

REVIEW

Biomarker discovery in asthma and COPD by proteomic approaches

Rossana Rossi¹, Antonella De Palma¹, Louise Benazzi¹, Anna Maria Riccio², Giorgio Walter Canonica² and Pierluigi Mauri¹

¹ Institute for Biomedical Technologies (ITB-CNR), Proteomics and Metabolomics Unit, Segrate, MI, Italy

² Allergy and Respiratory Diseases, Department of Internal Medicine, University of Genoa, IRCCS-IST AOU San Martino, Genoa, Italy

Asthma and chronic obstructive pulmonary disease (COPD) are multifactorial respiratory diseases, characterized by reversible and irreversible airway obstruction, respectively. Even if the primary causes of these diseases remain unknown, inflammation is a central feature that leads to progressive and permanent pulmonary tissue damage (airway remodeling) up to the total loss of lung function. Therefore, the elucidation of the inflammation mechanisms and the characterization of the biological pathways, involved in asthma and COPD pathogenesis, are relevant in finding new possible diagnostic/prognostic biomarkers and for the validation of new drug targets. In this context, current advances in proteomic approaches, especially those based on MS, provide new tools to facilitate the discovery-driven studies of new biomarkers in respiratory diseases and improve the clinical reliability of the next generation of biomarkers for these diseases consisting of multiple phenotypes. This review will report an overview of the current proteomic methods applied to the discovery of candidate biomarkers for asthma and COPD, giving a special emphasis to emerging MS-based techniques.

Received: October 11, 2013

Revised: July 1, 2014

Accepted: September 1, 2014

Keywords:

Asthma / Biomarkers / COPD / Inflammation / Proteomic approaches

1 Introduction

Asthma and chronic obstructive pulmonary disease (COPD) are the most common chronic respiratory diseases and also represent two of the principal leading causes of morbidity and mortality worldwide [1, 2]. Both these pathologies show airflow limitation [3–6], affecting primarily the airways, and

cause a structural remodeling of the pulmonary tissue that in the long run compromises normal lung function [7]. Even if the primary causes of these diseases remain unknown, inflammatory processes within the airways play a central role and are probably triggered by complex interactions between environmental (indoor and outdoor air pollution, occupational hazards, infections) and genetic factors [8, 9].

In particular, chronic inflammation of the airways is present in all clinical forms of asthma (allergic, nonallergic, occupational, and aspirin-induced) [3, 4, 10, 11] and it is characterized by a widespread but variable airflow obstruction that is often reversible, either spontaneously or with pharmacological treatment. On the contrary, the progressive and not fully reversible airflow limitation caused by COPD is associated with an abnormal inflammatory response that leads to emphysematous parenchymal destruction (emphysema), variable loss of lung elastic recoil, and abnormalities in gas diffusion [12] up to the total loss of lung function and/or the risk of developing lung cancer [13]. As described above, asthma and COPD present different physiological and anatomical features and distinct inflammatory profiles

Correspondence: Dr. Pierluigi Mauri, Proteomics and Metabolomics Unit, Institute for Biomedical Technologies—CNR, Via Fratelli Cervi, 93, 20090 Segrate, MI, Italy

E-mail: pierluigi.mauri@itb.cnr.it

Fax: +39-02-26422770

Abbreviations: 2DC-MS/MS, 2D chromatography coupled to MS/MS; 2DG, bidimensional gel electrophoresis; BALF, bronchoalveolar lavage fluid; CF, cystic fibrosis; COPD, chronic obstructive pulmonary disease; CRP, C-reactive protein; FEV1, forced expiratory volume in 1 s; IPA, ingenuity pathway analysis; IS, induced sputum; MMP, matrix metalloprotease; MW, molecular weight; NO, nitric oxide; PBMC, peripheral blood mononuclear cell; PIGR, polymeric immunoglobulin receptor; PRDX, peroxiredoxin; PYGM, glycogen phosphorylase; Q10, anion exchange at pH10; SAX2, strong anion exchange; WB, Western blotting; WCX2, weak cation exchange

Colour Online: See the article online to view Fig. 1 in colour.

[14–16]. However, especially in acute asthmatic forms and COPD exacerbations, it is possible to observe patients with asthma showing COPD-like phenotypes and vice versa. In clinical settings this condition is known as “overlap syndrome,” affecting a significant number of patients that share matching inflammatory pathways and combined to the multifactorial nature of asthma and COPD make the management of these disorders especially challenging.

In addition, traditional methods of diagnosing these respiratory diseases, essentially based on physical examinations, medical history, and airflow measurements [17–20], are completely inadequate in achieving an early diagnosis, assessment of disease staging and phenotype, and in the monitoring of therapeutic responses. However, in the clinical practice of respiratory diseases they are currently used for the lack of noninvasive procedures and tools that could reach the direct assessment of airway inflammation. Recently, measurements of inflammatory biomarkers are being increasingly explored, even if they are not yet considered as a diagnostic parameter by clinicians [21–28]. Finally, the limited sensitivity of current instrumentation and the low abundance of biomarkers in biological materials has provided another hurdle to the discovery and validation of new candidate biomarkers for asthma and COPD.

In the last decade, advances in proteomic approaches stimulated considerable interest among scientists and clinicians involved in respiratory disease studies. In particular, MS-based proteomic techniques show a relevant improvement over other methods, due to the selectivity and specificity with which it succeeds in following protein markers involved in inflammatory pathways and in asthma and COPD phenotyping. In this way, it could be possible to overcome the repeated failures of old techniques and unravel the complex molecular pathways responsible for these diseases, improve early diagnosis, and provide new strategies for their monitoring and drug discovery.

Herein, we provide an overview of the main proteomic approaches for the identification of potential diagnostic/prognostic protein biomarkers in asthma and COPD, with a specific focus on emerging MS-based techniques.

2 Test for diagnosis of asthma and COPD

Currently, the diagnosis of asthma and COPD is based on medical history, physical examination, and spirometry. A detailed medical history for a patient suspected of having asthma or COPD should address symptoms and the pattern of their occurrence, precipitating or aggravating factors, the severity and frequency of exacerbation episodes, family and social history, and assessment of illness by the patient [17, 29]. Asthma symptoms should be recurrent and may worsen in the presence of exercise, viral infection, or exposure to allergens, such as animal fur or hair, dust mites, mold, smoke, pollen, or airborne chemicals. Instead in COPD, the symptoms often do not appear until significant lung damage has occurred. Physi-

cal examination in both asthma and COPD should be focused on abnormalities of the nose, throat, upper airway, chest, and skin [8, 17].

As stated above, spirometry is the most widely noninvasive test used in clinical practice for the evaluation of lung function [30] and is considered the most practical and reliable tool for the diagnosis of asthma and COPD, as is applied routinely to establish the presence and severity of these two obstructive airway diseases [31, 32]. In particular, forced expiratory volume in 1 s (FEV1) and FEV1/forced vital capacity (FEV1/FVC) measurements are commonly used for the differentiation of asthma from COPD [17].

Another spirometric measurement that should be noted is bronchial provocation testing, such as the methacholine challenge [33]. Nevertheless, the complexity and the heterogeneity of asthma and COPD, combined with the inaccessibility and invasiveness of sampling, contribute in maintaining these spirometric parameters, as the only accepted tests, as reported by recent clinical guidelines, for the diagnosis of obstructive pulmonary diseases. However, in recent years, the inherited limitation of spirometry and medical history has prompted clinicians and scientists to search alternative strategies based on biomarkers of airway diseases [34].

Being airway inflammation a central feature of asthma and COPD, the search for new candidate biomarkers able to characterize the inflammatory phenotypes of these respiratory diseases is ongoing. Currently, the most validated tests to assess inflammation, in a relatively noninvasive manner and with the available clinical instrumentations, are represented by the measurement of carbon monoxide (CO) and nitric oxide (NO) in exhaled breath and by quantitative cell counts in sputum.

In fact, exhaled CO values have been evaluated as potential indicators of inflammation in asthma and stable COPD [15, 16, 28], but the predictive value of this measurement as a diagnostic tool remains unclear, since there are several confounding factors limiting its clinical usefulness.

The measurement of exhaled NO, as indirect indicator of airway inflammation [35], provides information about the severity of the disease, the prognosis and the response to treatment with anti-inflammatory drugs such as inhaled corticosteroids. In asthma, exhaled NO measurements can predict steroid responsiveness [27, 36–38]. For COPD instead, the levels of exhaled NO may have a role in the identification of patients with severe and unstable forms, which are characterized to have higher NO levels compared to the stable forms. However, the use of an oxidative stress-related biomarker, such as NO, has not yet been inserted in clinical routine because in some cases the results are ambiguous, such as in the case of smoking patients.

The presence of eosinophils in the airway is often an indicator of response to treatment with anti-inflammatory therapies (corticosteroids) in patients with asthma [39] or COPD [40]. The measurement of airway eosinophil abundance is extracted from the quantitative cell counts of sputum recovered from patients [41]. Pioneering therapeutic strategies, based

on the count of sputum eosinophils, for the treatment of asthma [42, 43] and COPD [44] patients, led to significantly better clinical outcomes than current guideline-based therapies. However, despite this enhanced efficacy, these assessments are not widely used in clinical practice because of the generally perceived view that they cannot be implemented in routine practice of most clinical settings [45]. Certainly, each of the emerging diagnostic strategies, described above, could help to improve in its own way the management of these multifactorial obstructive airway diseases. But the heterogeneous phenotypes and the known interplay of environmental and genetic variations of molecular and biochemical pathways within asthma and COPD, suggest the use of additional system-wide tools, such as proteomic approaches, to overcome the limitation of the diagnostic techniques so far applied.

