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REVIEW

CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Identification of biomarkers in COPD by metabolomics of exhaled breath condensate and serum/plasma

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) is the third cause of death worldwide, presenting poor long-term outcomes and chronic disability. COPD is a condition with a wide spectrum of clinical presentations because its pathophysiological determinants relate to tobacco smoke, genetic factors, alteration of several metabolic pathways, and oxidative stress. Consequently, patients present different phenotypes even with comparable degrees of airflow limitation. Because of the increasing social and economic costs of COPD, a growing attention is currently paid to "omics" techniques for more personalized treatments and patient-tailored rehabilitation programs. In this regard, the systematic investigation of the metabolome (*i.e.*, the whole set of endogenous molecules) in biomatrices, namely metabolomics, has become indispensable for phenotyping respiratory diseases. The metabolomic profiling of biological samples contains the small molecules produced during biological processes and their identification and quantification help in the diagnosis, comprehension of disease outcome and treatment response. Exhaled breath condensate (EBC), plasma and serum are biofluids readily available, with negligible invasiveness, and, therefore, suitable for metabolomics investigations. In this paper, we describe the latest advances on metabolomic profiling of EBC, plasma and serum in COPD patients.

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Chronic obstructive pulmonary disease (COPD) is already the third leading cause of death worldwide, although this was expected to happen by 2030.[1] The main risk factor for COPD is tobacco smoke, but other important pathophysiological determinants such as genetic factors, alterations of various metabolic pathways and oxidative stress, are necessary for COPD to manifest.[2] Presently, diagnostic and severity information on COPD is achieved by resorting to spirometry and symptoms' evaluation. However, a single physiological parameter, such as airflow obstruction, is unable to describe COPD heterogeneous nature and the complexity of the disease. Other diseases (chronic obstructive asthma, bronchiectasis with chronic airflow obstruction and chronic bronchiolitis associated with connective tissue diseases and lung development abnormalities) are also characterized by chronic airflow obstruction and need to be distinguished from cigarette smoking-induced COPD as they present a different course. Therefore, COPD diagnosis and treatment can become challenging. COPD patients are also burdened by a low-grade inflammation, fatigue or weakness, limited ability in exercise training and, at an advanced stage, cachexia, and long-term death.[3] For the above reasons, COPD patients are currently considered of primary public concern because of their health problems,[4] presenting long-lasting disability and necessitating constant rehabilitation treatment.[5] COPD is a complex pathology because its causative multiple mechanisms are not observed in all patients at a definite time, or in the single patient at different evolving times,[6] and this strongly suggests that COPD phenotype is time related. A phenotype can be operationally identified as "a cluster of features that leads to the separation of a specific group at a given time."[7] Consequently, the definition of phenotypes is essential for patients' classification in cohorts presenting equivalent features, prognosis, or personalized approaches.[8] Likely, the different phenotypes of a pathology are characterized by specific molecular differences. Because of the complexity and heterogeneity, differences in COPD phenotypes cannot be represented by a difference of a single biomarker but require instead a panel of molecules. Only a panel of biomarkers can accurately describe a disease-related phenotype. Currently, the protocol for GOLD stratification considers a combination of clinical, pathological, functional, and imaging data. However, it rarely can define the different COPD phenotypes characterized by several concomitant manifestations like airflow impairment, emphysema, chronic bronchitis, asthma-COPD overlap (ACO), exacerbations, etc.[9, 10] Actually, it has been reported that the same degree of airway obstruction may induce different phenotypes in COPD patients.[9] Therefore, mapping COPD phenotype specificity and the related endotypes is mandatory for organizing a tailored disease management, particularly for high-risk patients like recurrent exacerbators. Additionally, the current diagnostic strategy is often controversial in differentiating COPD from other obstructive pulmonary diseases. Accordingly, a multilevel evaluation of COPD should be considered.[11] Omics tools (*i.e.*, genomics, proteomics, and metabolomics) warrant the optimal resolution to recognize specific discrimination for complex chronic diseases like COPD.[12] Specifically, metabolomics, the study of the complete metabolite content of a biological matrix, has added new molecular details to the physiopathological mechanisms triggering COPD disease. Divergent molecular signatures have been associated to specific phenotypes and outcomes, supporting the presence of different pathological mechanisms.[12] Additionally, molecular profiling clearly discriminates COPD patients from healthy controls and other obstructive pulmonary diseases, linking molecular alterations with the degree of airway obstruction, exacerbation, emphysema severity, hospitalization, and response to external stimuli like physical rehabilitation. Amongst the biological matrices relevant for phenotyping, exhaled breath condensate (EBC), plasma and serum are readily available, with negligible invasiveness, and, therefore, appropriate for metabolomics investigations. In this paper, we describe the latest advances on metabolomic profiling of EBC, plasma and serum in COPD patients.

