of an additional set of lung cancer patient material [46,47]. Comparable to this, arsene-induced changes of protein expression profiles in rat lung epithelial cells were studied by surface-enhanced laser desorption/ionization time of flight MS (SELDI-TOF) [48]. This technology utilizes protein chip arrays to capture individual targets from a complex protein sample, which are subsequently resolved by MS [49]. These technologies can be applied in finding disease-related alterations in protein expression profiles, and are useful for diagnosis and disease monitoring. However, no identification of the proteins with altered expression is performed and thus these techniques do not deliver any clues on the molecular mechanisms underlying these diseases.

2.3. Proteome focus through the immune system

Detection of low abundance proteins remains a problem because their expression level is below the detection limit of the technique and methods for protein amplification analogous to PCR for DNA are not yet available. One strategy to circumvent this is to use the immune system for biological amplification [50]. Tumor proteins, (auto-)antigens, with upregulated expression can trigger the host immune system and elicit antibody-based immune responses. Protein extracts of lung tumors have been separated by 2DE followed by Western blotting. Incubation of these blots with sera of lung cancer patients revealed the presence of circulating antibodies against various tumor antigens, which proved to be potentially useful as diagnostic markers for screening of patient sera [51,52]. Additionally, NSCLC cDNA phage libraries, which express tumor proteins on their surface, were incubated with sera of NSCLC patients. Phages binding to reactive antibodies from patient sera were isolated and enriched by biopanning. This eventually resulted in the identification of 57 potential lung tumor biomarkers [53]. Besides triggering an (auto-)antibody response by upregulated cancer-related proteins, the latter are also degraded in all nucleated cells and the resulting peptides are transported by the major histocompatibility complex type I (MHC I) to the surface of the cell. There they are presented by MHC I to T cells that play a regulatory role in the immune system. Tumor proteins are often physiological proteins with altered, non-physiological, expression levels. T cells recognizing these ‘self’ proteins are down-regulated by peripheral tolerance mechanisms to prevent autoimmunity [54]. However, vaccination in the presence of a strong adjuvant helps to overcome tolerance by stimulating the ‘anti-self’ T cells through presentation of epitopes on activated dendritic cells [55]. These cancer peptide epitopes therefore have a potential as antigens for anti-cancer vaccination. Several studies have been employed to isolate these tumor-specific or tumor-associated antigens from MHC I on tumor cells and charac-

terize them by liquid-chromatography coupled to MS [56]. Because peptides of all expressed proteins in a cell are presented by MHC I, the analysis of all MHC I-bound peptides in a cell will generate an enormous amount of data. Filtering data through clustering of spectra will help to select the abundant cancer peptides from the 'noise' of proteins of unaltered expression [57].

3. Asthma

Of all lung pathologies, cancer is the one most abundantly studied by proteomics. This can be understood from the viewpoint of the lethality of lung cancer. However, when looking at incidence, cancer is by far outnumbered by asthma, which is one of the most common diseases of the airways in the Western industrialized world. According to the US Centers for Disease Control and Prevention, doubling of the number of asthma cases during the past two decades has resulted in a 5% incidence among the entire US population to date. During this same period, the incidence of asthma has even increased with approximately 250% worldwide.

3.1. Pathophysiology

The pathophysiology of asthma is complex and involves three components: chronic airway inflammation, intermittent airflow obstruction, and bronchial hyperresponsiveness to a variety of stimuli such as tobacco smoke, cold air and exercise [58,59]. The chronic inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment, usually steroids [60].

Although the clinical phenomena of asthma have been widely documented, the underlying mechanisms remain unclear. The tremendous increase in incidence of asthma over the last 50 years strongly suggests that besides a genetic basis, which has been extensively investigated [61–63], environmental factors do play an important role in the development of asthma. There also appears to be a strong link between asthma and allergy since more than 70% of patients with asthma have positive skin test reactions to common household allergens [64–66]. Epidemiological studies indicate that allergic diseases have their origin in childhood and are associated with lifestyle [61,67]. For example, increased hygiene in the Western world has been indicated as an important factor [68] since the incidence of allergies and asthma is the highest in well-developed Western countries. At present, a strong link between allergy and asthma is generally accepted, but recent data suggests that non-allergic mechanisms, also involving the immune system, may play a role [69,70].

Triggering of the immune system can lead to excessive release of various cytokines and inflammatory mediators in the lungs produced by T cells, infiltrated mononuclear cells and eosinophils, and local mast cells [71]. These mediators affect constituent airway cells, such as fibroblasts [72], endothelial cells [73], and epithelial cells [74,75] and cause airway edema and mucus secretion that contributes to airflow obstruction. Additional factors, such as adhesion molecules (e.g. selectins, integrins), are critical in modulation of the inflammatory changes in the airways [76]. Finally, cell-derived mediators can influence smooth muscle tone and lead to structural changes and remodeling of the airways.

