CHAPTER FIVE

Clinical metabolomics of exhaled breath condensate in chronic respiratory diseases

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Abstract

Chronic respiratory diseases (CRDs) are complex multifactorial disorders involving the airways and other lung structures. The development of reliable markers for an early and accurate diagnosis, including disease phenotype, and prediction of the response and/or adherence to treatment prescribed are essential points for the correct management of CRDs. Beside the traditional techniques to detect biomarkers, "omics" sciences have stimulated interest in clinical field as they could potentially improve the study of disease phenotype. Perturbations in a variety of metabolic and signaling pathways could contribute an understanding of CRDs pathogenesis. In particular, metabolomics provides powerful tools to map biological perturbations and their relationship with disease pathogenesis.

The exhaled breath condensate (EBC) is a natural matrix of the respiratory tract, and is well suited for metabolomics studies. In this article, we review the current state of metabolomics methodology applied to EBC in the study of CRDs.

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1. Introduction

Chronic respiratory diseases (CRDs) are complex multi-factorial disorders. They involve the airways and other structures of the lung, whose pathogenesis depends on the interplay between host and environmental factors [1]. Chronic obstructive pulmonary disease (COPD) and asthma are certainly the most common among CRDs, but others like allergic rhinosinusitis, occupational lung diseases, sleep apnea, lung cancer, cystic fibrosis, primary ciliary dyskinesia and pulmonary fibrosis, although less common, are often underdiagnosed. CRDs affect hundreds of millions people worldwide, and being multi-factorial diseases with both environmental and genetic influences [2], are characterized by a remarkable heterogeneity in the clinical course and in their pathophysiological phenotypes [3,4].

Phenotyping of human pathologies in general, and of CRDs in particular, has recently become a way toward precise and personalized medicine. The identification of reliable markers for early and accurate diagnosis, phenotype characterization, and prediction of response and adherence to treatment are essential points for the correct management of CRDs [5]. Because of the complexity, CDRs' phenotyping cannot be described by a single biomarker, and a biomatrix profile including several biomarkers has the potential to better characterize disease phenotypes [6]. Recently, "omics" sciences have become a fundamental research tool in the respiratory clinical field. In particular, metabolomics provides a powerful tool to map the perturbations originating from the altered metabolic and signaling pathways characterizing each CRD [7].

Metabolomics analyzes the whole set of low-molecular-weight metabolites present in cells, tissues, organs and bio-fluids as a manifestation of any exposure (including drug treatment), lifestyle, environmental issues and genetic mutations [8]. Therefore, metabolomics can be useful to define the disease onset and its prognosis and progression [8]. Furthermore, detection of unexpected/unknown metabolites can possibly drive new pathophysiological hypotheses [9].

The respiratory tract offers a natural matrix, the exhaled breath, which is well suited for metabolomics studies. Exhaled breath condensate (EBC) can be easily obtained by cooling exhaled air from spontaneous tidal breathing. It represents a non-invasive method of sampling the airway-lining fluid (ALF) [10], on which metabolomics methodology can be fruitfully applied.

2. Exhaled breath condensate (EBC)

The pulmonary inflammatory response releases many mediators into the ALF, and may mirror the severity of lung injury [10]. EBC essentially contains water (99.9%) but also inorganic compounds like nitric oxide and carbon monoxide, volatile organic compounds (VOCs) and non-volatile substances, and probably reflects the ALF composition. The non-volatile compounds include inorganic anions and cations, organic molecules (urea, organic acids, amino acids and their derivatives), peptides, proteins, surfactants and macromolecules [10].

Different mechanisms are responsible for the presence of volatile and non-volatile compounds in the EBC. While VOCs are present in EBC because of the partitioning between the gaseous and aqueous phases of the exhaled breath, the exact mechanism(s) explaining how the non-volatiles enter the gaseous phase is still debated. It is hypothesized that the non-volatile compounds can be released from ALF into the exhaled air as aerosolized particles and/or aerosols. A number of conditions such as age, sex, circadian rhythm, infection and other exogenous elements may interfere with the EBC composition, and they should be taken into account [11].

Because of the water excess, the EBC compounds are highly diluted. Often, the ratio between the concentrations of the non-volatile analytes in EBC and those in ALF is considered as the EBC dilution factor, which is reported to vary between 1000 and 50,000 [10]. Consequently, such a significant intra- and inter-individual variability of the concentration of non-volatile compounds must be considered when analyzing EBC samples and interpreting data. So far, no golden standard for dilution has been identified. The appropriate dilution standardization, the simultaneous measurement of dilution marker(s), the monitoring of ventilatory patterns, the measurement of the exhaled particles and the condensation temperature are all factors that need to be addressed for a successful application of metabolomic-based analysis of EBC in clinical and/or research area.

3. Metabolomics of EBC

Metabolomics is the comprehensive assessment of endogenous metabolites (metabolome). It systematically identifies and quantifies metabolites from a biological specimen in a global/targeted approach [12]. Metabolites are small molecules ($\leq$ 1000 Da), and include peptides, amino acids,

nucleotides, carbohydrates, organic acids, vitamins, polyphenols, alkaloids and inorganic species, which act as a signature of the functional phenotype in a cell, tissue or organism. The metabolome represents the expression of a multiparametric response of a living system to genetic modification, pathophysiological stimuli and influence of the environment [13]. Therefore, it is well suited to study a disease through a metabolic bioprofile because metabolites can potentially represent the history of the cellular response to past exposure, and offer potential diagnostic and/or prognostic application [12].

Currently, no single analytical platform leads to a comprehensive identification and quantification of the metabolome of a biological system. The intrinsic chemical diversity demands that different analytical techniques should be combined to generate complementary results useful to enhance metabolic analysis. The most used techniques in metabolomics are nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) [14], whose main characteristics are compared in Table 1.

Comparing NMR and MS, it should be underlined that NMR requires little pre-treatment of samples, is rapid (10–15 min for a 1D acquisition), is non-destructive, non-invasive, has a high degree of sensitivity (less than or equivalent to μmol L^{-1}), and provides highly reproducible results. However, NMR is a rather insensitive technique, and, therefore, requires concentrated metabolites. The major advantage of MS is represented by its significantly lower limit of detection, while disadvantages include the potential biases introduced by sample preparation and differential ionization effects that can affect the detection and quantization of metabolites.

NMR spectroscopy can be considered a general technique for metabolite detection. It studies molecules by recording the interaction of the nuclei forming the molecules with an electromagnetic radiation in the radiofrequency range when the sample is placed in a strong magnetic field. This energy is at a specific resonance frequency that depends on the strength of the magnetic field and the magnetic properties of the nucleus. The re-emission of the energy absorbed by the nuclei in the experiment is registered and an NMR spectrum is then obtained after a Fourier-transformation (Fig. 1). A single nucleus in a molecule can be "observed" by monitoring the corresponding line (resonance) in a spectrum, and the various parameters that characterize the line (frequency, splitting, line width and amplitude) can be used to determine the molecular structure, conformation and dynamics of biomolecules, whose size varies from small organic molecules to macromolecules.

NMR spectrometers operating at high static magnetic field detect more spectral details. The magnetic field strength available today has reached

Table 1 Main characteristics of nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) for metabolomics studies[a].