3 Proteomic approaches

In recent years, advances in proteomic approaches opened new opportunities for the discovery of potential diagnostic/prognostic protein biomarkers in asthma and COPD, with the aim to overcome the lack of specificity and sensitivity with the actual diagnostic tests.

In clinical practice, the interest on proteomic approaches is increasing, since they may help to understand the pathogenesis of respiratory diseases and improve patient care.

There are multiple advantages in studying protein expression rather than gene expression [46]. First, it is primarily proteins, not genes that determine how a cell functions. Second, a single gene or even a single mature messenger RNA may be associated with multiple proteins due to splicing, RNA editing, or PTMs, and there may also be a poor correlation between the levels of messenger RNA and protein expression. Third, an acellular compartment, such as plasma and lung epithelial lining fluid, have little DNA or RNA but may have abundant proteins that could be important markers of disease. Moreover, the cell's proteome expression changes in response to environmental and intracellular stimuli. A study of the proteome of an organism, tissue, or cells starts with the measurement of the relative quantities of the various proteins expressed in the sample of interest. This protein "fingerprint" can be used to compare the sample in various conditions (experimental, physiological, and pathological) and determine the presence of possible relevant changes, which become essential in understanding the relationship between function and modification. The development of high-throughput proteomic techniques make possible the detection of more than one protein simultaneously in a great variety of disease relevant samples. Samples generally used in proteomic studies of lung diseases include *in vitro* lung cell cultures, bronchial fluids (i.e. bronchoalveolar lavage fluid (BALF) and induced sputum (IS)), body fluids (i.e., serum, plasma, blood, or urine), and lung tissue biopsies or autopsies. All these features make proteomic approaches more suited in under-

standing the inflammation mechanisms involved in asthma and COPD pathogenesis and in discriminating the multiple phenotypes and endotypes of these two diseases.

The main proteomic approaches are summarized in Fig. 1 and may be classified, according to the separative and/or detection methods used, in

- (i) multiplex immunoassay or antibody based, involving the monitoring of proteins using an array of specific antibodies,
- (ii) peak profiling methods, involving the generation of "finger prints" for sample comparison (i.e., SELDI),
- (iii) gel-based methods, involving the separation of proteins by mono- or bidimensional electrophoresis and the identification of bands/spots by means of MS,
- (iv) gel-free methods, involving mono- or bidimensional LC coupled to MS for both identification and quantification of proteins.

Tables 1 and 2 summarize the main proteomic technologies that are providing new tools to facilitate the discovery-driven studies of new biomarkers for asthma and COPD and will be discussed in more depth.

3.1 Antibody-based methods

There are few studies (see Table 1A and Table 2A), where mAb-based methods are used as the main platform for both discovery and validation of asthma and COPD biomarkers [47–55].

In 2007, Csaky et al. [50] proposed one of the first applications of a mAb-based platform for the discovery of COPD-specific biomarkers. In particular, plasma pools from COPD patients and healthy controls were used to prepare the complex immunogen. Briefly, plasma samples were deprived of the most abundant and physiologically present proteins through a first step of affinity chromatography. Then, the remaining protein mixture was subjected to a second affinity purification step using a column containing immobilized polyclonal antibody prepared against normal human serum with the aim to enrich the disease-specific proteins. In this way, ten high-quality biomarkers were discovered and validated with a high degree of statistical significance in a single step, representing a good starting point for testing clinical validation paradigms.

Recently, the group of Ropcke et al. [51] applied the immunoassay platform to monitor about 100 potential COPD biomarkers in BALF, bronchial biopsies, serum and IS of healthy smokers, and COPD smoker's patients. The study provided useful information on the interrelationship between pulmonary and systemic COPD-related markers and allowed to discriminate smokers with or without COPD by means of serum markers. In fact, on the total number of markers monitored, 51 were identified in serum and resulted upregulated in the COPD group, such as C-reactive

Table 1. Summary of proteomics studies in asthma diseases for (A) antibody-based, (B) peak profiling MS-based, (C) gel-MS based, (D) LC-MS-based methods

Ref.	Biomarkers	Sample source	Method	Verification	Subjects
(A) Antibody-based methods					
[47]	FOXP3	PBMC	Real-time PCR, ELISA	Real-time PCR, ELISA	Asthma/control subjects
[48]*	SP-A, MPO, sRAGE, NGAL	Induced sputum, plasma	EIA/ELISA	EIA/ELISA	Asthma/COPD/COPD-asthma-smoker/nonsmoker
[49]*	CD31, VEGF-A, angiogenin, Bk receptors	Bronchial biopsies	Immunostaining ELISA	Immunostaining ELISA	Nonsmoking old patients with asthma/nonsmoking young patients with asthma/smoking patients with COPD/old control smokers/old control nonsmokers/young control nonsmokers
(B) Peak profiling MS-based methods					
[56]*	HNP1, HNP2, HNP3, three C-terminal amidated peptides	Induced sputum	MSB (mesoporous silica beads) MALDI TOF/TOF		Asthma/COPD
[57]	No	Plasma	SELDI-TOF		Healthy/asthmatic pregnant women
[58]*	Calgranulin A, calgranulin B, calgranulin C, CCSP, proline-rich salivary peptide	Induced sputum	SELDI-TOF	ELISA	Healthy controls/asthma/COPD/CF/bronchiectasis
[59]*	Hemoglobin subunit-beta	Serum, nasal epithelial cell, lung tissue	SELDI-TOF		Asthma/COPD/CF
(C) Gel-MS based methods					
[61]		Cell cultures of mesenchymal type from bronchial biopsies	2D gel MALDI TOF	Western blotting	Asthma/patients with chronic cough
[62]	AAT, complement component C7	Plasma during exacerbation and stable period	2D gel MALDI TOF	ELISA and nephelometry	Asthmatic children
[63]	ApoA1, α 2M, CP	Nasal lavage fluid	2D-DIGE MALDI TOF	ELISA and immunoCAP	AERD
[64]*	α 2M, CP, haptoglobin, hemopexin	Plasma peripheral blood	2D gel MALDI TOF	Immunoassay	Nonsmoking asthma/nonsmoking COPD/healthy
[65]	426 proteins	Peripheral blood eosinophil	2D gel MALDI-TOF/TOF		Nonsmokers donors/who showed neither asthmatic nor allergies symptoms
[66]	Fibrinogen complement component C3	Plasma	2D gel MALDI-TOF-MS	Western blotting	Nonsmoking mildly asthmatic patients
[67]*	Calciphosine, surfactant protein A, transthiretin, ApoA1, S100A8	BALF	2D-PAGE MALDI-TOF/TOF		Healthy and sulfur mustard exposed
[68]			2D gel MS		Nonsmoking asthma
[69]	No	PBMCs	2D-DIGE MALDI-TOF		Healthy volunteers

Table 1. Continued

Ref.	Biomarkers	Sample source	Method	Verification	Subjects
(D) LC-MS based methods					
[78]	Galectin-binding glycoproteins	BALF	LC-MS/MS	Immunohistochemistry	Controls/asthma nonsmokers
[79]	PDE7 and syntaxin	Plasma	LC-MS/MS	Multiplex immunoassay and WB	Asthma/NSCLC/healthy controls
[80]	Secretoglobin S100A8/A9 complement component 3a	Induced sputum	LC-MS/MS high-resolution ORBI	Western blotting	Healthy/asthma
[81]*	42 protein spots, 22 upregulated and 20 downregulated	T-help cell	nano-LC-MS/MS		
[82]*	Panel of proteins	Nasal cells	2DLC MS/MS		Volunteers
[83]	Citokeratin, albumin, actin, hemoglobin, lysozyme, dermicidin, and calgranul B	Exhaled breath condensate (EBC)	2D-nano-LC-MALDI-TOF/TOF + MS/MS		Healthy controls/asthma children
[84]	Lamin, cyclophilin A, annexin 5, α 2M, serpin, vimentin, aldolase A	Bronchial biopsies	iTRAQ labeling SCX off-line nano-LC-MS/MS		Nonsmoking asthma/nonsmoking controls

The references marked with asterisk (*) investigated both asthma and COPD.

MPO, myeloperoxidase; sRAGE, soluble receptor for advanced glycation endproducts; NGAL, neutrophil gelatinase-associated lipocalin; CCSP, Clara cell secretory protein; HNP1, human neutrophil peptide 1; HNP2, human neutrophil peptide 2; HNP3, human neutrophil peptide 3; ApoA1, apolipoprotein A1; α 2M, α 2-macroglobulin; AERD, aspirin-exacerbation respiratory disease; AAT, α -1-antitrypsin; VEGF-A, vascular endothelial growth factor A; Bk, bradykinin; EIA, enzyme immunoassay.

protein (CRP), interleukin-6 (IL-6), von Willebrand factor (vWF), and calprotectin. In particular, calprotectin and von Willebrand factor proteins were found to be interesting markers for further investigations because the first is related to neutrophilic inflammation, while the second is an indicator of early structural and functional changes in pulmonary vessels and accompanies the development of COPD. However, despite these correlations they did not seem to sufficiently reflect the disease-associated inflammatory processes within the airways.