The complex interaction of environmental factors and the host-dependent genetic background that leads to asthma as well as the phenotype asthma itself are very interesting subjects for proteomics. However, while numerous proteomics studies addressing lung cancer have been published, the amount of proteomics data on asthma is surprisingly scarce, probably because of a number of impracticalities and pitfalls of which some are discussed here.

3.2. Considerations for asthma proteomics

The clinical phenomena in asthmatics are apparent and application of proteomics to find diagnostic markers therefore seems unnecessary. However, analysis of the lung phenotype of the late stage of asthma at the proteome level has great potential for the development of new therapeutic strategies. An interesting topic in this respect is the posttranslational modification of proteins by nitric oxide (NO). This simple free-radical gas elicits a diverse range of physiologic and pathophysiological effects, and plays an important role in pulmonary diseases. NO-mediated nitrosylation may be responsible for steroid resistance in asthma and NO modulators may therefore have clinical benefit in asthma [77]. Alternatively, protein cystein nitrosylation was demonstrated to have a physiological role in a ventilatory response to hypoxia [78–80]. Although the latter function is not directly linked to asthma, it was recently studied through an interesting approach that is worth mentioning [81]. In this elegant study, biotin labeling of nitrosylated cysteines was achieved by a series of chemical reactions. This provided a tag for protein isolation by streptavidin and is an excellent example of a strategy for sample enrichment and simplification before proteomic analysis.

One of the problems associated with asthma research is that patient material is usually limited, in contrast to for example lung cancer tissue that is often available after surgery. One approach to evaluate cellular and protein components in the lower respiratory tract of the lungs is to perform a bronchoalveolar lavage. The latter is a diagnostic and therapeutic procedure conducted by placing a suction catheter into the lung of a patient and injecting sterile saline into the lung. Thereby, this relatively mild bronchoscopic procedure is a method by which cells, cellular secretions and proteins, inhaled particles, and pathogenic organisms can be acquired from the terminal bronchi. Analysis of the obtained bronchoalveolar lavage fluid (BALF) may reveal information on the inflammatory status of the cells lining the lung lumen and the influx of inflammatory cells [82,83]. A second tech-

nique to obtain samples from asthma patients is bronchial brushing, in which a protected brush catheter in the bronchoscope is used to gently brush material from the airways. This method has proven to be a safe and effective technique to collect viable bronchial epithelial cells for morphological and functional studies or to establish primary cultures [84]. The first two-dimensional map displaying the major soluble proteins present in BALF was published in 1979 [85] and current work still aims at the construction and improvement of an exhaustive 2DE reference database of bronchoalveolar lavage fluid proteins. Proteomic maps of BALF in various disease states using 2DE/MS have been constructed [86–88] including cystic fibrosis [89], pulmonary fibrosis [90], hypersensitivity pneumonitis [91], immunosuppression [92], but not asthma. One disadvantage of the use of BALF is that soluble proteins are very diverse and can originate from a broad range of sources, both endogenous as well as exogenous. Therefore, detected differences in the amount of lung specific proteins in the BALF may result from different kinds of phenomena. BALF proteomics also harbors two practical problems, namely low protein concentration and high salt concentration, which requires special sample handling procedures analysis. Moreover, when studying asthma-correlated changes of protein expression profiles in BALF or samples obtained by brushing, it can be expected that one should be aware of the fact that the observed effects are correlated with the downstream effects of the inflammation.

Although the application of proteomics to analyze downstream effects of the chronic inflammation may prove invaluable for the development of new therapeutic strategies for symptomatic relief, in our opinion the most intriguing part of asthma is formed by the underlying mechanisms that trigger the upstream onset of the chronic inflammation.

As indicated, the molecular basis for the development of asthma is probably located in the immune system. This biological system works with very potent bioactive agents, such as cytokines and chemokines, which are present in amounts that belong to the lower end of the dynamic range of expression of all proteins. To study these low abundance proteins, protocols for enrichment and sample concentration, and a reasonable amount of starting material are required. Another approach to solve the problem of limited material is by using one of the animal models for asthma that have been developed [93]. These models can be used to study the onset of the inflammation as well as the inflammation-induced alteration of lung tissue. However, mouse models have mainly predictive values and several discrepancies with human asthma have been reported [94].

One issue that is still under debate is the location of the onset of the inflammatory response leading to asthma. This could take place in one of the immunological organs, such as spleen or lymph nodes, or locally within the lung tissue. Therefore, the choice of the proper target tissue to study is extremely relevant for the focus of the research.