	NMR	MS
Detection limit	Nanomolar (with cryoprobes)	Picomolar (much lower with special techniques)
Metabolite detection	All metabolites detected	Usually needs a targeted approach with pre-analytical conditions based on chemical classes
Analysis	Whole sample analyzed in one measurement	Different experimental conditions for different chemical classes
Quantity	200–400 μL	Few μL
Recovery	nondestructive	Destructive (but uses small amounts)
Tissue analysis	Yes (MAS-NMR)	Yes (MALDI protein tissue imaging; DESY metabolite imaging)
Reproducibility	Very high	Targeted: high; Untargeted: fair
Sample preparation	Minimal	Variable but can be extensive (protein precipitation, solid-phase extraction, liquid-liquid, etc.)
Difficulty in molecular identification	Low, from databases and analysis of multidimensional spectra	Depending on instruments (MS/MS, MS^n, GC-MS, etc.)
Acquisition time	10–15 min for 1D spectra	10 min for UPLC-MS
Quantification	1–5%	5% intraday
Instrument robustness	High	High
Databases	Available and increasing	Available and increasing

[a]Adapted from M.C.A. Maniscalco, D. Paris, D.J. Melck, A. Molino, S. Fuschillo, Motta A, Metabolomics of exhaled breath condensate by nuclear magnetic resonance spectroscopy and mass spectrometry: a methodological approach, Curr. Med. Chem. (2018) in press.

28.2 T (that is, 1.2 GHz for proton frequency). However, most applications in NMR-based metabolomics are obtained using spectrometers operating at 600 MHz (i.e., 14.1 T), equipped with a CryoProbe. Such technology brings about a sensitivity increase while the level of the thermal noise

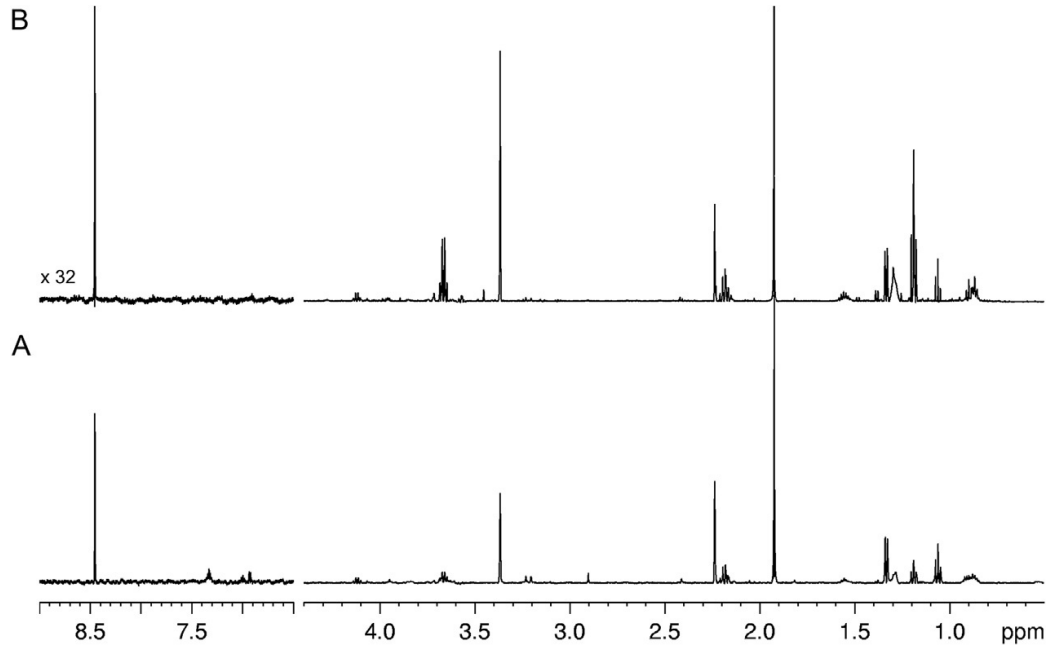

Fig. 1 NMR spectra of EBC samples. Representative one-dimensional ^{1}H spectra of an asthmatic (A), and a COPD (B) patient. The region between 9.0 and 6.5 ppm has a 32-fold vertical expansion. All signals were assigned to single metabolites by resorting to two-dimensional NMR experiments and referring to published data on metabolite chemical shifts. Absorption (related to the intensity) is plotted on the *y*-axis, and magnetic field strength is plotted on the *x*-axis, which usually ranges from 0 to 12 ppm.

produced by electronic circuits and components is efficiently reduced, therefore increasing the signal-to-noise ratio of the experiments. NMR spectrometers present an amplitude response linearly dependent on sample concentration, and this permits a straightforward quantification of the metabolite concentration. In modern spectrometers, all steps involved in the acquisition and processing of NMR data, including machine setting and exchange of samples, are fully automated for dozens of samples without human intervention.

NMR-based metabolomics offers several advantages for a rapid and accurate metabolic analysis of the sample with minimal sample pretreatment. Furthermore, since the technique is nondestructive, the samples can be repeatedly investigated when the metabolic stability is preserved.

Biological molecules are mainly composed by hydrogen, carbon, oxygen, nitrogen and fluorine atoms, which all present an NMR-detectable isotope. Since proton has a high natural abundance and inherent sensitivity, ^{1}H (the "proton") is the nucleus of choice used for metabolomics. In principle, the identification (i.e., the "assignment") of a resonance originating from a specific metabolite is obtainable by a comparison with published reference

data, which tabulate the chemical shifts (i.e., the position of the line in a spectrum with respect to a reference line) of metabolites found in several specimens. Since proton signals are spread over a chemical shift range of ca. 14 ppm, signal overlap is often observed, and this generates specificity problems because resonances can be hidden under signals from chemically similar species. This problem can be (partially) overcome by recording spectra for nuclei with a larger chemical shift range (for example, ^{13}C), or for nuclei with a limited presence in the molecules (for example, ^{15}N, ^{19}F, and ^{31}P).

Identification of metabolites in crowded spectral regions can be helped by acquiring two-dimensional (2D) spectra, which separate signals in two dimensions. Used experiments connect the same ["homonuclear" correlations like ^{1}H–^{1}H clean total correlation spectroscopy (TOCSY)], or different ["heteronuclear" correlations like proton and carbon, like ^{1}H–^{13}C single-quantum coherence (HSQC) experiments] nuclei. The built-in pulse sequence library of each spectrometer allows for a panoply of 2D experiments, but in-house personalization for dedicated experiments is also possible.

MS is a technique that ionizes molecules and classifies the ions according to their mass-to-charge ratio. Accordingly, a mass spectrum determines the masses of the molecules that are present within a sample. MS methods vary with respect to throughput (i.e., time of analysis), sensitivity and selectivity, as well as robustness, ease of use and cost. Mass spectrometers determine the molecular mass of molecules using mass analyzers, which broadly exist in distinct formats of increasing mass resolution and accuracy: quadrupole, ion trap, time of flight, Orbitrap and Fourier Transform Ion Cyclotron Resonance.