Metabolomics

Metabolites are molecules of *ca.* 1500 Dalton molecular weight, and comprise amino acids, short polypeptides, nucleic acids, amino acids, carbohydrates, organic acids, inorganic species, etc. Since metabolites are the final yields of gene expression, they are considered the functional expression of a phenotype.[13, 14] Moreover, metabolites are implicated in homeostasis and disease, and therefore they are involved in several biological processes like redox balance, oxidative stress, signaling, apoptosis and inflammation.[13] Metabolomics systematically studies the whole set of endogenous molecules (*i.e.*, the metabolome) ubiquitous in organs, tissues, cells, and biological fluids. It can identify and quantify markers in a global and targeted mode[15] and can highlight the multiparametric reactions of a living organism to genetic perturbations, and pathophysiological and environmental stimuli.[16] The metabolome comes after proteome and transcriptome, and therefore contributes a high number of molecular determinants that can be used for an accurate description of a pathology.[17] Several analytical instruments can generate metabolomic profiling, but the most used are Nuclear Magnetic Resonance (NMR) spectroscopy and mass spectrometry (MS), both showing advantages and disadvantages. Collected data are next analyzed with multivariate statistical methods. Technical details and the statistical procedures used to interpret the data are reported elsewhere[18-20] and will not be described here. In this study, we summarized the metabolomics profiling of EBC and plasma/serum to describe the pathophysiology of COPD. While application of metabolomics to follow-up of COPD patients under pharmacological treatment is very interesting, it will not be considered here (see for example[21]).

Exhaled breath condensate

General aspects

EBC is a biological medium efficaciously used in metabolomics for phenotyping airway diseases.[22] It is easily collected in a non-invasive way and can be done repeatedly by cooling exhaled air from spontaneous tidal breathing. Subjects, sitting comfortably and wearing a nose-clip, are asked to breathe through a mouthpiece and a two-way nonrebreathing valve, which acts as a salivary trap, at normal frequency and tidal volume, for *ca.* 15 min. They preserve a dry mouth throughout the operation by swallowing excess saliva from time to time. Volatile substances, probably of extrapulmonary origin,[23, 24] are removed from collected samples (2-3 mL) by using a gentle stream of nitrogen before sealing the samples. Nitrogen also favors oxygen removal, which, together with freezing of the samples, instantaneously "quenches" metabolism at the time of collection, therefore preventing metabolic alteration.[25] Samples are then stored at -80°C until data acquisition. In addition to water (99.9%), EBC contains inorganic molecules like nitric oxide and carbon monoxide, volatile organic compounds (VOCs) and non-volatile substances. The latter comprise inorganic anions and cations, organic molecules like urea, organic acids, amino acids, and their derivatives), peptides, proteins, surfactants, and macromolecules.[26] EBC is influenced by several parameters like age, gender, circadian rhythm, and infections, which all require control.[27] Additionally, alcohol consumption, exercise, nasal contamination, environmental temperature and humidity, exogenous contamination, ammonia, and sulfur-containing compounds from the oral cavity also affect EBC variability.[26] As a confirmation, the levels of 12 EBC metabolites in three groups of smokers (current, former, and never smokers), were considerably different amongst the groups.[28]

Applications

EBC profiling delineates an unambiguous fingerprint that represents each COPD patient and permits to group patients in distinct phenotypes. For examples, NMR-based metabolomics consistently separates COPD from healthy controls and other respiratory diseases. Figure 1A reports a representative NMR spectrum of an EBC sample from a COPD patient. The profile refers to a single patient and is characterized by sharp lines (resonances), which can be identified and attributed to specific metabolite by using two-dimensional experiments.[18-20] The absence of saliva contamination, one of the methodological problems with EBC collection, can be confirmed by checking the presence of signals originating from salivary carbohydrates observed between 3.3 and 6.0 ppm. Their absence indicates that no saliva contamination is present in the collected EBC sample (Figure 1A). De Laurentiis *et al*.[29] found that NMR investigation of EBC differentiated COPD from healthy controls, with a reduced pyruvate signal in COPD as compared with healthy controls, in which it was very strong. The healthy subjects presented small succinate and glutamine signals, both absent in COPD. Furthermore, choline, phosphorylcholine and trimethylamine-N-oxide (TMAO) were not observed in COPD. Other metabolites that favor the separation between COPD and healthy controls are acetoin, ethanol, fatty acids, lactate, propionate, and threonine.[30] Stable COPD with respect to non-obstructed controls presented reduced concentration of acetone, lysine and valine, and increased concentrations of acetate, lactate, proline, propionate, serine, and tyro-