In our laboratory, a mouse model was employed to gain insight into the underlying molecular mechanisms of non-allergic asthma, using 2DE [45]. Analysis of total lung tissue revealed significant up- or down-regulation of 23 proteins following antigen challenge, which was consistent with the expectations and bearing in mind the limitations of 2DE. Twenty proteins were identified by MS, of which 18 could be linked to asthma-related symptoms, such as stress and inflammation, lung detoxification, plasma exudation and/or tissue remodeling. We concluded that the proteins found in our model probably do not play a role in the immunological mechanisms leading to asthma and were found because they were abundantly expressed. There is a physical limitation to the amount of proteins that can be visualized on a single 2DE page gel. A silver staining method was used, which was stopped whenever an acceptable amount of separated spots were observed. The first proteins to appear using the staining method, represent the proteins expressed at high abundance, and thereby mask the low abundance proteins. In fact, the intrinsic shortcoming of 2DE to detect low-abundance proteins by 2DE has been excellently shown by Gygi et al. [95]. To enhance the resolution we applied the same lung sample parallel to three adjacent, partially overlapping pH gradients to construct 32DE gels from which a composite zoom gel could be built in silico (Fig. 2, unpublished data). In this way, we were able to increase the amount of separated and visualized proteins from ∼2000 in our initial experiment to ∼8000 in the constructed zoom gel. Thus, application of zoom gels combined with strategies for sample simplification provides a potential way to generate more detailed protein maps in future investigations.

In summary, one may conclude that the pathophysiology of asthma is more complex than that of lung cancer. While the latter is often the result of uncontrolled growth of a single cell type, asthma is the result of a disturbed interplay of many different, mostly immune, cells leading to a pathology that affects lung tissue and function. Altered protein expression levels in cancer cells are often more apparent than the initial subtle changes in the immune system during the development of asthma. Therefore, development of a strategy to study asthma using proteomics requires careful considerations regarding the choice of ‘study object’ or model, sampling and method of analysis. A promising additional approach for sample analysis with enormous potential for future proteomics research, namely microarray technology, is discussed below.

4. Array-based proteomics

Microarrays provide the potential for determining thousands of different binding events in a single experiment in a massively parallel fashion. The microarray format was originally designed for studying DNA dot blots and later mRNA expression levels and is currently being extrapolated for applications to study protein expression and function [96,97]. Miniaturization of systems allows for less analyte consumption. But perhaps more importantly, reduced spot size associated with the area devoted to capture molecules on the array