MS presents higher intrinsic sensitivity and specificity compared with NMR, although it usually requires a previous separation step such as gas chromatography (GC), high-performance liquid chromatography (HPLC) or ultra-performance liquid chromatography (UPLC) and capillary electrophoresis (CE) (Table 1). Both VOCs and non-volatile organic components of EBC (NVOCs) are analyzed in clinical studies. Separation techniques coupled to MS are important to reduce sample complexity and to minimize ionization suppression effects, thus enhancing the detection sensitivity and increasing the metabolome coverage [15]. Differently from NMR, which requires limited sample preparation, pre-concentration and/or pre-purification steps are often required before MS analysis can be carried out, and they may be quite demanding, but the MS sensitivity improves after clean-up and chromatographic separation of the biological sample

[16]. MS sample preparation includes lyophilization, liquid-liquid extraction (LLE), solid-phase extraction (SPE), and micro-extraction (SPME).

MS-based metabolomics allows for untargeted and targeted approaches. The untargeted strategy evaluates the whole molecular content of the samples, and may favor a deeper and more complete knowledge of physiological and pathological biochemical processes. On the contrary, targeted methodology can reveal the biological mechanism(s) responsible for pathology. It can identify and quantify specific metabolites, or class of metabolites, according to a hypothesis-driven strategy, but it disregards potential new or unknown disease biomarkers.

GC-MS can be usefully applied to detect volatile and semi-volatile thermally stable compounds. Furthermore, after appropriate chemical derivatization, some non-volatile compounds can be transformed into volatile at the analytical temperature. Identification of VOCs in EBC by GC-MS or GC coupled to Tandem Mass Spectrometry (GC/MS/MS) is typically obtained on samples pre-enriched by SPME [17–24]. Unidentified mass values require mass spectral databases and libraries to match molecular ion and ion fragment patterns. Since databases do not contain all metabolites, to avoid tentative identification reference standards should be used for structural validation.

LC-MS/MS uses a wide array of platforms that allow for simultaneous identification and quantification of targeted metabolites like 8-isopostaglandin F2α in human EBC samples [25], with high degree of sensitivity and specificity. Often it does not require sample derivatization, and therefore thermally unstable molecules that cannot be safely analyzed by GC-MS-based methodologies can be investigated. In EBC, LC-MS/MS has been chiefly used to assess the presence of biomarkers of inflammation like alkenals [26], leukotrienes [27,28], isoprostanes [29] and eicosanoids [30,31] in healthy, asthmatic and pneumoconiosis subjects. Although not described in this chapter, LC-MS/MS is also extremely useful in analyzing proteins present in EBC samples [32], as demonstrated for lung cancer screening [33] and asthma monitoring [34].

NMR and MS spectral data derived from the entire metabolome in biofluids are extremely complex for the presence of hundreds of low-molecular-weight compounds. A biological matrix (EBC, blood, urine, saliva, cells, tissue, etc.) comprises not only the endogenously formed and metabolized compounds, but may also include xenobiotic molecules together with metabolites produced by viruses and bacteria. Furthermore, sample manipulations and biological fluctuations of the metabolic profile

may increase the sample variability. Multivariate analysis techniques provide three of the most popular regression algorithms, the principal component analysis (PCA), the partial least squares projection to latent structures (PLS) [26,27], together with the "filtered versions" orthogonal projection to latent structures (OPLS, O2PLS) [28–30]. The new factors that are generated are called latent variables or principal components. The subsequent data projection into the corresponding latent space results in dimensionality reduction, and in an easily and intuitive data visualization. Therefore, multivariate analysis is an integrated part of metabolomics, due to its ability to provide interpretable models for complex intercorrelated data [31].

The raw metabolic profiles acquired with NMR or MS techniques can be processed and arranged to form a data matrix containing N observations in rows and K variables in columns, consisting of NMR spectral bins or m/z intensities each (coupled with the retention time). The chemical profile dataset can be transformed into a matrix through a binning/bucketing procedure that defines chemical shift or m/z bins sizes and integrates the bin intensities [32,33].

After NMR/MS spectral processing, which transforms row spectral data into clean spectra for the matrix, data analysis proceeds via multiple steps that include alignment, normalization and scaling. All these procedures are widely described in literature [34–36] and performed with dedicated commercial software, or available from on line resources and free platforms (for example, Metaboanalyst [http://www.metaboanalyst.ca/] or OpenMS [37]).

Statistical analysis is carried out by applying unsupervised or supervised procedures and discriminant methods. The difference between them refers to the regression algorithms: in the unsupervised, the dimensionality reduction is carried out without a-priori knowledge of sample categories or related metadata; while, in the supervised the reduction is obtained together with extra information on the sample. The choice of the regression method depends upon the experimental conditions of the data collected and the research purpose.

The most popular unsupervised method to analyze metabolomic data is PCA. The regression strategy obtains a small number of orthogonal factors, called *principal components* (PCs), gradually explaining the largest variance within the dataset while adding components stepwise. The dataset projection into the obtained low-dimensional PC space visualizes sample distribution, possibly discerning groups without referring to class information. Two matrices, known as *scores* and *loadings*, are obtained from the original matrix.

Scores are the new coordinates associated to each EBC sample, which is represented as a point in the *scores plot*, while *loadings* represent the role of metabolites responsible for samples distribution in the PC space. In the scores plot, points close to each other present comparable concentration variation in the metabolic profiles, while points placed distant to each other imply different metabolic levels. In the loadings plot, variables (metabolic compounds) close to each other are positively correlated for the samples placed in the corresponding position in the *scores plot*, while negatively correlated variables will appear diagonally at the opposite side.

PCA is often applied as an initial step prior to the application of a supervised method, as it can anticipate likely sample clustering that PLS or OPLS can further highlight. Supervised methods such as PLS guide the matrix decomposition to disclose new latent factors by resorting to additional sample information [26]. Recently, OPLS has been introduced to further enhance the ability of data modeling [28,29]. The prediction ability of PLS and OPLS and their discriminant version (PLS-DA, OPLS-DA) is extremely valuable in data explanation and sample classification, but proper validation is fundamental. Model performance could be assessed with several dedicated methods [38,39], but projection in the calculated model of an external dataset, not included in the calculations of the primary model, is considered the most valid.

4. Metabolomics in respiratory disorders

Metabolomics applied to EBC in CRDs allows for: (a) the assessment of biomarker profiles, resulting in a specific "fingerprint" of a disease (Fig. 1); (b) identification of specific metabolites characterizing the disease; (c) discrimination of specific diseases and their phenotypization (Fig. 2); (d) the possible metabolic pathways involved in the separation obtained by using dedicated software (e.g., MetaboAnalyst 3.0) [40] (Fig. 3).