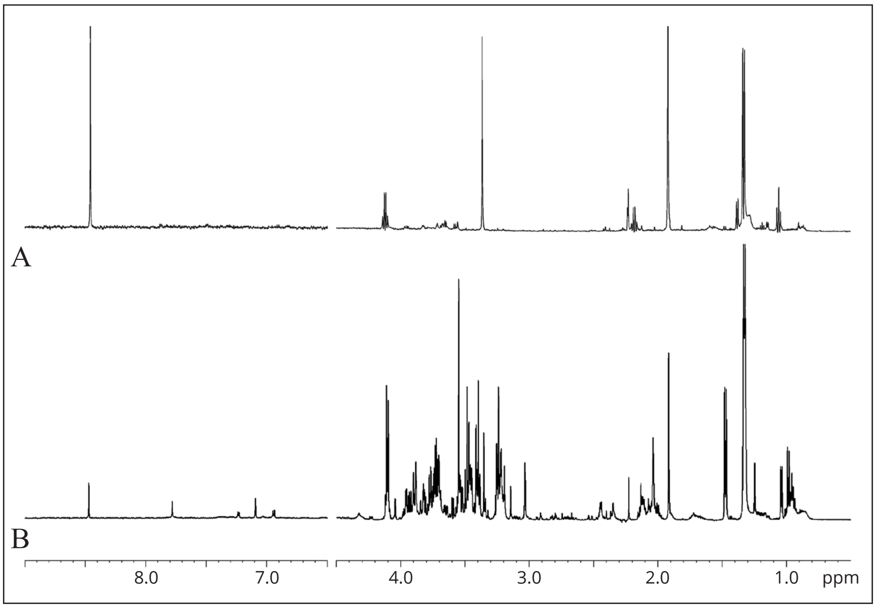

Figure 1.—Representative one-dimensional ^1H spectra of a COPD patient: A) EBC sample; and (B) serum sample. The region between 9.0 and 6.5 ppm in (A) has a 16-fold vertical expansion. All signals in both spectra can be assigned to single metabolites by resorting to two-dimensional NMR experiments and 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.

sine.[31] The biomarkers differentiating COPD from related pathologies discussed in this review are reported in Supplementary Digital Material 1 (Supplementary Table I). The reported metabolites describe a distinct COPD "metabotype" (*i.e.*, a metabolic phenotype) referred to respiratory inflammation. Possible relationships with clinical parameters usually require more than a single fluid (like EBC), adding for example serum/plasma and/or urine. However, "average" biomatrices like serum/plasma and urine are reported to reveal systemic rather lung inflammation,[32] while the bronchoalveolar lavage (BAL) and EBC seem to better reflect respiratory metabolism in comparison with urine and plasma and urine.[33, 34] EBC profiling was also useful to differentiate COPD from other chronic obstructive pulmonary diseases related to smoking, namely asthma and Langerhans cell histiocytosis (PLCH).[35] With respect to asthma, ethanol and methanol were elevated in COPD, while formate and acetone/acetoin decreased. In comparison with PLCH, elevated 2-propanol was observed in COPD while isobutyrate concentration diminished, whereas both metabolites in PLCH presented an opposite behavior. Furthermore, increased acetate and decreased 1-methylimidazole separated COPD and PLCH from current smokers devoid of COPD.[35] The found metabolites indicate that COPD and PLCH present different metabotypes, signifying that, although they share similar smoking behavior, the molecular response is different. Accordingly, metabolomics profiling of EBC via NMR can carefully detail the specific disease boundaries even in the presence of similar insults. This conclusion is very important, as NMR profiling is a fully untargeted approach that makes no a-priori assumptions on the molecular determinants of a disease.[35] Additionally, since a multiparametric approach is used to analyze the samples, metabolomics can reveal unique and apparently unconnected networks between pathological state/evolution and altered metabolic pathways in a diseased state. The distribution of networks' alteration could help in discriminating pathologies that share common molecular phenotypic background. As an example, COPD and PLCH patients could be efficaciously differentiated, even though they share common tobacco exposure. Figure 2[19, 20, 36] reports the metabolic pathways possibly altered in COPD-PLCH discrimination. From a biological point of view, asthma and COPD are two nosological entities with different airway inflammatory cells recruitment, mediators' production, and responses to therapy.[37] However, from a clinical point of view, asthma and COPD are characterized by nonspecific and