Still focusing on the monitoring of serum markers, El-Deek and co-workers [52] compared the expression levels of high-sensitive CRP (hs-CRP), soluble intercellular adhesion molecule-1 (sICAM-1), and surfactant protein D (SP-D) of healthy individuals and COPD patients, with the aim to assess the correlation between the upregulation of these indices and the degree of COPD severity.

The mAb-based platform was also applied to follow inflammatory mediators for the tentative classification of patients with COPD, which suffer from acute exacerbation (AECOPD) [53, 54] and for the study of COPD–asthma overlap syndrome [48]. In particular, Gao et al. [53] employed a range of serum mediators and sputum inflammatory cells to classify AECOPD patients into four groups compared with healthy controls. These authors observed by ELISA test that the serum and sputum levels of CRP, IL-6, serum amyloid A (SAA), and matrix metalloproteinase-9 (MMP-9), together with the predominant type of sputum inflammatory cells (eosinophilic, neutrophilic, mixed granulocytic, and paucigranulocytic), were excellent biomarkers for judging the severity of AECOPD. The difference among groups was determined by statistical tests such as ANOVA, Kruskal–Wallis, Mann–Whitney, and *U*-test (only *p*-value of <0.05 was considered statistically significant).

On the contrary, Chen et al. [54] developed a method for the evaluation of plasma inflammatory biomarkers in COPD patients with or without acute exacerbation. They integrated the proteomic profiles of inflammatory mediators obtained by antibody microarray assays with data of clinical informatics achieved by a Digital Evaluation Score System (DESS), in order to assess the disease severity. In this way, 20 significantly different mediators were identified among patients with stable COPD, AECOPD, and healthy controls (see Table 1A and Table 2A). The COPD–asthma overlap syndrome is instead the subject of the work proposed by Iwamoto et al. [48], in which, using an enzyme immunoassay/ELISA test, plasma and sputum levels were evaluated for four inflammatory and lung injury related biomarkers in patients affected by COPD, asthma, or COPD–asthma overlap syndrome. Plasma surfactant protein A (SP-A) and sputum myeloperoxidase (MPO) resulted upregulated in patients with COPD or overlap syndrome compared with asthma patients, while soluble receptor for advanced glycation endproducts (sRAGE) resulted downregulated in plasma from patients with overlap and COPD but not from those with asthma. In contrast, only sputum levels of neutrophil gelatinase-associated lipocalin

Table 2. Summary of proteomics studies in COPD diseases for (A) antibody-based, (B) peak profiling MS based, (C) gel-MS based, (D) LC-MS-based methods

Ref.	Biomarkers	Sample source	Method	Verification	Subjects
(A) Antibody-based methods					
[48]*	SP-A, MPO, sRAGE, NGAL	Induced sputum, plasma	EIA/ELISA	EIA/ELISA	Asthma/COPD/COPD-asthma-smoker/nonsmoker
[49]*	CD31, VEGF-A, angiogenin, Bk receptors	Bronchial biopsies	Immunostaining ELISA	Immunostaining ELISA	Nonsmoking old patients with asthma/nonsmoking young patients with asthma/smoking smokers/old control nonsmokers/young control nonsmokers
[50]	10 high-quality biomarkers	Plasma	mAb-based proteomics	mAb-based proteomics	COPD/control
[51]	51 protein marker. CRP, IL-6, vWF, calprotectin	BALF/biopsies/serum/induced sputum	ELISA	ELISA	COPD smokers/control smokers
[52]	hs-CRP, sICAM-1, SP-D	Serum	ELISA	ELISA	COPD/control (nonsmokers-smokers)
[53]	CRP, IL-6, SAA, MMP-9	Induced sputum/serum	ELISA	ELISA	COPD/AECOPD/healthy
[54]	20 protein mediators	Plasma	Microarray assay: an antibody-based membrane array for measuring inflammatory factors	Microarray assay: an antibody-based membrane array for measuring inflammatory factors	Healthy/COPD/AECOPD
[55]	CRP, IL-6, procalcitonin, TNF- α	Blood			COPD
(B) Peak profiling MS-based methods					
[56]*	HNP1, HNP2, HNP3, three C-terminal amidated peptides	Induced sputum	MSB (mesoporous silica beads) MALDI TOF/TOF		Asthma/COPD
[58]*	Calgranulin A, calgranulin B, calgranulin C, CCSP, proline-rich salivary peptide	Induced sputum	SELDI-TOF	ELISA	Healthy controls/asthma/COPD/CF/bronchiectasis
[59]*	Hemoglobin subunit-beta	Serum, nasal epithelial cell, lung tissue	SELDI-TOF		Asthma/COPD/CF
[60]	SAA	Plasma	SELDI-TOF	ELISA	COPD stage II-IV/patients with acute and severe AECOPD
(C) Gel-MS based methods					
[64]*	α 2M, haptoglobin, CP, hemopexin	Plasma peripheral blood	2D gel MALDI TOF	Immunoassay	Nonsmoking asthma/nonsmoking COPD/healthy
[67]*	Calciphosine, surfactant protein A, transthyretin, ApoA1, S100A8	BALF	2D-PAGE MALDI-TOF/TOF		Healthy and sulfur mustard exposed
[70]	Human fibrinogen fragment D, ApoE, ITIH4, CO3, RETB, GPX1, C4B3	Plasma	2D gel imaging MALDI-TOF nano-LC-MS/MS		Healthy smokers/COPD smokers patients
[71]	MMP-13, thioredoxin-like 2	Lung tissue	2D gel MALDI-TOF	Western blotting and immunohistochemistry	Nonsmokers healthy/smokers healthy/COPD smokers
[72]	CAT, PRDX1, PRDX2, PRDX5, CAPS2, CFL1	BAL	2D gel MALDI TOF/TOF	Western blotting	Smokers and nonsmokers: COPD, LC, LC + COPD, no LC, and no COPD
[73]	Hsp 27, cyclophilin A	Lung tissue	2D gel MALDI-TOF	Western blotting and immunohistochemistry	Healthy nonsmokers/non-COPD smokers/COPD smokers

Table 2. Continued

Ref.	Biomarkers	Sample source	Method	Verification	Subjects
[74]	Lipocalin, ApoA1	Induced sputum	2D gel QTOF+	Western blotting and ELISA	Smokers with mild, moderate, and severe COPD/smokers with chronic bronchitis/smokers with low transfer factor of the lung for CO/control subject group consisting of healthy smokers, nonsmokers/ nonsmoking subjects with asthma
[75]	PIGR	Lung, induced sputum, and plasma	2D-DIGE MS	Western blotting and ELISA	COPD mild to moderate smokers/ healthy smokers and nonsmokers
[76]		Peripheral blood, epithelial lining fluid, urine, bronchial brushes and biopsies, exhaled breath condensate (EBC) induced sputum	2D DIGE, iTRAQ, 2LC MALDI-TOF/TOF		Young susceptible and not susceptible for COPD, old control smokers and COPD patients
(D) LC-MS based methods					
[81]*	42 protein spots, 22 upregulated and 20 downregulated	T-help cell	nano-LC-MS/MS		Volunteers
[82]*	Panel of proteins	Nasal cells	2DLC MS/MS		Male and female otherwise healthy
[85]	Proteins complement (complement C3, C6, C7) and coagulation cascade (fibrinogen)	Plasma	LC SCX off-line MS/MS		cigarette smokers, aged 35–60, with mild or moderate COPD
[86]	12 proteins complement and coagulation cascade	Plasma	LC SCX off-line FTICR-MS		Male and female otherwise healthy
[87]	Salivary proline-rich peptides, neutrophil defensins 1 and 2, S100A8/A9	BALF	SELDI-TOF, MALDI-TOF, and nano-LC-MS/MS	ELISA	cigarette smokers, aged 35–60, with mild or moderate COPD during 1986–1989
[88]	Vitamin D and vitamin D binding prot (DBP)		LC MS/MS		Asymptomatic smokers/COPD smokers patients
[89]	Vitamin D binding prot, inter-alpha-trypsin inhibitor heavy chain H3, complement component 8 alpha, beta, gamma, mannose binding prot C	Plasma	iTRAQ labeling 2DLC-MS/MS	WB	Control smoker and nonsmoker/COPD with severity stage 2, 3, 4
[90]	HMGB1 alpha, cofilin1, and lactoferrin	BALF	chipLC MS/MS		Smokers and nonsmokers healthy

The references marked with asterisk (*) investigated both asthma and COPD.

IL-6, interleukin-6; vWF, von Willebrand factor; hs-CRP, high-sensitive CRP; siCAM-1, soluble intercellular adhesion molecule-1; SP-D, surfactant protein D; SAA, serum amyloid A; SP-A, surfactant protein A; MPO, myeloperoxidase; sRAGE, soluble receptor for advanced glycation endproducts; NGAL, neutrophil gelatinase-associated lipocalin; CCSP, Clara cell secretory protein; HNP1, human neutrophil peptide 1; HNP2, human neutrophil peptide 2; HNP3, human neutrophil peptide 3; ApoA1, apolipoprotein A1; ApoE, apolipoprotein E; α 2M, α 2-macroglobulin; CO3, complement component 3; RETB, retinol-binding protein; GPX1, antioxidant glutathione peroxidase 1; C4B3, complement component 4B; ITIH4, inter-alpha-trypsin inhibitor heavy chain H4; VEGF-A, vascular endothelial growth factor A; TNF- α , tumor necrosis factor alpha; Bk, bradykinin; EIA, enzyme immunoassay.