4.1 COPD

COPD is one of the leading causes of mortality and morbidity worldwide, and is expected to be the third leading cause by 2030 [41]. It is characterized by incompletely reversible airflow limitation that results from small airway disease (obstructive bronchiolitis) and parenchymal destruction (emphysema). It is often very difficult to differentiate COPD from other diseases such as asthma particularly in adult smokers or in the outpatient clinical practice, as the symptoms may be very similar [42]. Metabolomic investigation of

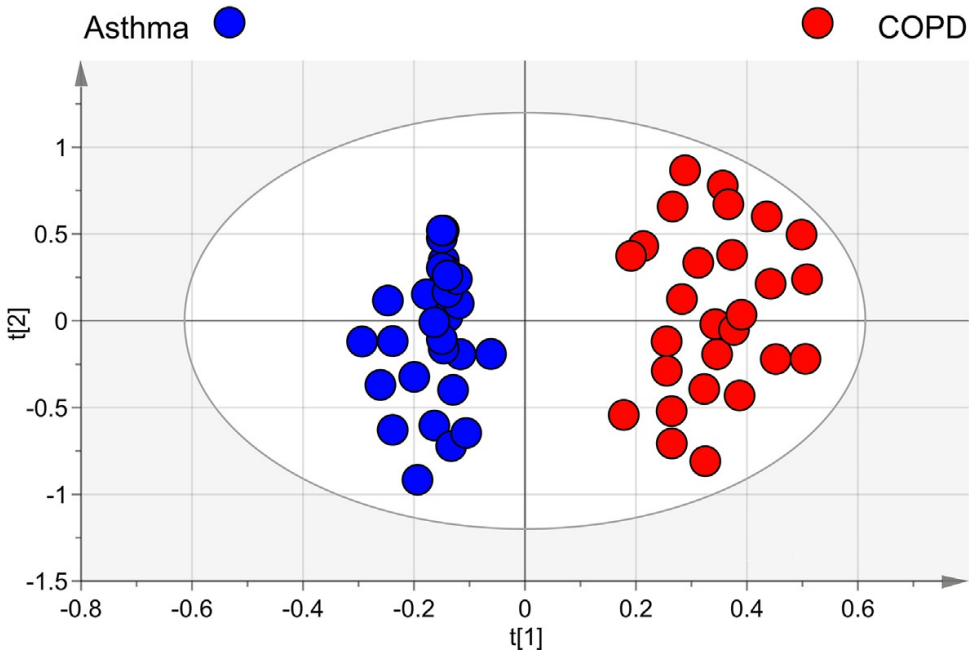

Fig. 2 Example of a scores plot obtained from MS data for the discrimination of asthmatic and COPD patients: asthmatics, blue circles; COPD, red circles. The labels *t*[1] and *t*[2] along the axes represent the scores (the first 2 partial least-squares components) of the model, which are sufficient to build a satisfactory classification model.

EBC by NMR and statistical analysis has been proved particularly able to reliably separate patients with COPD from other pulmonary diseases.

We have recently reported that patients with a new diagnosis of asthma or COPD can be separated by NMR-based metabolomics of EBC samples [43] (Fig. 1). We initially built a model in which the COPD patients, in comparison with those obtained from the asthmatic subjects, show a statistically significant increase in ethanol and methanol levels and significantly lower levels of both formate and acetone/acetoin (Fig. 4). Next, we tested a separate, different cohort of EBC samples obtained from asthmatic and COPD patients that were tested blindly.

In the validation study, the asthmatics were clearly separated from COPD patients, identifying 12 of 13 asthmatic patients and 19 out of 20 COPD subjects. The results confirm that, by using an NMR-based profiling of the EBC metabolites, it is possible to easily discriminate between asthma COPD patients with high sensitivity and specificity.

de Laurentiis et al. [44] evaluated the possibility of distinguishing COPD patients from healthy (HS) and laryngectomized subjects by means of NMR analysis of EBC and saliva samples from 12 COPD, 12 HS and 12 laryngectomized subjects. They first showed that EBC spectra were not contaminated by saliva. By applying PLS-DA analysis, it was possible to obtain a

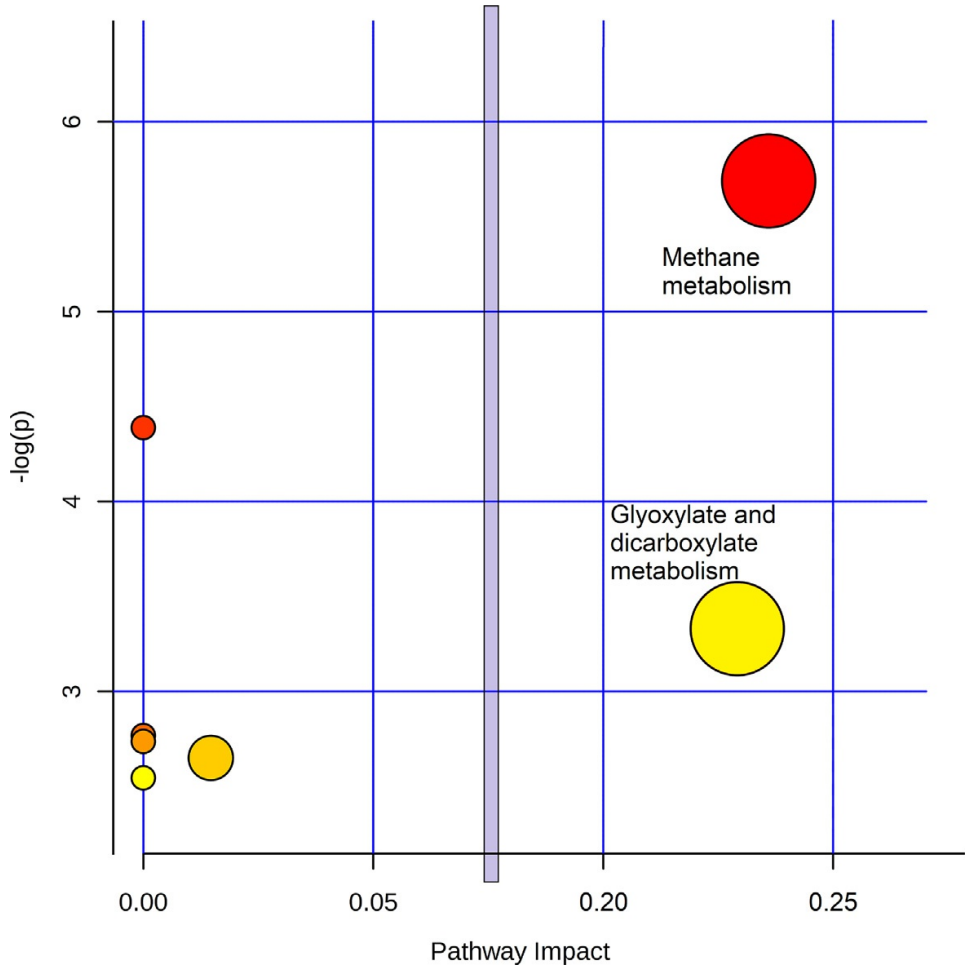

Fig. 3 Example of MetaboAnalyst (http://www.metaboanalyst.ca/) pathway impact based on selected and statistically ($P < 0.05$) representative metabolites responsible for the class separation between asthmatic and COPD subjects. Circles represent all metabolic pathways potentially involved in class separation. The vertical bar highlights the 2 metabolisms with higher impact located on the right side of the bar, namely, methane metabolism (24%) and glyoxylate and dicarboxylate metabolism (23%). The impact [the x—(horizontal) coordinate] is the pathway impact value calculated from pathway topology analysis.

sample classification of ∼95% (samples correctly classified into different regions). In particular, the saliva from HS, laryngectomized and COPD patients were all different from EBC and from each other. Such a separation comes mostly from metabolites present within the 3.5–2.9 and 2.1–1.7 ppm regions of the NMR spectra. In addition, EBC and saliva were stable as samples collected at times 0 and 12 h demonstrated good within-day repeatability, showing no evident spectral difference.