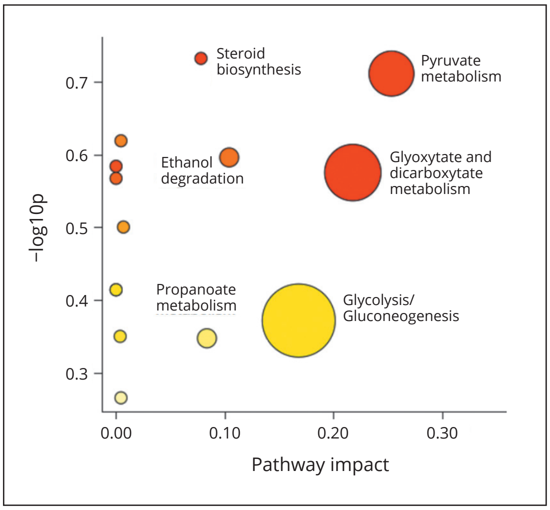

Figure 2.—Altered pathways identified with MetaboAnalyst software using metabolites responsible for the separation between COPD and pulmonary Langerhans cell histiocytosis (PLCH). Circles symbolize the metabolic pathways possibly altered in class discrimination. Labels indicate pathways with statistically significant dysregulation (impact and P value).[19, 20, 36]

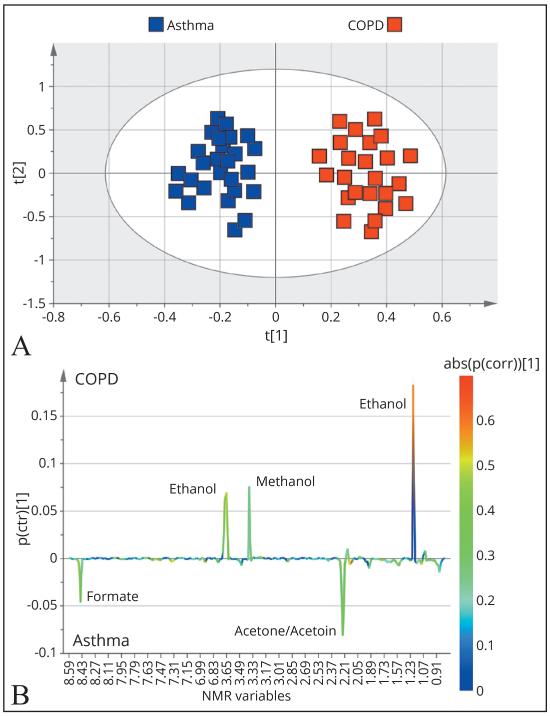

Figure 3.—Asthmatic *versus* COPD patients: orthogonal partial least squares discriminant analysis (OPLS-DA) model of EBC NMR data. A) Scores plot showing the degree of separation between asthmatic (blue squares) and COPD (red squares) patients. t[1] and t[2] along the axes represent the scores (the first 2 partial least-squares components) of the model. B) S-line plot between 8.6 and 0.5 ppm; positive signals indicate metabolites with an increased concentration in COPD patients, whereas negative signals refer to those with a decreased concentration in COPD patients with respect to asthmatic patients. The x-axis identifies NMR signals; the y-axis p(ctr)[1] indicates the loading value for each variable, whereas abs (p[corr])[1] refers to the absolute correlation value.[19, 20]