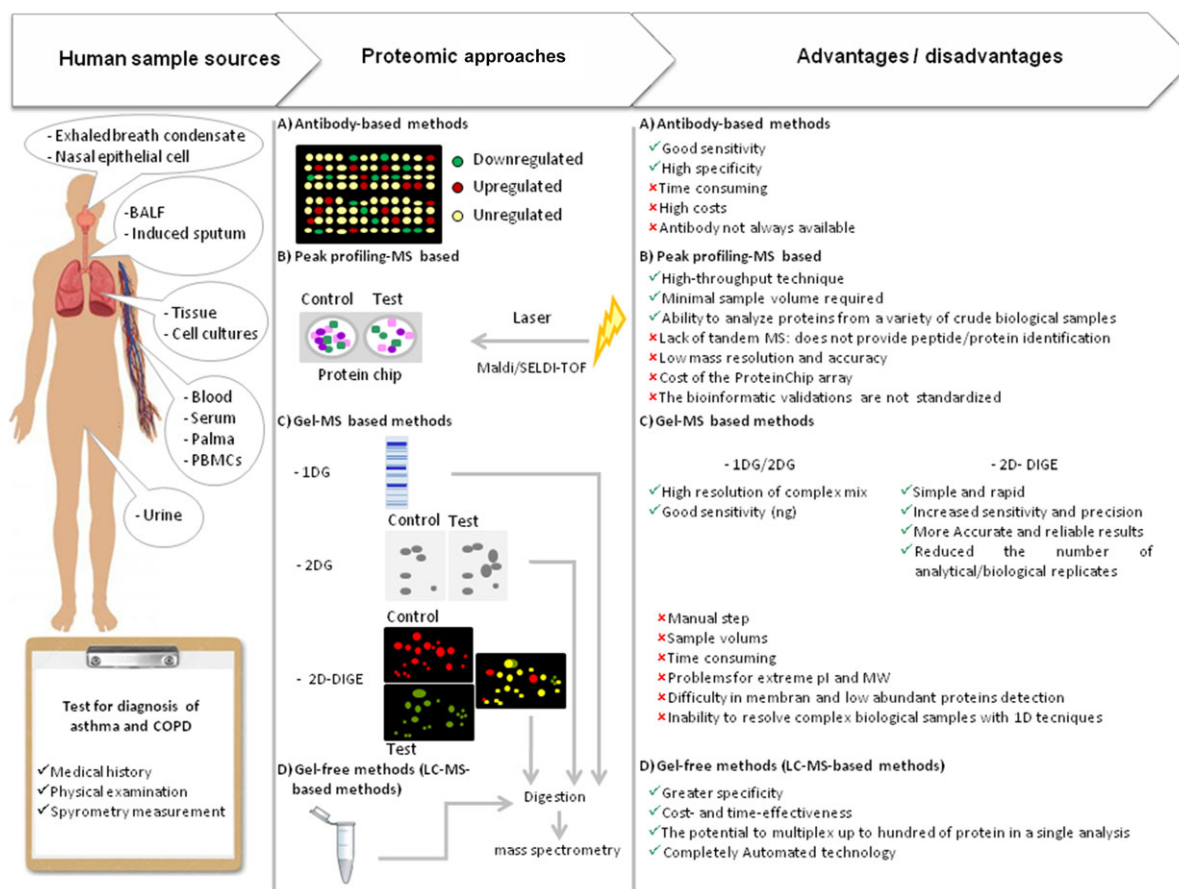


Figure 1. Overview of proteomics strategies.

(NGAL) were found significantly increased only in patients presenting overlap syndrome.

However, it is easy to observe that in proteomics the antibody-based approach is usually used to validate a subset of proteins previously identified and quantified with MS-based techniques. This is mainly due to the fact that the antibodies are very expensive and not always available for all the markers under investigation, limiting their development as an independent proteomic platform.

3.2 Peak profiling

Peak profiling methods, mainly based on SELDI technology, do not directly lead to the identification of proteins but to the generation of a “fingerprint” that should be compared among samples and/or individuals.

These profiling approaches were applied to various sample sources, in order to discover potential target proteins involved in airway inflammation mechanisms and remodeling, which characterize asthma and COPD (see Table 1B and Table 2B) [56–60].

In one of the earliest studies, Murphy et al. [57] applied the SELDI-TOF technique to screen the plasma of asthmatic and nonasthmatic pregnant women for monitoring possible changes in asthma during this condition. The samples collected at 18 and 30 weeks of gestation were applied on the surfaces of chips made up of strong anion exchange (SAX2), weak cation exchange (WCX2), and immobilized metal affinity capture with Cu^{2+} matrices. The experiment resulted in 91 and 51 plasma peaks of different intensities based on the Mann–Whitney test ($p > 0.05$), between asthmatic and nonasthmatic women at 18 and 30 weeks of gestation, respectively. Moreover, only two of these peaks were expressed with a significant variation and similar intensity levels at both time points, but the identity of the corresponding proteins were not determined.

In a subsequent study, Gray et al. [58] employed a similar approach to investigate the presence of potential inflammation biomarkers in sputum samples from patients with asthma, COPD, cystic fibrosis (CF), and bronchiectasis compared to healthy controls. The proteomic profiles of sputum were analyzed by SELDI-TOF using three ProteinChip Arrays: a WCX2 at pH 4 (CM10), a SAX2 at pH 10 (Q10), and an immobilized metal affinity surface activated with nickel.

In this way 105, 113, 381, and 377 peaks were identified in asthma, COPD, CF, and bronchiectasis, respectively (Mann–Whitney test, $p > 0.01$). On the basis of these results, selected peaks were subjected to gel electrophoresis and identified, after tryptic digestion, by mass fingerprint analysis and, when available, by Western blotting (WB). In particular, calgranulin A, B, and C were found to be increased in the inflammatory states of the different airway diseases investigated, whereas Clara cell secretory protein (CCSP) and proline-rich salivary peptide resulted to be decreased.

In 2009, Gomes-Alves et al. [59] demonstrated that protein signatures obtained by SELDI analysis could be useful for the diagnosis of diseases such as CF, asthma, and COPD, which share common clinical features and/or as follow-up markers of therapeutic intervention. The study concerned the screening of serum and nasal epithelial cell samples to establish the proteomic profiles of patients with CF, asthma, and COPD, using two different chip surfaces: a WCX2 at pH 4 (CM10) and a SAX2 at pH 10. Profiling analysis revealed a large number of peaks that, considered in their entirety, could differentiate between healthy and diseased individuals both in serum and nasal brushing samples. These findings led the authors to state that, even if the SELDI approach is fast and can reveal patterns representative of each pathology, the interpretation of results is complicated, require caution, and the use of multivariate analysis is mandatory.

An unconventional application of SELDI is offered by Bozinovski and co-workers [60], who analyzed plasma samples from patients with COPD with a different perspective, which consisted in validating instead of discovering known biomarkers. In fact, they confirmed serum amyloid protein (SAA) as a biomarker of acute exacerbations in COPD.

Another particular example of SELDI application is represented by Terraciano et al. [56]. They characterized the low molecular weight (MW) proteome (peptidome) of IS of patients with asthma and COPD, coupling the fractionation by mesoporous silica beads to MALDI-TOF-MS. The dimensions and porosity of beads are specifically designed to detect only peptides of <4500 MW. With this approach, the authors were able to identify more than 400 peptides, but only six peaks emerged as potential diagnostic peptidic patterns. In particular, it was possible to identify three α -defensins (human neutrophil peptide, HNP1, HNP2, HNP3) and C-terminal amidated peptides of unknown identity, which were able to discriminate asthma, COPD, and healthy groups by virtue of *T*-test ($p < 0.05$).

This approach could represent a high-throughput screening MS-based platform for monitoring key peptidic biomarkers of inflammatory respiratory diseases, but it is limited to low MWs and does not permit an unambiguous protein identity.

Definitely SELDI-TOF technology has significant advantages as a “first line” approach in biomarker discovery, due to its relative simplicity and its high-throughput potential, but it needs to be assisted by other proteomic instruments (such as 2DE and MALDI-TOF/TOF) in order to produce a clear iden-

tification and validation of proposed biomarkers. In this way, relevant proteomic signatures could be identified in different sample types, setting up potential diagnostics and/or prognostics instruments for various pulmonary diseases. Success in validation has already been achieved in some cases, yet the overall conclusion is that SELDI technology represents one component of a broader spectrum of versatile investigation technologies that may finally lead to setting up a more “clinical friendly” set of dedicated diagnostics tools.

3.3 Gel-based methods

Gel-based methods represent the most widely used proteomic approach and are based on mono- or bidimensional gel electrophoresis (1DG or 2DG) combined with the isolation and identification of the protein bands/spots of interest by means of MS. In particular, as shown in Tables 1 and 2, asthma and COPD proteomic investigations are mainly supported by gel-based approaches, among which a high percentage is represented by 2DG and its variant, 2D-DIGE [61–76].

The 2D-DIGE addresses some of the drawbacks with the original technique, which consists of variation between gels that often leads to multiple repeat runs. This technique has also the advantage of running an internal standard as a part of each separation, providing more accurate and reliable results.