The authors also reported some guidelines on EBC manipulation (how to avoid possible contamination of the EBC samples by the disinfectant when a condenser with reusable collecting parts is used, how to remove

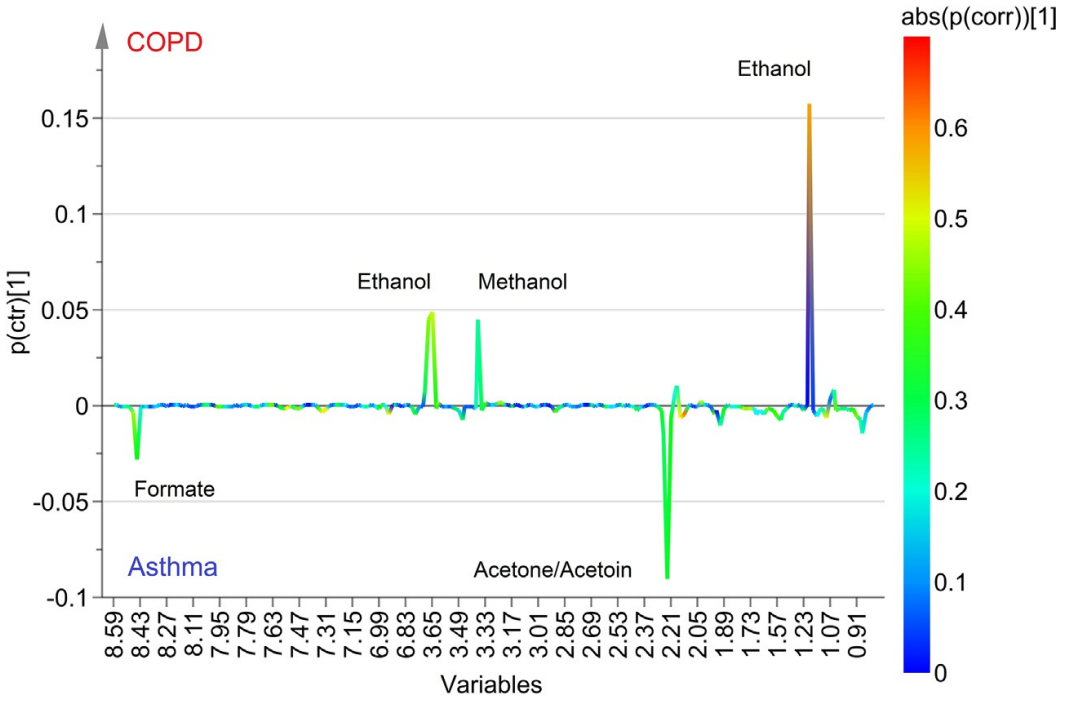

Fig. 4 S-line plot between 8.6 and 0.5 ppm corresponding to Fig. 1 NMR spectra of asthmatic and COPD patients. Positive signals correspond to metabolites that present an increased concentration in COPD patients, whereas negative signals correspond to those that show an increased concentration in asthmatic patients with respect to COPD subjects (i.e., a reduction in COPD). The buckets (i.e., the sequentially integrated spectral regions) are labeled on the *x*-axis according to metabolite assignment with variable identity. The *y*-axis p(ctr)[1] indicates the loading value for each variable according to the centering, whereas abs (p[corr])[1] refers to the absolute correlation value.

oxygen from the samples by nitrogen degassing, storage at −80 °C to quench metabolism at the collection time and prevent any metabolic decay, why lyophilization should not be carried out to prevent metabolite precipitation, etc.). Based on qualitative and quantitative spectral differences, together with acetate, four additional signal variations were found to differentiate COPD from healthy and laryngectomized subjects with an overall accuracy of 94%. The observed signal variations were linked to the increased oxidative stress that is observed in COPD.

The same authors [13] studied 54 EBC samples from 27 COPD subjects and 27 healthy controls. Compared to healthy controls, the corresponding spectrum from a COPD patient showed some differences located in the regions centered at 2.2 and 1.2 pm. These regions include propionate αCH_2, acetoin αCH_3, fatty acid $(CH_2)_2COO$, propionate βCH_3, ethanol CH_3, fatty acid CH_2CH_3, lactate βCH_3 and threonine γCH_3. Unfortunately, in that study no interpretation of the metabolic pathways involved was provided.

Another study from the same authors [45] demonstrated that NMR profile of EBC samples from COPD subjects is different from that of subjects affected by pulmonary Langerhans cell histiocytosis (PLCH). An inverse behavior of 2-propanol and isobutyrate characterized COPD with respect to PLCH (high/low in COPD, low/high in PLCH). Furthermore, NMR was able to identify COPD and PLCH subjects compared to current smokers without COPD. Indeed, COPD and PLCH samples presented a profile different from that of smokers without COPD, showing acetate increase and 1-methylimidazole reduction. The method unambiguously recognized metabolites responsible for between-group differences, strongly suggesting that the biomarker signature characterizing PLCH and COPD is independent from the "common background" due to smoking habit. A limited number of metabolites identify molecular changes in smoking-related diseases such as COPD and PLCH. Acetate, acetoin, ethanol, formate, methanol, 1-methylimidazole, 2-propanol, propionate, and isobutyrate were identified as responsible for intergroup separation and, because of their different concentrations, identified specific trends. In particular, high acetate characterizes COPD and PLCH, representing a key metabolite to differentiate them from health subjects, suggesting an involvement of lipid metabolism related to energy requirements. Indeed, cholesterol molecule is formed from acetate units. Unlike acetate and propionate, no butyrate was reported in COPD and PLCH. Butyrate is oxidized in the mitochondria, forming two acetate molecules for each butyrate molecule. It is possible that the acetate increase in COPD and in PLCH is due to the result of butyrate beta-oxidation.

In a study by Bertini et al. [46] involving 37 COPD patients and 25 healthy subjects, NMR spectroscopy applied to EBC yielded an accurate discrimination between normal subjects and COPD subjects, thereby suggesting that COPD features a distinct metabolomic fingerprint. In particular, COPD subjects displayed significantly lower levels of acetone, valine and lysine, and significantly higher levels of lactate, acetate, propionate, serine, proline and tyrosine. The reported lower levels of valine and lysine could be related to an enhanced metabolic demand. The high levels of lactate observed in COPD could be the result of a persistent subclinical airway inflammation, while the increased levels of acetate could be related to acetylation of pro-inflammatory proteins in the airway lining fluid.