overlapping symptoms. Although a careful history evaluation about age of onset, persistence or periodicity of symptoms, social and occupational risk factors (particularly smoking exposure) and response to treatment can help defining the etiology of airflow obstruction, in the clinical practice it can often be very difficult, particularly in adult smokers, to achieve an accurate differential diagnosis without performing specific lung function tests. COPD and asthma differential diagnosis has important implications in both management and life expectancy. Because of overlapping symptoms, EBC metabolic content may be similar in both pathologies. How can they be distinguished? It should be kept in mind that, notwithstanding the metabolic overlap, it is the different relative variation among the metabolites that "labels" a pulmonary pathology by building a specific "molecular map" of the pathology. EBC profiling by NMR can be useful to differentiate COPD from asthma, as shown in the scores plot of Figure 3A.[19, 20, 38] It was found that in comparison with asthma, COPD presents increased concentrations of ethanol and methanol, and considerably reduced formate and acetone/acetoin levels (Figure 3B).[19, 20] A blind test using cohorts of patients not considered in the building of the main model (Figure 3A)[19, 20] estimated model consistency. Twelve out of 13 patients with asthma (92.3% accuracy) and 19 out of 20 COPD patients (95.0% accuracy, 5.0% false-positive results) were correctly identified, with a sensitivity (true positive rate) of 92.3%, and a specificity (true negative rate) of 95.0%. The probability that positivity corresponds to the actual presence of the disease ("positive predictive value") was 92.3%, while the probability that negativity actually indicates the absence of the disease ("negative predictive value") was 95.0%. Such results well agree with those reported for an electronic nose.[39] In addition, the EBC profiling together with the found metabotype discriminated asthma and COPD better than, for example, a panel of sputum cytokines.[40] Notwithstanding the common clinical features, asthma and COPD can be well separated by the metabolomics approach.[38] Similarly, application of unsupervised (*i.e.*, no prior group knowledge is used in the calculation) principal component analysis (PCA) to inflammatory cells (eosinophils or neutrophils) also separated COPD and asthma,[41] and the presence of an overlapping region in the model was related to the inclusion of patients with possible asthma and COPD overlap (ACO). ACO is a controversial clinical condition mainly present in older heavy-smoker asthmatic patients with irreversible airflow obstruction and suggested to display mixed characteristics of asthma and COPD.[42, 43] Non-smoking asthmatics with airway hyperresponsiveness (AHR) can be easily distinguished from smokers with COPD but without AHR. As compared with patients with asthma or COPD alone, patients with asthma and COPD present more recurrent and critical exacerbations, poor quality of life, augmented comorbidities, and a quick decline of lung functions.[43] Ghosh *et al*.[44] investigated the metabolic profiles of asthma-COPD patients with respect to asthma and COPD *via* NMR of EBC. ACO presented dysregulation of fatty acid, propionate, isopropanol, lactate, acetone, valine, methanol, and formate in comparison with only asthma or COPD. Multivariate receiver operating characteristic (ROC) curves obtained from the above EBC metabolites produced a strong classification model clearly differentiating ACO from asthma and COPD. ACO has also been studied using serum. Obstructive sleep apnea (OSA) syndrome affects about 10-20% of COPD patients, and because of similar comorbidities, their differentiation is often rather challenging. COPD was also investigated in comparison with OSA by applying NMR-based metabolomics to EBC, serum and urine.[45] The reported results indicated that serum or urine metabolites allow for a more reliable prediction with respect to EBC. The EBC-based model correctly identified OSA patients but presented a very low probability of a right identification of COPD. Consequently, a COPD patient has a high risk of being identified as OSA. A model combining EBC, and urine/serum metabolites again misclassified some COPD patients as OSA. This is quite surprising because a biological system should be better described by a combination of biofluids. One possible problem related to misclassification between COPD and OSA could be related to the different severity grade of the enrolled patients. Explicitly, a safe comparison should be between the A grade in COPD and mild OSA, avoiding OSA severe grade. That is, when pathologies present similar metabolic profiles, a strict classification of patients is required to build the initial model.[46] Hence, the poorer specificity of a model could be due to a different evolution of the patients: the profiles may be comparable at the pathology onset and evolve differently during pathology progression. Alternatively, it should also be considered the possibility that COPD and OSA share the same molecular phenotype and that the severity grade is irrelevant.

Blood plasma and serum

General aspects

Plasma and serum are useful biomatrices for a timely diagnosis of COPD and a successful discrimination of COPD phenotypes. Plasma and serum contain several molecules, namely, proteins, peptides, nutrients, electrolytes, organic wastes, and several other small organic molecules. A typical NMR spectrum is reported in Figure 1B. The main difference between plasma and serum involves compounds participating in the clotting process,[47] and both metabolic contents have been widely studied.[48] Several physical factors like dysfunctions of organs, body lesions, different pathophysiological states, and xenobiotic toxic exposure (principally cigarette smoke) can affect plasma and serum molecular compositions. This was observed in mice exposed to cigarette smoke with mild emphysema. With respect to unexposed mice, exposition induced alterations in amino acidic, purinic, lipidic, fatty acid and steroid concentrations. Furthermore, liquid chromatography/mass spectrometry (LC/MS) indicated that *ca.* 40% of the concentrations of the above metabolites remained altered even 2 months after smoke exposure ended.[49]