Verrillis et al. [64] applied 2D-DIGE and MALDI-TOF to analyze plasma of subjects with asthma and COPD and determined that iron metabolism, coagulation cascade and acute-phase response could be involved in the pathogenesis of airway diseases. Among 20 unique proteins, which resulted differentially expressed and positively identified, four (α 2-macroglobulin (α 2M); haptoglobin; ceruloplasmin (CP); hemopexin) were selected and able to discriminate subjects with asthma, COPD, and healthy controls. These findings are supported by other authors. For example, Choi et al. [63] identified CP, apolipoprotein A1 (ApoA1), and α 2M as upregulated in nasal lavage fluid of aspirin-exacerbation respiratory disease patients. Nishioka et al. [62] confirmed that proteins up- or downregulated in asthma were involved in responses to stress and in complement and coagulation cascades. In fact, they validated the upregulation of α -1-antitrypsin (AAT) and complement component C7 in asthma exacerbation by ELISA. Fibrinogen and complement C3 resulted as asthma biomarkers in the study performed by Rhim and co-workers [66].

In the study of Ohlmeier et al. [75], a cysteine-specific 2D-DIGE technique was combined with MS for the proteomic screening of human sputum samples from nonsmokers, healthy smokers, and patients with COPD, revealing 15 COPD-related proteins. The polymeric immunoglobulin receptor (PIGR) was selected for further investigation. Elevated, smoking-dependent levels of a secreted PIGR were detected in human lung, sputum, and plasma from healthy, smokers, and mild to moderate COPD patients, with plasma PIGR

showing a direct correlation of its expression with airway obstruction and with the onset of an early COPD stage in healthy smokers.

Moreover, the recent introduction of immobilized pH gradients and advanced bioinformatics has vastly improved the reproducibility and comparability of this technique, although its low-throughput remains a serious obstacle in becoming a routine method of analysis in clinical practice.

In fact, as highlighted by Bandow et al. [70], three issues are holding back gel-based methods by large-scale proteomics studies: low-throughput, high technical variation, and a study design lacking statistical power. With this in mind, the authors developed an improved image analysis workflow, based on the creation of a consensus spot pattern for the quantitation of all acquired gel images. Image analysis coupled with a statistical test, allowed to perform a quantitative analysis of all protein spots and to determine which spots were differentially expressed and would be processed by MS. To this aim they combined 2DG separation and nano-LC-MS/MS identification to discover candidate biomarkers in plasma samples from COPD smokers and healthy smokers. Eight potential biomarkers were identified, with statistical significance (*T*-test), in patients with COPD compared to control subjects. Apolipoprotein E (ApoE), complement component 3 (C3), and human fibrinogen fragment D resulted upregulated in COPD patients, while retinol-binding protein (RTB), antioxidant glutathione peroxidase 1 (GPX1), complement component 4B (C4B3), and two inter-alpha-trypsin-inhibitor heavy chain H4 (ITIH4) were downregulated in the patient group. No other tests were applied to validate these potential biomarkers.

Recently, bioinformatic tools have been introduced as support to proteomics analysis, which allow to improve the significance of the findings obtained by gel-based methods. For example, in Pastor et al.'s [72] study, using 2DG in combination with MALDI-TOF/TOF for the analysis of BALF from patients with COPD and lung cancer, 123 spot were detected and 40 of them resulted differentially expressed between patients with COPD and lung cancer (test INCA, $\geq$ twofold, $p < 0.05$). Specifically, aldehyde dehydrogenase (ALDH3A1), aldo-keto reductase family 1 member B10 (AKR1B10), aldo-keto reductase family 1 member C3 (AKR1C3), aldolase A (ALDOA), cathepsin D preprotein (CTSD), cofilin 1 (CFL1), ezirin (EZR), fructose-1,6 bisphosphatase (FBP1), glycogen phosphorylase (PYGM), peptidylprolyl isomerase A, pyruvate kinase 2 (PKM2), transketolase (TKT) were upregulated in lung cancer. Instead, catalase (CAT), peroxiredoxin 1 (PRDX 1), peroxiredoxin 5 (PRDX 5), peroxiredoxin 2 isoform a (PRDX 2) were upregulated in COPD while calcyphosin 2 (CAPS2) and CFL1 were downregulated in this disease. Some proteins such as PKM2, AKR1B10, PRDX1 were also validated by WB. Moreover, the multiple biomarkers found were subjected to computational analysis to predict pathways that they could collectively regulate. In fact, the authors applied ingenuity pathway analysis (IPA), based on gene ontology to plot the proteins identified to be differentially expressed

in COPD and lung cancer patients. It was observed that the main networks involved were related to inflammatory signaling, free radical scavenging, oxidative stress response, and glycolysis plus gluconeogenesis pathways. This integrative approach contributed to elucidate the underlying pathogenic pathway of both diseases, highlighting NF- κ B pathway as a link between them.

A final remark that arises from this study and underlines a limit of the 2DG approach is the almost exclusive cytoplasmic origin of the identified proteins. The gel-based approach has a relatively high resolution, which is however limited by the difficulty in detecting certain classes of proteins. These include membrane proteins due to their low solubility in gel electrophoresis buffer, proteins with an either low (<10 kDa) or high (>200 kDa) MW, as well as those with an extreme isoelectric point ($pI < 4$ or >9) [77]. This has led to a limited application for the analysis of complex matrices, such as lung tissue, which is also reflected by the few published studies [71, 73]. Lee et al. [71] investigated the proteins expressed in lung tissue of COPD smokers and healthy smokers and nonsmokers, using 2DG and MALDI-TOF analysis. MMP-13 and thioredoxin-like 2 resulted upregulated in COPD patients compared to healthy smokers and nonsmokers. MMP-13, a proteolytic enzyme implicated in tissue damage and remodeling, was predominantly expressed in alveolar macrophages and type II pneumocytes. Thioredoxin-like 2 is an antioxidant protein whose expression is mainly located within the bronchial epithelium. Therefore, thioredoxin-like 2 synthesis could be induced by the oxidative burden that plagues the airways of smokers and the exposure to airborne pollutants. These proteins were validated by WB and immunohistochemistry. The difference among groups was determined by statistical tests such as Kruskal–Wallis, Wilcoxon rank sum with Bonferroni adjustment, considering a p -value < 0.05 as statistically significant. Instead, Hu et al. [73] used a proteomic strategy based on 2DE coupled with MALDI-TOF to study human lung tissue samples obtained from three groups of subjects, including never-smokers, non-COPD smokers, and COPD smokers, respectively. Twenty-four proteins were identified and found to be involved in basic metabolism, oxidation/reduction, coagulation/fibrinolysis, protein degradation, signal trasduction, inflammation, and cell growth/differentiation/apoptosis. Moreover, in the COPD smokers group, the authors observed an upregulation of the heat shock protein 27 (Hsp27) and cyclophilin A, which are associated with the COPD pathogenesis. Also these results were determined by variance analysis (ANOVA test) and confirmed by immunochemistry and WB.

As further confirmation of the above assumptions, Maes et al. [69] investigated the possible drawbacks of experimental variance in the gel-based study of peripheral blood mononuclear cells (PBMCs), considered by many as “surrogate tissue.” The aim of this study was to assess markers that exercise important roles in inflammatory processes involved in different diseases including asthma and COPD. The PBMCs were isolated from blood and used to obtain protein extracts,

which were analyzed by 2D-DIGE and MALDI-TOF approach. This paper also emphasizes the importance of paying great attention to the washing steps employed to remove plasma proteins during the PBMCs isolation procedure and generally to the whole preparation of the adopted workflow, in order to avoid possible causes of false-positive changes among the investigated groups. Finally, an interesting aspect that this work also revealed was the importance of considering variance of human population for a good experimental biomarker discovery design. Despite the improvement in the inflammatory biomarker discovery represented by gel-based methods, this last consideration is to be taken into account as one of the bottlenecks to be overcome.

3.4 LC-MS based methods

LC-MS based proteomics offers several advantages over the approaches discussed previously, including greater specificity, cost- and time effectiveness, and the potential to identify up to hundreds of proteins in a single analysis.

It is also important to emphasize that this proteomic approach, involving the combination of two of the most high-throughput and innovative techniques, is considered by scientists and clinicians as the most promising and suitable in the challenge of analyzing multifactorial respiratory diseases [78–90].

The problems of low-throughput and high technical variations, encountered with other proteomic techniques, are solved using an advanced proteomic methodology based on 2D chromatography coupled to MS/MS (2DC-MS/MS) [82, 85, 86]. It involves the generation of peptides from enzymatic digestion of a complex protein mixture, their separation by means of 2DC-MS/MS that combines off-line or online ion exchange with RP separation of peptide mixtures. York et al. [85] applied off-line strong cation exchange fractionation with nano-RP-LC-ESI-MS/MS and robust database searching to understand molecular mechanisms underlying lung function decline. The authors analyzed plasma samples pooled and depleted of the top 12 most abundant proteins from healthy smokers and mild to moderate COPD subjects. By reducing the heterogeneity within the group and increasing the yield of low abundance proteins, they identified 1758 peptides belonging to proteins involved in complement (complement C3, C6, C7), coagulation systems (fibrinogen), and with antiprotease and metabolic functions (inter- α -inhibitor). Simultaneously, the same authors [86] proposed a work in which they interrogated the data obtained by the multidimensional approach discussed above to populate the putative mass and tag (PMT) database and generate accurate mass and tag (AMT) results. In this way, combining capillary LC separations with FT-ICR accurate mass measurements and statistical evaluation of accurate mass and tag data, they were able to increase the number of identified peptides and proteins (3549 and 533, respectively). In particular, 33 proteins, belonging to the complement and coagulation pathways, resulted differ-

entially expressed, dividing the COPD smokers in rapid and slow declines in FEV. An application of online 2DC-MS/MS is proposed by the work of Simoes et al. [82], in which the proteome profile of human nasal epithelium (NE) was exhaustively characterized, allowing the identification of approx 1500 distinct proteins. This study, conducted on volunteers and using noninvasive and easily collectable specimens, has provided the most complete NE proteome profiling to date, unraveling the mechanism, which is intimately correlated with lung function and has demonstrated the usefulness of these cells in lower airway epithelia research. Online 2DC was also combined to iTRAQ technology and MALDI-TOF/TOF in order to compare plasma proteins between healthy smokers and nonsmokers [89]; 16 proteins, associated with immunity and inflammatory responses, resulted differentially expressed and useful to discriminate the two groups examined.