Zabek et al. [47] investigated whether it is possible to diagnose a patient with either COPD or obstructive sleep-apnea syndrome (OSAS) and, simultaneously, to determine whether, to correctly diagnose a patient, it

is necessary to study metabolites present in one, two or three biofluids (serum, exhaled breath condensate or urine). Urine metabolites presented the highest probability to correctly identify patients with COPD and the lowest probability for an incorrect identification of the OSA syndrome. In this study changes in EBC metabolite levels did not appear to be specific enough to differentiate between patients with COPD and OSAS.

4.2 Asthma

Asthma is a serious health and socioeconomic issue all over the world [48]. The effort of researchers has focused on the identification of key metabolites useful for diagnosis, monitoring and treatment of asthma. In that regard, early studies on metabolomics have opened encouraging perspectives for patients [49,50]. In fact, asthma should no longer be considered a single disease and efforts should be made to identify the different biochemical and inflammatory profiles behind asthma symptoms in order to treat them with specifically targeted therapies [51]. Objective measurements of airway inflammation allowed a better customization of therapy than traditional measures alone [52]. Metabolomics has been used in discrimination among asthma, healthy subjects and other diseases both in children and adults, and for the identification of specific pathways that could lead to the development of targeted therapies thereby improving the outcome of patients.

Carraro et al. published a proof-of-concept study on the metabolomics analysis of EBC in relation to asthma [50]. Twenty-five children with asthma (with or without ICS treatment) and 11 healthy controls were enrolled to perform exhaled nitric oxide (eNO), spirometry and EBC collection. They found that selected signals from NMR spectra from two regions, namely, 1.7–2.2 and 3.2–3.4 ppm, corresponding to oxidized and acetylated compounds, were more successful in identifying asthma than the combination of eNO and forced expiratory volume in 1-s (86% versus 81%). In that study, the NMR signals were not identified, but the authors speculated that because of the increased inflammation present in asthmatic airways, the high motility group box protein 1 were acetylated and released in the airways. The presence of oxidized compounds is in line with other studies showing higher concentration of oxidative stress markers in EBC from asthmatic subjects.

In a successive study, Sinha et al. [53] found that the presence or absence of a trident peak at 7 ppm during NMR spectroscopy reliably distinguished between EBC samples collected from healthy and asthmatic

subjects, respectively. This peak probably represents ammonium ion, whose loss in asthma is consistent with a reduced expression of glutaminase, an enzyme that converts glutamine to glutamate and ammonia leading to impaired acid neutralization.

In a number of studies metabolomics approach has showed to be able to discriminate between asthma sub-phenotypes.

In 2013, Carraro et al. [54] applied the metabolomics approach to EBC from 42 asthmatic children to discriminate asthma phenotypes based on disease severity. They found that compounds related to retinoic acid, adenosine and vitamin D were relevant for the discrimination between different groups and a specific metabolite fingerprint emerged in the characterization of severe asthma. These results are consistent with previous literature data. Retinoic acid and its metabolites are involved in both inflammation and airway remodeling in asthma and particularly in the more severe forms [55]. Adenosine presents a number of pro-inflammatory effects and has been involved in asthma especially in those with worsening symptoms [56]. Finally, vitamin D is implicated in the onset of asthma and there are reports suggesting an inverse relationship between its serum levels and asthma severity [57]. Metabolomics data analysis leads to a robust model also when the three groups of children (no asthma, non-severe asthma and severe asthma) were considered altogether, indicating that each group is characterized by a specific metabolomics profile. Furthermore, severe asthma phenotype could be fully discriminated, suggesting that this approach may be very well suited to develop new-targeted therapies [58].

Ibrahim et al. [59] used NMR-based metabolomics of EBC to discriminate asthmatics from controls, and to observe whether these profiles could be used to distinguish asthmatic phenotypes based on sputum eosinophilia, neutrophilia, asthma control and inhaled corticosteroid use. In this investigation, 82 asthmatics and 35 healthy controls were recruited. Five NMR spectral regions were different between patients and healthy volunteers, and demonstrated good accuracy in between-group discrimination. In addition, the model was validated in an independent set of subjects. Additional regions were able to discriminate between sputum neutrophilia and use of inhaled corticosteroids, but this method was not able to differentiate the asthma phenotypes of sputum eosinophilia and asthma control with good accuracy.

Motta et al. [60] studied the EBC from 35 mild asthmatic patients and 35 healthy subjects at two different condensing temperatures, at −27.3 °C and −4.8 °C. Blind validation of the models was obtained from 20 asthmatic

and 20 healthy different subjects not included in the primary analysis. Samples were initially investigated separately according to the collection temperature, and the within-day, between-day, and technical repeatabilities were assessed. Next, samples were interchanged, and, finally, all samples were analyzed together, disregarding the condensing temperature. Partial least-squares discriminant analysis of NMR spectra correctly classified samples, without any influence from the temperature. To build the models the authors used either integral bucket areas (spectral bucketing) or metabolite concentrations (targeted profiling), obtaining strong regression (95%) with high quality parameters for spectral ($R^2 = 0.84$ and $Q^2 = 0.78$) and targeted ($R^2 = 0.91$ and $Q^2 = 0.87$) profilings. In particular, the authors showed that all models were able to reproduce the metabolic variations responsible for class separation notwithstanding the differences in the devices and the condensing temperature. This suggests that cross comparisons of data originating from different condensers are reliable and that NMR-based metabolomics could attenuate some specific problems linked to standardization of EBC collection.

A number of epidemiologic studies indicate that obesity and asthma are linked. Obesity is a risk factor for incident asthma and also affects its severity, control and medication response [61]. A distinct "asthma-obesity" phenotype was suggested in unbiased cluster analysis [62].

In a study by Paris et al. [63], NMR-based metabolomics applied to EBC correctly discriminated samples from obese and lean subjects. In particular, with respect to lean controls, obese subjects presented an increased concentration of ethylene, glycol, ethanol, *n*-valerate short-chain fatty acid, and hydroxybutyrate, and a decreased concentration of formate, methanol, succinate, acetone, acetoin, propionate, acetate, and lactate. As these metabolites are involved in energy homeostasis and inflammatory processes, it was concluded that obesity constitutes a specific respiratory phenotype (metabotype) in which discriminating metabolites are linked to energy homeostasis and inflammation.

In another study [64], the same authors showed that obese asthmatic (OA), lean asthmatic (LA), and obese non-asthmatic subjects (ONA) can be discriminated by NMR-based metabolomics. OA patients were characterized by a respiratory metabolic fingerprint fully different from that of patients independently affected by asthma or obesity. In that study methane, pyruvate, and glyoxylate and dicarboxylate metabolisms were the most probable pathways involved in the class separation. In particular, as compared with ONA, both OA and LA presented reduction of methanol,

formate and acetate, where OAs show a smaller reduction of formate, methanol and acetate with respect to ONA. Since methane metabolism is an important pathway involved in the production of cellular energy, the authors suggested that the energy requirement characterized the two models. The progression from obese to OA seems to require lower energy, consuming a relatively low amount of formate, methanol and acetate. On the contrary, the obesity-asthma co-morbidity involves additional energy, consuming more substrates of methane metabolism. Furthermore, many of the altered metabolites are also suggestive of inflammation. In fact, reduced methanol and formate in OA with respect to LA and, to a lesser extent, with respect to ONA, might indicate a tentative to reduce pulmonary inflammation.