Applications

Despite plasma and serum variability, adding biomarkers to clinical data can ameliorate the prognosis. The prognostic models of death risk in COPD patients were improved by considering parameters like WBC counts, IL-6, CRP, IL-8, fibrinogen, CCL-18/PARC and SP-D together with clinical data presently evaluated for prediction of mortality [age, FEV_1, BODE (body mass, airflow obstruction, dyspnea and exercise capacity) index, and hospitalizations due to exacerbation].[50] A connection between serum profiling and obstruction, degree of emphysema, systemic inflammation and BMI was observed in the ECLIPSE COPD cohort study patients.[50] A study using both NMR and tandem MS reported that COPD patients with respect to control subjects showed reduction of lipoproteins, *N*,*N*-dimethylglycine, and augmented glutamine, phenylalanine, 3-methylhistidine and ketone bodies, with a parallel reduction of branched-chain amino acids (BCAAs) for GOLD stage IV patients. BCAAs and the corresponding degradation products, 3-methylhistidine, ketone bodies, and triglycerides were associated negatively with cachexia and positively with systemic inflammation. In addition, emphysemic patients presented a reduction of serum creatine, glycine and *N*,*N*-dimethylglycine.[50] A distinctive metabolomic profile was reported for the emphysematous COPD phenotype related to smoking. Investigation of plasma samples from 38 subjects with different phenotypes (healthy non-smokers, smokers, and emphysemic smokers) by Ultra High-Performance Liquid Chromatography (UHPL)/quadrupole-Time-of-Flight (Q-TOF) MS identified 3,534 discriminating metabolites, and smokers with emphysema were discriminated with high sensitivity and specificity from other classes by unsupervised and supervised clustering analysis.[51] The changes in phenotypes of high-resolution computed tomography (HRCT) COPD with phenotypes emphysema without (E) and with (M) bronchial wall thickening were investigated by NMR of sera in comparison with normal subjects, and with other COPD phenotypes of patients under pre- and postpharmacological tiotropium bromide therapy.[52] The E phenotype reacted to therapy better than the M phenotype showing increased pulmonary function and test scores for COPD assessment. Metabolic differences were observed in the comparison COPD *vs.* normal controls and treated COPD *vs.* untreated patients. Main alterations were detected for lactate, phenylalanine, fructose, glycine, asparagine, citric acid, pyruvic acid, proline, acetone, ornithine, lipid, pyridoxine, maltose, betaine, and lipoprotein. These metabolites participate in the metabolic pathways of amino acids, carbohydrates, lipids, genetic materials, and vitamin. With respect to normal controls, the E phenotype showed a decrease of asparagine and pyridoxine, with an increase of lactate, fructose, glycine, creatine, citric acid, pyruvate, proline, acetone, glutamine, proline, ornithine, lipids, 2-hydroxyisobutyrate, threonine, isopropyl alcohol, maltose, threonine, valine, glutamic acid, beta-alanine, cyclopentane, and 2-aminoisobutyric acid. The M phenotype presented a decline of ornithine, guanosine, and lipoproteins, with increased fructose, glycine, pyruvate, proline, acetone, lipids, threonine, isopropyl alcohol, betaine, and N-acetylcysteine (NAC). Compared to the M phenotype, in the E phenotype glutamine and alanine presented higher levels. Finally, in the serum of phenotypes E and M a different panel of metabolites was found upon treatment with tiotropium bromide.[52] The sera of 118 subjects including healthy smokers, COPD smokers and non-smokers were investigated by LC/MS global untargeted metabolomics, detecting some 1181 molecular ions in up to 95% of samples.[53] With respect to healthy smokers, in COPD smokers the concentrations of 23 biomarkers resulted altered and could be used to build a prediction model that was able to identify COPD patients with 87.8% sensitivity and 86.5% specificity. The panel comprised myoinositol, glycerophopshoinositol, fumarate, cysteinesulfonic acid, a modified form of fibrinogen peptide B (mFBP), and three other peptides that correlated statistically with mFBP concentrations.[53] Glycoprotein acetyls (GlycA), which can predict several chronic diseases, are also linked with COPD.[54] In an NMR study on plasma samples from 5557 subjects[55] increased concentrations of GlycA, 3-hydroxybutirate, HDL and acetoacetate were detected, and they were related with a high occurrence of COPD. However, by applying a correction threshold and adjusting for smoking, GlycA was the only biomarker statistically related to COPD, suggesting for it the role of a prodromal COPD indicator.[55] However, contrarily to what observed in a study involving 17,345 persons,[56] GlycA did not significantly correlate with mortality in COPD.[55] The NMR analysis of sera and urines from COPD patients and healthy controls found metabolic changes for both fluids.