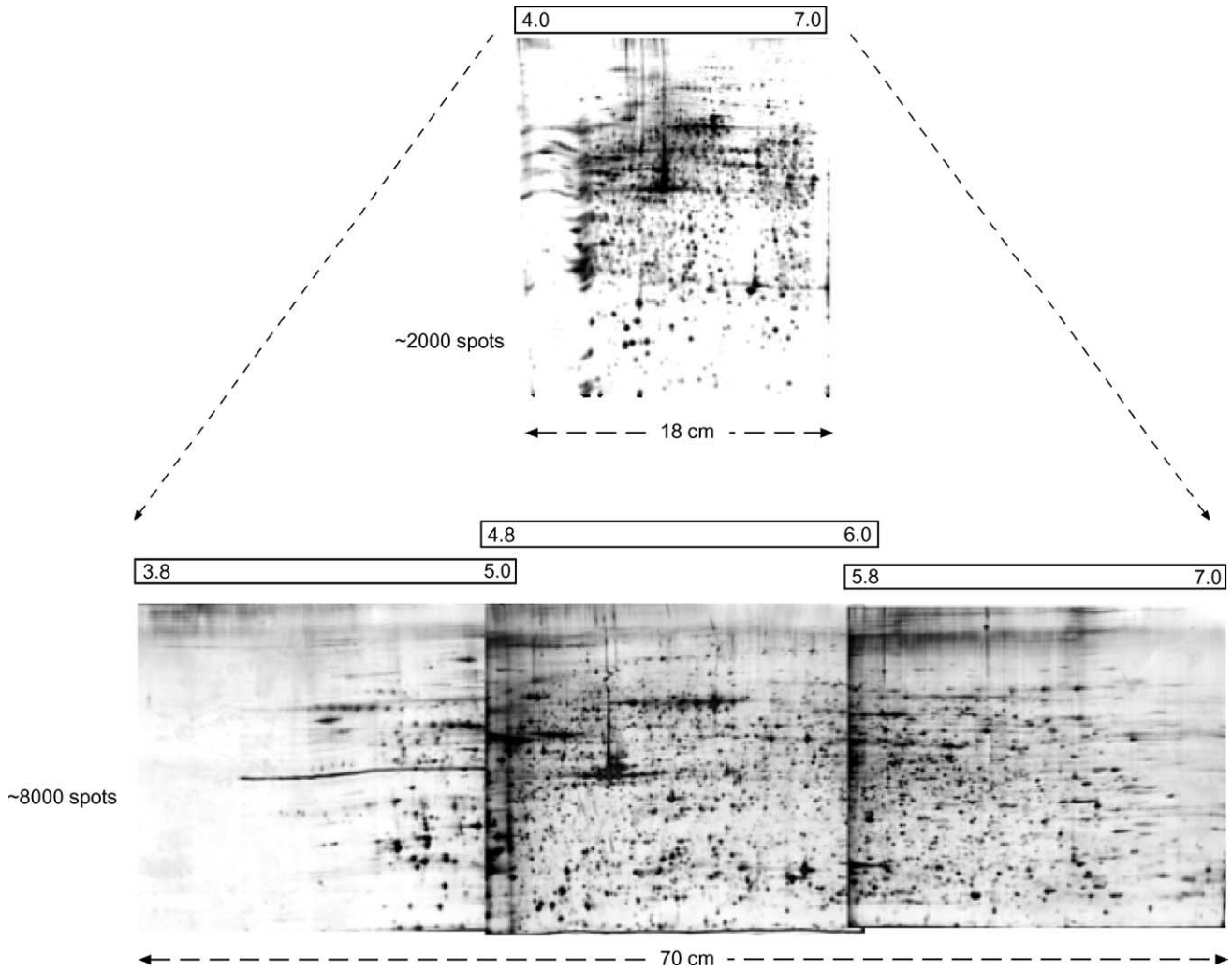

Fig. 2. The concept of zoom gels. To enhance resolution, identical samples are separated in parallel on different 2DE gels with adjacent, partially overlapping, narrow pH ranges. After protein visualization, gels are 'stitched' together in silico. Separation of total mouse lung tissue on a zoom gel, constructed from three partially overlapping narrow pH-range gels, increased the number of separated proteins four-fold compared to separation of the same sample on a single wide pH-range 2D gel.

will concomitantly allow for microspots with high overall density of binding sites. This results in enhanced sensitivity compared to the use of larger spot areas, i.e. microspots. This phenomenon was previously explained by Ekins and co-workers and is referred to as the 'ambient analyte theory' [98–100].

Whereas microarrays are widely applied in gene-expression analysis, it is not easily extrapolated to protein expression analysis. A number of practical problems are associated with the setup of high throughput parallel binding assays involving proteins. Proteins are far more diverse with respect to their biochemical properties than their nucleic acid counterparts, whereby each amino acid combines to rapidly produce an incredibly large potential variety as to properties of the final polypeptide.

For the detection of a certain DNA or RNA molecule in a sample, the antisense sequence to this target can serve as capture molecule when immobilized on the array. However, generation of capture molecules for proteins requires much more effort. Various strategies for the generation of capture molecule libraries using phages, aptamers, and antibodies with a variety of high affinity binding to various protein targets are being investigated [100–105].

To date, protein biochips have been employed to examine a wide variety of assay types. These include detection of interactions between antibody–antigen, protein–protein, protein–nucleic acid, protein–small molecule, membrane-bound receptors and target, domain screening, and analysis of enzymatic function. In addition, a parallelized format has been adopted for a variety of other applications in proteomics. These applications include tissue arrays, epitope mapping via peptide arrays, reverse arrays for the examination of naturally-occurring protein isoforms found in cellular extracts as eluted from liquid chromatography,

cellular arrays for bioassays and immobilization of various chromatography affinity capture reagents. The latter are now being used extensively to track biomarkers associated with the progression, prognosis and precocious detection of disease and are also being applied to patient cohorting during drug trials, i.e. pharmacoproteomics. Protein arrays equally hold great potential to improved lead development and lead optimization as a means of verifying target specificity in the presence of numerous potential recombinant binders and thereby working towards reducing adverse drug effects as a direct spin-out of increased knowledge of the human genome.

5. Future perspectives and conclusions

Several proteomics applications to study lung pathologies have been discussed in this review. Additionally, various difficulties associated with proteomics-based unraveling of molecular basis of asthma have been mentioned. At present, asthma may be looked upon as the ugly duckling of lung proteomics research, since the vast majority of proteomics studies are focusing on other lung pathologies such as lung cancer. However, considering the incidence of asthma and the resulting consequences for healthcare in the decades to come, we would like to stress that the importance of intensification of asthma proteomics is highly desirable. Tackling the numerous facets of this disease requires implementation of multiple strategies and technology platforms, with a possible role for the Human Proteome Organization (HUPO). Joining of forces and close collaboration between different scientific disciplines will eventually lead to the identification of an increased number of biomarkers associated with the onset and development of asthma. Multifactor analysis of these markers through array-based proteomics and bioinformatics will contribute to the unraveling of the underlying mechanisms of this pathology and will promote the development of novel therapeutic strategies against one of the fastest growing diseases of the Western world.

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