4.3 Cystic fibrosis and primary ciliary dyskinesia

Mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator protein cause cystic fibrosis (CF) [58]. It promotes alterations in the transport of chloride and sodium ions in epithelial secreting cells, producing more viscoid mucus (respiratory tract secretions), chronic respiratory tract infection, dysregulated and heightened inflammatory responses, and progressive lung tissue destruction [65]. Although CF affects multiple organs, over 90% of patients dies from progressive pulmonary disease and subsequent respiratory failure [66]. The natural course of CF is characterized by acute pulmonary exacerbations triggered by viral infections, pollutants or increased bacterial load and, often, requires hospitalization.

CF drives metabolomics alterations in plasma, broncho-alveolar lavage fluid, sputum and serum [67–69]. Robroeks et al. [70] recruited 48 children with CF and 57 controls and analyzed their EBC by GC-MS. They reported a classification rate of 100% for CF patients by using 22 compounds present in exhaled breath. The discrimination between CF and controls was mainly based on C5–C16 hydrocarbons and *N*-methyl-2-methylpropylamine.

In a study by Montuschi et al. [71], EBC metabolomics by NMR discriminated between 29 subjects with stable CF, 24 subjects with unstable CF and 31 age-matched healthy controls. Correct classification rate of CF versus healthy subjects was 96%. The classification rate of stable CF versus unstable CF subjects was 95%. Ethanol, acetate, 2-propanol and acetone were most discriminant between patients with CF and healthy subjects. Acetate, ethanol, 2-propanol and methanol were the most important

metabolites for discriminating between patients with stable and unstable CF. The authors suggested that the elevated EBC ethanol concentrations in patients with CF may be related to the reduced capacity of *Pseudomonas aeruginosa* to oxidize ethanol to acetate, whereas the elevated EBC 2-propanol levels might be due to bacterial metabolism or to increased lipolysis and lipid peroxidation. These data suggest that a limited number of metabolites can be used to pinpoint some of the metabolic changes in CF. A strength of this study is the external validation of these results in independent datasets.

15-F_{2t}-Isoprostane, a reliable biomarker of oxidative stress, has been reported elevated in EBC of CF patients. Azithromycin has antioxidant properties in experimental models of CF, but its effects on oxidative stress in CF patients are largely unknown. Recently, Montuschi et al. [72] reported a proof-of-concept pharmacological study in which they investigated the potential antioxidant effects of azithromycin in CF patients as reflected by EBC 15-F_{2t}-isoprostane, and the effect of azithromycin on EBC and serum metabolic profiles, and on serum 15-F_{2t}-isoprostane. No change was detected in EBC 15-F_{2t}-isoprostane concentrations compared with baseline values after 8-week treatment or 2 weeks after treatment suspension. Likewise, no differences in serum 15-F_{2t}-isoprostane concentrations were observed in either study group. On the contrary, NMR-based metabolomics of EBC showed that suspension of both azithromycin plus vitamin E and vitamin E alone had a striking effect on metabolic profiles, but no effect was observed in serum. Between-group comparisons indicated that EBC metabolite distribution after treatment and 2 weeks after treatment suspension was different. Quantitative differences in ethanol, saturated fatty acids, acetate, acetoin/acetone, and methanol were responsible for these differences. The study suggested that EBC NMR-based metabolomics might be used for assessing the effects of pharmacological treatment suspension in stable CF patients.

Distinct inflammatory and metabolic processes generate different metabolites that may be found in the EBC of subjects with CF and primary ciliary dyskinesia (PCD) [73]. NMR-based analysis of EBC found that acetoin, lactate, methanol, acetate, ethanol, saturated fatty acid, and formate discriminated PCD from CF with 85% sensitivity and 88% specificity. Ethanol, methanol, saturated fatty acids, formate, lactate, acetate, leucine/isoleucine, isobutyrate, and glutamine/glutamic acid separated subjects with PCD from control subjects with 90% of sensitivity and 96% specificity.

Ethanol, methanol, and SFAs represent a sufficient set for subjects' clustering. Ethanol is implicated in the cilia beating with stimulatory effects at low concentrations. SFAs modulate neutrophil production of proinflammatory mediators, and their decreased concentration might be associated with a lack of inflammation suppression in PCD and CF. Similarly, increased methanol in PCD and CF may be associated with airway inflammation. Methanol, present in human breath, is a breakdown product of formaldehyde, which exacerbates airways inflammation. Acetoin is involved in inflammatory processes and its reduction in PCD compared with CF might be associated with a different airway inflammation pattern in these disorders [73].

Recently, Zang et al. [74] applied UPLC-MS to profile metabolites in EBC from 17 clinically stable CF subjects, 9 CF subjects with an acute pulmonary exacerbation, 5 CF subjects during recovery from an acute exacerbation, and 4 CF subjects who were clinically stable at the time of collection but developed an acute exacerbation in the subsequent 1–3 months. Untargeted UPLC-MS metabolomics method coupled to multivariate analysis allowed identification of EBC metabolites related to an acute exacerbation event in CF. 4-Hydroxycyclohexylcarboxylic acid and pyroglutamic acid differentiated EBC of exacerbated subjects from stable CF samples with 84.6% accuracy [74].

Lactic acid was identified as key biomarker for predicting an oncoming acute exacerbation pulmonary event (APE). Lactic acid levels in the studied cohort possibly reflect the status of different stages preceding and following an APE event. The higher levels of lactic acid in the pre-APE and APE patients compared to stable CF patients could possibly result from the increasing hypoxic environment in CF lungs due to poorly cleared thick mucus developing on epithelial surfaces, which leads to an increased lactate conversion from pyruvate in anaerobic glycolysis. Lactate is also a glucose precursor in gluconeogenesis, and elevated gluconeogenesis has been found in CF-related diabetes.

Pyroglutamic acid is a known intermediate in the γ-glutamyl cycle, a pathway for the biosynthesis and degradation of glutathione, and is thus related to redox imbalance.

4-Hydroxycyclohexylcarboxylic acid is a rare organic acid involved in gut microbial mammalian metabolism. This type of metabolic gut-lung crosstalk has also been found to be associated with inflammatory bowel disease in which the pulmonary inflammation is reported to accompany the main inflammatory process in the bowel [74].

4.4 Lung cancer

In the carcinogenetic process cancer cells develop new biochemical adaptations with quantitative changes in endogenous metabolites [75]. A number of enzymatic activities are affected through the alteration of oncogenes and onco-transcription factors, contributing to drive the metabolic shifts observed in cancer cells [76].

Metabolomics, due to its ability to simultaneously detect changes in metabolite profile, is currently one of the fastest developing disciplines in cancer research. In cancer studies, untargeted metabolomics has potential application in biomarker discovery and interventional studies to evaluate the effect of the treatment or search for novel therapeutic targets [76]. Over the last decade, numerous articles on cancer metabolomics have been reported. They involve several cancer types and use different kinds of samples (cells, tissues, biofluids, etc.), and aim at discovering specific metabolic signatures or biomarkers applying NMR and MS techniques [77].