Moreover, the possibility to combine micro- and/or nano-LC techniques with the powerful and innovative MS instrumentations permit high sensitivity, efficiency, speed, and accuracy in MS acquisition, thus increasing the confidence and the amount of peptide identifications [84, 90]. The identification of the corresponding proteins is then obtained by the use of appropriate software, which automates the process of matching experimental to theoretical mass spectra. As an example, the microfluidic-based chip nano-LC-MS/MS was used by Franciosi et al. [90] to successfully identify and quantify about 300 proteins of the epithelial lining fluid (ELF). Healthy and COPD subjects were compared and, applying iTRAQ labeling, the main differentially expressed proteins resulted being lactoferrin, cofilin-1, serpin, and calgranulins. In addition, O'Neil et al. [84] demonstrated the potentiality of a workflow based on the interplay of nano-LC high-resolution MS/MS, iTRAQ quantification, and IPA. They were able to identify and quantify more than 1800 proteins from endobronchial biopsies of asthma, without the need of pooling samples. The IPA analysis of the proteome profiles obtained by patients before and after glucocorticoid inhalation highlighted distinct pathophysiological features of asthma (such as collagen accumulation, angiogenesis, and structural changes) and how their expression changed with treatment. The authors correlated some proteins to asthma (lamin, cyclophilin A, and annexin A5) and to glucocorticoid effect (α 2M, serpin, vimentin, and aldolase A). Moreover, as stressed in the paper, the availability of well-characterized phenotypes of asthma is mandatory for obtaining good results.

Thus, the LC-MS based approach provides a significant improvement over 2DG, as it represents a fully automated technology that simultaneously allows separation of digested peptides, their sequencing, and the identification of the corresponding proteins. In this way, it is possible to concurrently identify both membrane and soluble proteins and to characterize quantitatively the protein mixtures, with label or label-free strategies and in a wide pI and MW range. In addition, to simplify the management and the interpretation of the

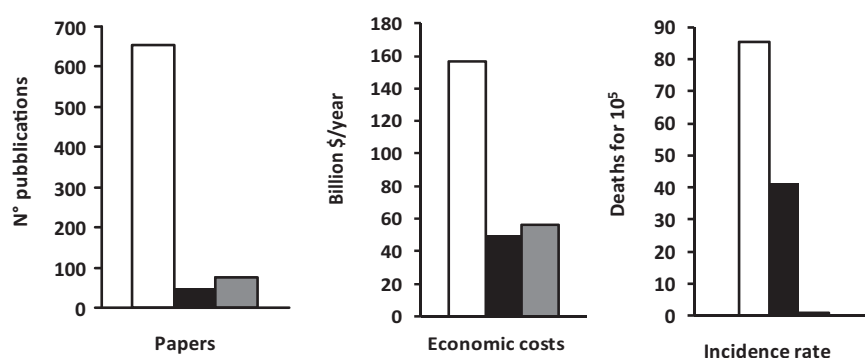


Figure 2. Bar charts show the statistical data concerning the scientific publications, the economic costs, and the incidence rates related to lung cancer (white bars), COPD (black bars), and asthma (gray bars).

large size of data output of LC-MS based experiments, specific bioinformatic tools and programs for statistical analysis and protein classification were usually adopted. Multivariate analysis is also employed to perform a correlation between the differentially expressed proteins and the metabolic pathways in which they are involved. To this end, Gharib et al. [80] integrated a label-free shotgun proteomic approach with unsupervised hierarchical clustering and network analysis to identify mechanisms activated in asthma. The analysis of sputum samples from asthmatic patients was enriched in selected processes, involved in immunity and defenses response, inflammation and protease inhibitory activity, as confirmed by differential expression of serpins, CO3, phospholipase A2, ApoA1, and S100A family.

Even if there are only a few papers involving LC-MS based proteomic methods, daily technological advancements make this approach the most promising in the discovery of a new generation of biomarkers for asthma and COPD diagnosis.

4 Conclusions and perspectives

Chronic obstructive respiratory diseases are becoming a growing global health concern, affecting people of any age, culture, and economic level, and have a significant social and economic impact. Among the respiratory diseases, asthma and COPD are certainly the most widespread and represent the principal leading causes of morbidity and mortality worldwide. In fact, in 2009 in the United States, the deaths due to asthma and COPD are 1.1 and 41.2 people per 100 000 populations, respectively (Fig. 2). Although the deaths for asthma and COPD are lower than that of lung cancer (85.5 people per 100 000 populations), the economic burden of these chronic obstructive respiratory diseases is considerable. In fact, the medical cost in the United States is estimated for asthma and COPD at 56 and 50 billions, respectively, while for respiratory cancer is 157 billions of dollars.

Another aspect to be considered is that both asthma and COPD are highly variable and not precisely defined because of their heterogeneous pathophysiological phenotypes and a not well-defined clinical course. The complexity and the heterogeneity of asthma and COPD pose many problems in

the development of highly reliable tests for their diagnosis and management. In this context, current advances in the proteomic approaches, especially those based on MS, provide new tools to facilitate the discovery-driven studies of new biomarkers in respiratory diseases.

Although the majority of publications on proteomic studies involving respiratory diseases concern lung cancer (Fig. 2), in this review the few preliminary proteomic studies applied to COPD and asthma are summarized. It is already possible to observe the emergence of interesting candidate biomarkers and the first attempts to understand the principal pathways involved in COPD and asthma pathogenesis. Today there are different proteomic approaches, the choice of which should be made considering the final goal, the source of samples, and the available tools. Certainly, because of the constant progress in MS-based techniques, it will soon be possible to increase clinical reliability and statistical confidence needed for the validation of biomarkers under proteomic investigation and their diagnostic employment.

The authors have declared no conflict of interest.

5 References

- [1] Global Strategy for Asthma Management and Prevention. December 2012 update. Available at <http://www.ginasthma.org/guidelines-gina-report-global-strategy-for-asthma.html>, accessed January 7, 2013.
- [2] Vestbo, J., Hurd, S. S., Agustí, A. G., Jones, P. W. et al., Global strategy for the diagnosis, management and prevention of chronic obstructive pulmonary disease, GOLD executive summary. *Am. J. Respir. Crit. Care Med.* 2012, **187**, 347–365.
- [3] Bousquet, J., Jeffery, P. K., Busse, W. W., Johnson, M. et al., From bronchoconstriction to airways inflammation and remodeling. *Am. J. Respir. Crit. Care Med.* 2000, **161**, 1720–1745.
- [4] Hamid, Q., Song, Y. L., Kotsimbos, T. C., Minshall, E. et al., Inflammation of small airways in asthma. *J. Allergy Clin. Immunol.* 1997, **100**, 44–51.
- [5] Jeffery, P. K., Structural and inflammatory changes in COPD: a comparison with asthma. *Thorax* 1998, **53**, 129–136.