[57] In comparison with serum of healthy controls, COPD samples displayed decreased lipoproteins and amino acids, including BCAAs, and amplified levels of glycerolphosphocholine. In urine, in comparison with healthy controls, COPD presented lower concentrations of 1-methylnicotinamide, creatinine and lactate. On the contrary, acetate, ketone bodies, carnosine, m-hydroxyphenylacetate, phenylacetyglycine, pyruvate and α-ketoglutarate were amplified in COPD patients with respect to controls. Taken together, the above data put forward alterations in the metabolism of amino acids, suggesting augmented protein turnover and degradation in emphysema and cachexic patients.[58] An NMR investigation on sera from male Chinese Han patients[59] highlighted that in COPD, with respect to non-COPD-smokers, decreased levels of creatine, glycine, histidine, and threonine were detected. These variations inversely correlated with IL-6 levels, and therefore were linked to the COPD inflammatory phenotype. Furthermore, COPD also showed, in comparison with non-COPD-smokers, elevated levels of histamine levels percent basophils. The latter data suggested that COPD is characterized by dysregulation of the histidine-histamine and creatine metabolic pathways. Increased respiratory exacerbations in ever-smokers with and without COPD have been linked to reduced concentrations of amino acids.[60] Moreover, the levels of tryptophan could be inversely connected with BODE index, and a significant relationship was found between reduced tryptophan level and poorer lung function, % emphysema on chest CT and functional capacity (namely, ability to walk, stand, carry, push, pull and lift).[60] By using LC/MS, different COPD phenotypes (namely, airflow obstruction, emphysema, and exacerbation) were investigated to uncover metabolomics and transcriptomics pathways involved in COPD outcomes.[61] Plasma profiling of current and former smokers with or without COPD derived from the COPD Gene cohort indicated that reduced airflow obstruction and intensification of COPD exacerbations were linked to glycerophospholipid metabolism, while poor pulmonary function outcomes and exacerbations that need hospitalization could be connected to metabolism of sphingolipids. Likewise, arginine and proline metabolism could be associated with exacerbation severity and emphysema with oxidative phosphorylation. The uncovered molecular targets were indicated as possible prodromal biomarkers depicting the COPD pathology.[61] Dysregulation of histidine, ornithine, phenylalanine, and leucine discriminated COPD patients with dissimilar BODE stages from reference normal subjects. Reduced leucine and increased ornithine concentrations were observed for COPD patients. Additionally, COPD patients from BODE 1 to BODE 4 were characterized by a significant increase of histidine, ornithine, and phenylalanine levels.[62] Serum samples from subjects enrolled in the Atherosclerosis Risk in Communities study, and in the Cooperative Health Research in the Region of Augsburg study were analyzed using gas chromatography (GC)/MS and LC/MS-based metabolomics.[61] The authors investigated 368 metabolites related to FEV_1 (forced expiratory volume in 1s), FVC (forced vital capacity), their ratio (FEV_1/FVC) and COPD. Specifically, 95 markers were linked to FEV_1, and 100 to FVC (73 overlapping), showing negative correlation with BCAAs and positive correlation with glutamine. Ten markers related to FEV_1 and/or FVC, whereas 17 correlated with COPD. It was suggested that the above circulating metabolites may become biomarkers to control the COPD onset and its progression.[61] Muscle dysfunction in COPD patients were investigated by NMR plasma metabolomics before starting exercise training and after an 8-week cycle.[63] In comparison with healthy subjects, patients before training had reduced concentrations of valine, alanine, and isoleucine, while after the cycle COPD presented a substantial reduction of lactate. Reference subjects showed decreased levels of some amino acids (namely, glutamine, tyrosine, alanine, valine, and isoleucine), creatine, creatinine, citrate and glucose, whereas lactate, succinate and pyruvate concentrations augmented. It was inferred that dysregulated levels of amino acids in plasma in COPD at rest can be connected with fat free mass index (FFMI), but variations observed after trainings are not associated with FFMI or GOLD stages.[63] Survival of COPD patients can also be investigated by plasma metabolomics profiling. Plasma from patients with COPD who died on average 2 years after enrollment (non-survivors) was compared with those who survived and with age-matched controls by using LC/MS, LC/MS/MS, and GC/MS.