Searching for articles on metabolomics-based analysis of EBC in lung cancer, we identified three articles. In the first work by Peralbo-Molina et al. [78], metabolomics analysis of EBC was applied to discriminate between patients with lung cancer and those at risk for lung cancer. Untargeted analysis, using GC-TOF-MS, was conducted in a cohort of patient with lung cancer ($n = 48$), risk factor individuals (active smokers and ex-smokers, $n = 130$) and control healthy individuals ($n = 61$) in order to detect the EBC metabolic signature within risk and cancer affected individuals. Five compounds were significant in the comparison of the lung cancer patients versus the risk-factor group. Among these compounds, it is worth noting the presence of two saturated monoacylglycerols (monopalmitin ad monostearin) and an acyclic triterpenoid (squalene). Monopalmitin and monostearin were characterized by different behaviors: monopalmitin was more concentrated in the risk factor group than the cancer group. On the other hand, monostearin offered an inverse profile, as the risk factor group showed a lower relative concentration than the lung cancer patients. Seven compounds were shown to discriminate between the lung cancer patients and the risk factor group. Among them, triethyl citrate and indole are related to cigarette smoke. No information about the role played by the other compounds was reported.

Subsequently, the same authors [79] identified metabolomics panels for potential lung cancer screening. Metabolite profiles were obtained from lung cancer patients, which were compared to those provided by two additional cohorts: a risk factor group formed by active smokers with at least

20 pack-years of exposure and ex-smokers, and a second group including healthy non-smoker individuals. EBC collected from the three groups was analyzed by GC. The best panel of metabolites with capability of discrimination between the risk factor cohort and healthy individuals was formed by combination of five metabolites (monopalmitin, monostearin, benzyl alcohol, 2,4-diphenyl-4-methyl-2E-penten and *p*-cresol) to provide 90.3% specificity, 77.9% sensitivity and 85.1% AUC. Discrimination of lung cancer patients versus the risk factor individuals resulted in one other five-compound panel characterized by sensitivity close to 90%, and composed by 2,4-bis-dimethylbenzyl-6-*t*-butylphenol, monostearin, spiro-2,4-heptane-1,5-dimethyl-6-methylene, 13-heptadecyn-1-ol and methyl stearate, with 67.5% specificity, 86.8 sensitivity and an AUC of 77.5% [79]. From a biological point of view, some of the compounds identified can be associated to smoking habit; others can derive from systemic circulation or from airways microbiome.

Furthermore, in this study, the subjects with the most common lung cancer diagnosis (squamous cell carcinoma and adenocarcinoma) were selected to compare their EBC composition with the aim of identifying metabolomics differences among lung cancer stages. Ten subjects belonging to early stages I and II and 28 belonging to advanced stages III and IV were compared with the risk factor cohort [79]. A test was applied to each compound to find statistical differences among the three groups. Six compounds were statistically significant among the three groups under study: cumylalcohol, benzoic acid methyl ester, 2,4,6-triisopropylphenol, 2,6-bis-(1,1dimethylethyl)-4-(1-methyl-1-phenylethyl)phenol, 2,4-bis-(1-methyl-1-phenylethyl) phenol and 2,4-bis-(dimethylbenzyl-6-*t*-butylphenol). All compounds were detected at higher concentrations in lung cancer patients at advanced stage. Five of these compounds had benzenoic structure with alkyl groups, which could be related to tobacco smoke or to metabolic variations ascribed to airways microbiome.

The third study by Ahmed et al. [80] aimed to determine if ^{1}H NMR of sputum and EBC could identify biomarkers of lung cancer. In the EBC samples, median concentrations of propionate, ethanol, acetate, and acetone were higher in lung cancer patients compared to the patients with benign conditions. Median concentration of methanol was lower in lung cancer patients (0.028 mM) than in patients with benign conditions (0.067 mM; $P = 0.028$). Methanol is primarily produced in the gut by the interaction of bacteria with the unabsorbed carbohydrates, and the

reduced levels observed in the EBC of lung cancer patients might indicate a possible alteration in its metabolic pathway triggered by the malignant transformation.

4.5 Miscellanea

EBC metabolomics has also been used to investigate other CRDs. In a pilot study, Fermier et al. [81] investigated the metabolomics signature of EBC from patients in shock with acute respiratory failure. They compared the EBC profiles of 12 patients with shock with those from 14 controls with LC-HRMS. Using a non-targeted approach together with multivariate analysis, they were able to differentiate the two enrolled groups of subjects. More than 1000 ions were detected, but an excellent model of differentiation could be obtained after inclusion of only 6 ions. Although ion identification was missing, the authors claimed that their findings may enable the characterization of endophenotypes of patients, and may improve our knowledge of shock pathogenesis.

Carraro et al. [82] compared EBC metabolomics signature from 20 adolescents with bronchopulmonary dysplasia (BPD) and 15 healthy controls. OPLS-DA model clearly showed a discrimination between patients and healthy controls. In particular, the adolescents with BPD showed an altered complex lipid profile, which persists in the lung of survivors of BPD as a long-term metabolic abnormalities.

In a pilot study, Airoldi et al. [83] reported an NMR-based metabolomics analysis of EBC from patients with α_1-antitrypsin deficit as compared with healthy subjects. NMR profiles were different from both a qualitative and a quantitative point-of-view. Acetoin, propionate, acetate, and propane-1,2-diol were the metabolites presenting the largest difference between the two group of subjects. Most of the metabolites identified derive from pyruvate metabolism pathway, and many of them are bacterial fermentation products.

Li et al. [84] used metabolomics of EBC to study the lymphangioleiomyomatosis (LAM), a progressive neoplastic disorder that leads to lung destruction and respiratory failure, particularly in women. They identified 15-epi-lipoxin-A_4 in EBC from LAM subjects, and noted that its level was increased by aspirin treatment, indicating a functional COX-2 expression in the LAM-patients airway. Then, they evaluated that in vitro 15-epi-lipoxin-A_4 reduced the proliferation of LAM patient-derived cells

in a dose-dependent manner. Therefore, they concluded that targeting COX-2 and prostaglandin pathways might have therapeutic value in treatment of LAM subjects.

5. Summary and future perspectives

Metabolomics represents a major and rapidly evolving component of the new biology. EBC holds a promise to become one of the preferred biomatrices for diagnostics, management and follow-up of various respiratory diseases, including inflammation phenotyping [85]. The separate and/or joint use of NMR and MS allows accurate measurement of small molecules in EBC. This could help our pathophysiological understanding of CRDs, and could possibly identify early metabolic changes of disease, favoring the development of predictive biomarkers that can trigger earlier interventions. In particular, metabolomics of EBC can: (1) add information about physiopathogenetic mechanisms of CRDs; (2) identify prognostic, diagnostic, and surrogate markers for a CRD disease state; (3) classify specific CRD phenotypes/endophenotypes; (4) recognize biomarkers related to drug responsiveness and evaluation of side effects (pharmacometabolomics), correcting/suggesting individually tailored therapeutic approaches in CRDs.

Although many efforts have been made to optimize protocols, many methodological issues still need to be addressed and require thorough validation in large cohorts before NMR- and MS-based metabolomics of EBC can be used in clinics. However, the published data are of remarkable interest, and may currently support clinical data in an unbiased way.

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