- [6] Barnes, P. J., Chronic obstructive pulmonary disease. *N. Engl. J. Med.* 2000, **343**, 269–280.
- [7] Jeffery, P. K., Remodeling in asthma and chronic obstructive lung disease. *Am. J. Respir. Crit. Care Med.* 2001, **164**, S28–S38.
- [8] Hasselgren, M., Arne, M., Lindahl, A., Janson, S. et al., Estimated prevalence of respiratory symptoms, asthma and chronic obstructive pulmonary disease related to detection rate in primary health care. *Scand. J. Prim. Health Care* 2001, **19**, 54–57.
- [9] Mannino, D. M., Buist, A. S., Global burden of COPD: risk factors, prevalence, and future trends. *Lancet* 2007, **370**, 765–773.
- [10] Battaglia, S., den, H. H., Timmers, M. C., Lazeroms, S. P. et al., Small airways function and molecular markers in exhaled air in mild asthma. *Thorax* 2005, **60**, 639–644.
- [11] Montuschi, P., Barnes, P. J., Roberts, L. J., Isoprostanes: markers and mediators of oxidative stress. *FASEB J.* 2004, **18**, 1791–800.
- [12] Global Initiative for Chronic Obstructive Lung Disease. 2002. Global initiative for chronic obstructive lung disease. Global strategy for the diagnosis, management and prevention of chronic obstructive pulmonary disease NHLBI/WHO Workshop Report [online]. NIH Publication, No. 2701. Available at <http://www.goldcopd.com>, accessed December 12, 2007.
- [13] Punturieri, A., Szabo, E., Croxton, T. L., Shapiro, S. D. et al., Lung cancer and chronic obstructive pulmonary disease: needs and opportunities for integrated research. *J. Natl. Cancer Inst.* 2009, **101**, 554–559.
- [14] Mauad, T., Dolhnikoff, M., Pathologic similarities and differences between asthma and chronic obstructive pulmonary disease. *Curr. Opin. Pulm. Med.* 2008, **14**, 31–38.
- [15] Boulet, L. P., Turcotte, H., Hudon, C., Carrier, G. et al., Clinical, physiological and radiological features of asthma with incomplete reversibility of airflow obstruction compared with those of COPD. *Can. Respir. J.* 1998, **5**, 270–277.
- [16] Fabbri, L. M., Romagnoli, M., Corbetta, L., Casoni, G. et al., Differences in airway inflammation in patients with fixed airflow obstruction due to asthma or chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* 2003, **167**, 418–424.
- [17] National Asthma Education and Prevention Program: Expert panel report III: Guidelines for the diagnosis and management of asthma. Bethesda, MD: National Heart, Lung, and Blood Institute, 2007. (NIH publication no. 08-4051). Available at www.nhlbi.nih.gov/guidelines/asthma/asthgdln.htm, accessed January 5, 2012.
- [18] Global Initiative for Chronic Obstructive Lung Disease. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. 2010. Available at http://www.goldcopd.org/uploads/users/files/GOLDReport_April112011.pdf, accessed June 20, 2011.
- [19] Boulet, L. P., Asymptomatic airway hyperresponsiveness: a curiosity or an opportunity to prevent asthma?. *Am. J. Respir. Crit. Care Med.* 2003, **167**, 371–378.
- [20] Ramsdale, E. H., Morris, M. M., Roberts, R. S., Hargreave, F. E., Bronchial responsiveness to methacholine in chronic bronchitis: relationship to airflow obstruction and cold air responsiveness. *Thorax* 1984, **39**, 912–918.
- [21] Ho, L. P., Wood, F. T., Robson, A., Innes, J. A. et al., Atopy influences exhaled nitric oxide levels in adult asthmatics. *Chest* 2000, **118**, 1327–1331.
- [22] Silvestri, M., Spallarossa, D., Frangova Yourukova, V., Fregonese, B. et al., Orally exhaled nitric oxide levels are related to the degree of blood eosinophilia in atopic children with mild-intermittent asthma. *Eur. Respir. J.* 1999, **13**, 321–326.
- [23] Fahy, J. V., Kim, K. W., Liu, J., Boushey, H. A., Prominent neutrophilic inflammation in sputum from subjects with asthma exacerbation. *J. Allergy Clin. Immunol.* 1995, **95**, 843–852.
- [24] Pizzichini, M. M., Popov, T. A., Efthimiadis, A., Hussack, P. et al., Spontaneous and induced sputum to measure indices of airway inflammation in asthma. *Am. J. Respir. Crit. Care Med.* 1996, **154**, 866–869.
- [25] Dweik, R. A., Boggs, P. B., Erzurum, S. C., Irvin, C. G. et al., On behalf of the American Thoracic Society Committee on Interpretation of Exhaled Nitric Oxide Levels (FeNO) for clinical applications. An official ATS clinical practice guideline: interpretation of exhaled nitric oxide levels (FeNO) for clinical applications. *Am. J. Respir. Crit. Care Med.* 2011, **184**, 602–615.
- [26] Kharitonov, S., Alving, K., Barnes, P. J., Exhaled and nasal nitric oxide measurements: recommendations. The European Respiratory Society Task Force. *Eur. Respir. J.* 1997, **10**, 1683–1693.
- [27] Kostikas, K., Koutsokera, A., Papiris, S., Gourgoulanis, K. I. et al., Exhaled breath condensate in patients with asthma: implications for application in clinical practice. *Clin. Exp. Allergy* 2008, **38**, 557–565.
- [28] Saydain, G., Beck, K. C., Decker, P. A., Cowl, C. T. et al., Clinical significance of elevated diffusing capacity. *Chest* 2004, **125**, 446–452.
- [29] Global Initiative for Chronic Obstructive Lung Disease. 2013. At-a-glance outpatient management reference for chronic obstructive pulmonary disease (COPD) based on the global strategy for diagnosis management and prevention of COPD. Updated February 2013. Available at <http://www.goldcopd.com>.
- [30] Pierce, R., Spirometry: an essential clinical measurement. *Aust. Fam. Physician* 2005, **34**, 535–539.
- [31] Global Initiative for Chronic Obstructive Lung Disease. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. Available at <http://www.goldcopd.com/Guidelineitem.asp?l1=2&l2=1&intld=989>, accessed April 14, 2008.
- [32] Miller, M. R., Hankinson, J., Brusasco, V., Burgos, F. et al., ATS/ERS Task Force. Standardisation of spirometry. *Eur. Respir. J.* 2005, **26**, 319–338.
- [33] Birnbaum, S., Barreiro, T. J., Methacholine challenge testing: identifying its diagnostic role, testing, coding, and reimbursement. *Chest* 2007, **131**, 1932–1935.
- [34] Leung, J. M., Bir, D., Biomarkers in airway diseases. *Can. Respir. J.* 2013, **20**, 180–182.

- [35] Choi, J., Hoffman, L. A., Rodway, G. W., Sethi, J. M., Markers of lung disease in exhaled breath: nitric oxide. *Biol. Res. Nurs.* 2006, 7, 241–255.
- [36] Corradi, M., Pesci, A., Casana, R., Alinovi, R. et al., Nitrate in exhaled breath condensate of patients with different airway diseases. *Nitric Oxide* 2003, 8, 26–30.
- [37] Alving, K., Weitzberg, E., Lundberg, J. M., Increased amount of nitric oxide in exhaled air of asthmatics. *Eur. Respir. J.* 1993, 6, 1368–1370.
- [38] Kharitonov, S. A., Chung, K. F., Evans, D., O'Connor, B. J. et al., Increased exhaled nitric oxide in asthma is mainly derived from the lower respiratory tract. *Am. J. Respir. Crit. Care Med.* 1996, 153, 1773–1780.
- [39] Kelly, M. M., Leigh, R., Jayaram, L., Goldsmith, C. H. et al., Eosinophilic bronchitis in asthma: a model for establishing dose-response and relative potency of inhaled corticosteroids. *J. Allergy Clin. Immunol.* 2006, 117, 989–994.
- [40] Leigh, R., Pizzichini, M. M., Morris, M. M., Maltais, F. et al., Stable COPD: predicting benefit from high-dose inhaled corticosteroid treatment. *Eur. Respir. J.* 2006, 27, 964–971.
- [41] Pizzichini, E., Pizzichini, M., Efthimiadis, A., Hargreave, F. et al., Measurement of inflammatory indices in induced sputum: effects of selection of sputum to minimize salivary contamination. *Eur. Respir. J.* 1996, 9, 1174–1180.
- [42] Green, R. H., Brightling, C. E., McKenna, S., Hargadon, B. et al., Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial. *Lancet* 2002, 360, 1715–1721.
- [43] Jayaram, L., Pizzichini, M. M., Cook, R. J., Boulet, L. P. et al., Determining asthma treatment by monitoring sputum cell counts: effect on exacerbations. *Eur. Respir. J.* 2006, 27, 483–494.
- [44] Siva, R., Green, R. H., Brightling, C. E., Shelley, M. et al., Eosinophilic airway inflammation and exacerbations of COPD: a randomised controlled trial. *Eur. Respir. J.* 2007, 29, 906–913.
- [45] D'Silva, L., Hassan, N., Wang, H. Y., Kjarsgaard, M., et al., Heterogeneity of bronchitis in airway diseases in tertiary care clinical practice. *Can. Respir. J.* 2011, 18, 144–148.
- [46] Gygi, S. P., Rochon, Y., Franza, B. R., Aebersold, R., Correlation between protein and mRNA abundance in yeast. *Mol. Cell. Biol.* 1999, 19, 1720–1730.
- [47] Provoost, S., Maes, T., van Durme, Y. M., Gevaert, P. et al., Decreased FOXP3 protein expression in patients with asthma. *Allergy* 2009, 64, 1539–1546.
- [48] Iwamoto, H., Gao, J., Koskela, J., Kinnula, V. et al., Differences in plasma and sputum biomarkers between COPD and COPD-asthma overlap. *Eur. Respir. J.* 2013, 43, 421–429.
- [49] Ricciardolo, F. L., Sabatini, F., Sorbello, V., Benedetto, S. et al., Expression of vascular remodelling markers in relation to bradykinin receptors in asthma and COPD. *Thorax* 2013, 68, 803–811.
- [50] Csanky, E., Olivova, P., Rajnavolgyi, E., Hempel, W. et al., Monoclonal antibody proteomics: discovery and prevalidation of chronic obstructive pulmonary disease biomarkers in a single step. *Electrophoresis* 2007, 28, 4401–4406.
- [51] Röpcke, S., Holz, O., Lauer, G., Müller, M. et al., Repeatability of and relationship between potential COPD biomarkers in bronchoalveolar lavage, bronchial biopsies, serum, and induced sputum. *PLoS One* 2012, 7, e46207.
- [52] El-Deek, S. E., Makhoul, H. A., Saleem, T. H., Mandour, M. A. et al., Surfactant protein D, soluble intercellular adhesion molecule-1 and high-sensitivity C-reactive protein as biomarkers of chronic obstructive pulmonary disease. *Med. Princ. Pract.* 2013, 22, 469–474.
- [53] Gao, P., Zhang, J., He, X., Hao, Y. et al., Sputum inflammatory cell-based classification of patients with acute exacerbation of chronic obstructive pulmonary disease. *PLoS One* 2013, 8, e57678.
- [54] Chen, H., Wang, Y., Bai, C., Wang, X., Alterations of plasma inflammatory biomarkers in the healthy and chronic obstructive pulmonary disease patients with or without acute exacerbation. *J. Proteomics* 2012, 75, 2835–2843.
- [55] Huerta, A., Crisafulli, E., Menéndez, R., Martínez, R. et al., Pneumonic and nonpneumonic exacerbations of COPD: inflammatory response and clinical characteristics. *Chest* 2013, 144, 1134–1142.
- [56] Terracciano, R., Preianò, M., Palladino, G. P., Carpagnano, G. E. et al., Peptidome profiling of induced sputum by mesoporous silica beads and MALDI-TOF MS for non-invasive biomarker discovery of chronic inflammatory lung diseases. *Proteomics* 2011, 11, 3402–3414.
- [57] Murphy, V. E., Johnson, R. F., Wang, Y. C., Akinsanya, K. et al., Proteomic study of plasma proteins in pregnant women with asthma. *Respirology* 2006, 11, 41–48.
- [58] Gray, R. D., MacGregor, G., Noble, D., Imrie, M. et al., Sputum proteomics in inflammatory and suppurative respiratory diseases. *Am. J. Respir. Crit. Care Med.* 2008, 178, 444–452.
- [59] Gomes-Alves, P., Imrie, M., Gray, R. D., Nogueira, P. et al., SELDI-TOF biomarker signatures for cystic fibrosis, asthma and chronic obstructive pulmonary disease. *Clin. Biochem.* 2010, 43, 168–177.
- [60] Bozinovski, S., Hutchinson, A., Thompson, M., Macgregor, L. et al., Serum amyloid A is a biomarker of acute exacerbations of chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* 2008, 177, 269–278.
- [61] Malmström, J., Tufvesson, E., Löfdahl, C. G., Hansson, L. et al., Activation of platelet-derived growth factor pathway in human asthmatic pulmonary-derived mesenchymal cells. *Electrophoresis* 2003, 24, 276–285.
- [62] Nishioka, T., Uchida, K., Meno, K., Ishii, T. et al., Alpha-1-antitrypsin and complement component C7 are involved in asthma exacerbation. *Proteomics Clin. Appl.* 2008, 2, 46–54.
- [63] Choi, G. S., Kim, J. H., Shin, Y. S., Ye, Y. M. et al., Eosinophil activation and novel mediators in the aspirin-induced nasal response in AERD. *Clin. Exp. Allergy* 2013, 43, 730–740.
- [64] Verrills, N. M., Irwin, J. A., He, X. Y., Wood, L. G. et al., Identification of novel diagnostic biomarkers for asthma and chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* 2011, 183, 1633–1643.
- [65] Straub, C., Pazdrak, K., Young, T. W., Stafford, S. J. et al., Toward the proteome of the human peripheral blood eosinophil. *Proteomics Clin. Appl.* 2009, 3, 1151–1173.

- [66] Rhim, T., Choi, Y. S., Nam, B. Y., Uh, S. T. et al., Plasma protein profiles in early asthmatic responses to inhalation allergen challenge. *Allergy* 2009, **64**, 47–54.
- [67] Mehrani, H., Ghanei, M., Aslani, J., Golmanesh, L., Bronchoalveolar lavage fluid proteomic patterns of sulfur mustard-exposed patients. *Proteomics Clin. Appl.* 2009, **3**, 1191–1200.
- [68] Ko, Y. C., Hsu, W. H., Chung, J. G., Dai, M. P. et al., Proteomic analysis of CD4+ T-lymphocytes in patients with asthma between typical therapy (controlled) and no typical therapy (uncontrolled) level. *Hum. Exp. Toxicol.* 2011, **30**, 541–549.
- [69] Maes, E., Landuyt, B., Mertens, I., Schoofs, L., Interindividual variation in the proteome of human peripheral blood mononuclear cells. *PLoS One* 2013, **8**, e61933.
- [70] Bandow, J. E., Baker, J. D., Berth, M., Painter, C. et al., Improved image analysis workflow for 2-D gels enables large-scale 2-D gel-based proteomics studies—COPD biomarker discovery study. *Proteomics* 2008, **8**, 3030–3041.
- [71] Lee, E. J., In, K. H., Kim, J. H., Lee, S. Y. et al., Proteomic analysis in lung tissue of smokers and COPD patients. *Chest* 2009, **135**, 344–352.
- [72] Pastor, M. D., Nogal, A., Molina-Pinelo, S., Meléndez, R. et al., Identification of proteomic signatures associated with lung cancer and COPD. *J. Proteomics* 2013, **89**, 227–237.
- [73] Hu, R., Ouyang, Q., Dai, A., Tan, S. et al., Heat shock protein 27 and cyclophilin A associate with the pathogenesis of COPD. *Respirology* 2011, **16**, 983–993.
- [74] Nicholas, B. L., Skipp, P., Barton, S., Singh, D. et al., Identification of lipocalin and apolipoprotein A1 as biomarkers of chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* 2010, **181**, 1049–1060.
- [75] Ohlmeier, S., Mazur, W., Linja-Aho, A., Louhelainen, N. et al., Sputum proteomics identifies elevated PIGR levels in smokers and mild-to-moderate COPD. *J. Proteome Res.* 2012, **11**, 599–608.
- [76] Lo Tam Loi, A. T., Hoonhorst, S. J., Franciosi, L., Bischoff, R. et al., Acute and chronic inflammatory responses induced by smoking in individuals susceptible and non-susceptible to development of COPD: from specific disease phenotyping towards novel therapy. Protocol of a cross-sectional study. *BMJ Open* 2013, **3**, pii: e002178.
- [77] Reisdorph, N. A., Reisdorph, R., Bowler, R., Broccardo, C., Proteomics methods and applications for the practicing clinician. *Ann. Allergy Asthma Immunol.* 2009, **102**, 523–529.
- [78] Cederfur, C., Malmström, J., Nihlberg, K., Block, M. et al., Glycoproteomic identification of galectin-3 and -8 ligands in bronchoalveolar lavage of mild asthmatics and healthy subjects. *Biochim. Biophys. Acta* 2012, **1820**, 1429–1436.
- [79] Izbicka, E., Streeper, R. T., Michalek, J. E., Loudon, C. L. et al., Plasma biomarkers distinguish non-small cell lung cancer from asthma and differ in men and women. *Cancer Genomics Proteomics* 2012, **9**, 27–35.
- [80] Gharib, S. A., Nguyen, E. V., Lai, Y., Plampin, J. D. et al., Induced sputum proteome in healthy subjects and asthmatic patients. *J. Allergy Clin. Immunol.* 2011, **128**, 1176–1184.
- [81] Rosengren, A. T., Nyman, T. A., Lahesmaa, R., Proteome profiling of interleukin-12 treated human T helper cells. *Proteomics* 2005, **5**, 3137–3141.
- [82] Simões, T., Charro, N., Blonder, J., Faria, D. et al., Molecular profiling of the human nasal epithelium: a proteomics approach. *J. Proteomics* 2011, **75**, 56–69.
- [83] Bloemen, K., Van Den Heuvel, R., Govarts, E., Hooyberghs, J. et al., A new approach to study exhaled proteins as potential biomarkers for asthma. *Clin. Exp. Allergy* 2011, **41**, 346–356.
- [84] O’Neil, S. E., Sitkauskienė, B., Babusyte, A., Krisiukienė, A. et al., Network analysis of quantitative proteomics on asthmatic bronchi: effects of inhaled glucocorticoid treatment. *Respir. Res.* 2011, **12**, 124.
- [85] York, T. P., van den Oord, E. J., Langston, T. B., Edmiston, J. S. et al., High-resolution mass spectrometry proteomics for the identification of candidate plasma protein biomarkers for chronic obstructive pulmonary disease. *Biomarkers* 2010, **15**, 367–377.
- [86] Rana, G. S., York, T. P., Edmiston, J. S., Zedler, B. K. et al., Proteomic biomarkers in plasma that differentiate rapid and slow decline in lung function in adult cigarette smokers with chronic obstructive pulmonary disease (COPD). *Anal. Bioanal. Chem.* 2010, **397**, 1809–1819.
- [87] Merkel, D., Rist, W., Seither, P., Weith, A. et al., Proteomic study of human bronchoalveolar lavage fluids from smokers with chronic obstructive pulmonary disease by combining surface-enhanced laser desorption/ionization-mass spectrometry profiling with mass spectrometric protein identification. *Proteomics* 2005, **5**, 2972–2980.
- [88] Berg, I., Hanson, C., Sayles, H., Romberger, D., Vitamin D, vitamin D binding protein, lung function and structure in COPD. *Respir. Med.* 2013, **107**, 1578–1588.
- [89] Bortner, J. D., Jr., Richie, J. P., Jr., Das, A., Liao, J. et al., Proteomic profiling of human plasma by iTRAQ reveals down-regulation of ITI-HC3 and VDBP by cigarette smoking. *J. Proteome Res.* 2011, **10**, 1151–1159.
- [90] Franciosi, L., Govorukhina, N., Fusetti, F., Poolman, B. et al., Proteomic analysis of human epithelial lining fluid by microfluidics-based nanoLC-MS/MS: a feasibility study. *Electrophoresis* 2013, **34**, 2683–2694.