[64] The results indicated that metabolomics prediction of the risk of death compares with that obtained from clinical parameters. The metabolites characterizing survivors were increased alpha-ketoglutarate, succinate, fumarate, malate, lactate, glycerate and fructose. Furthermore, alteration of pentose phosphate pathway and glyoxylate and dicarboxylate metabolism and glycerolipid pathway was also found. Taken together, the data suggested dysregulation in mitochondrial oxidative energy generation. With respect to survivors, the non-survivors showed an increased level of circulating polypeptides associated with Factor XII and fibrinogen cleavage, and des-Arg-9 bradykinin,[64] an active metabolite of the endogenous vasodilator bradykinin. Sphingolipids are reported to participate in COPD pathogenesis. Using MS, specific plasma phospholipids were identified by Bowler *et al*. in current and former smokers taken from the COPDGene cohort.[65] In particular, five sphingomyelins are specific for emphysema, while four trihexosylceramides and three dihexosylceramides were connected with COPD exacerbations. These data suggest that sphingomyelins characterize COPD emphysema and glycosphingolipids define COPD exacerbations phenotypes.[65] Differences in lipid profiles are present between COPD patients and healthy controls. By applying UHPLC coupled with Q-Exactive MS, Zhou *et al*. reported that 142 metabolites and 688 lipids were altered in the diverse COPD stages.[66] Specific lipids differentiated COPD from healthy controls and dissimilar stages within COPD patients. Specifically, compared to healthy controls, in COPD a reduction of lysophosphatidylcholine was observed, while phosphatidylethanolamines and ether-linked phosphatidylethanolamines increased. It was also reported that in exacerbation of COPD (ECOPD), glutamylphenylalanine and taurine were greatly upregulated in the control group and downregulated in ECOPD, supporting their role as main biomarkers expressed in the disease onset and evolution.[66] Patients with respiratory exacerbation necessitating noninvasive mechanical ventilation related to COPD (ECOPD), chronic heart failure, and pneumonia were compared with stable COPD by profiling serum samples *via* NMR.[67] With respect to stable COPD, glycine level was reduced in ECOPD, formate declined in respiratory failure patients, and histidine decreased in ECOPD and pneumonia. Likewise, citrate, glutamate, proline, and creatine phosphate decreased in ECOPD and pneumonia. The results indicated that serum (and urine) profiling could distinguish sable COPD patients from those with respiratory failure needing ventilation, chronic heart failure or pneumonia, but no specific biomarker was identified to univocally identify ECOPD. However, taken together, the data indicated that the levels of definite metabolites were dysregulated in hypermetabolic conditions like ECOPD and pneumonia.[67] Conversely, specific indications for classifying ECOPD were reported by profiling plasma samples from 33 patients at hospital admission (day 0) and 30 days later, which were compared with 65 matched controls.[68] By using MS, 377 metabolites were mapped, which discriminated ECOPD at day 0 and controls, and 31 separating ECOPD at 0 and 30 days. Moreover, a reduction of tryptophan concentration was detected at day 0 for ECOPD with respect to controls, pointing to an upregulation of indoleamine 2,3-dioxygenase activity. According to the findings, the authors determined that ECOPD patients present a typical metabolomic profile that includes diminished tryptophan levels originating from an upregulation of indoleamine 2,3-dioxygenase activity.[68] A stated above, metabolomics of serum/plasma has also been used to study the controversial asthma-COPD overlap. ACO patients seem to be characterized by increased metabolic and energetic functions.[69, 70] This would justify the poorer life quality, the faster lung function decline, the higher exacerbation frequency, the higher morbidity, and mortality in comparison with patients with either asthma or COPD.[71] In comparison with asthma and COPD sera, GC-MS analysis identified ACO dysregulation of serine, threonine, ethanolamine, glucose, cholesterol, 2-palmitoylglycerol, stearic acid, lactic acid, linoleic acid, D-mannose, and succinic acid. Furthermore, in ACO dysregulation also involved TNFα, IL-1β, IL-17E, GM-CSF, IL-18, NGAL, IL-5, IL-10, MCP-1, YKL-40, IFN-γ, IL-6 and TGF-β patterns. They all exhibited strict relationships with each other, and with clinical parameters of pulmonary functions. These data could shed some light on the nature of ACO, highlighting molecular parameters to be used for a differential diagnosis with respect to asthma and COPD.[72] NMR profiling of serum samples from moderate and severe asthma patients, moderate and severe COPD, ACO patients and healthy controls indicated dysregulation of lipids, isoleucine, N-acetylglycoproteins (NAG), valine, glutamate, citric acid, glucose, leucine, lysine, asparagine, phenylalanine, and histidine in ACO patients in comparison with asthma and COPD.[72, 73] This confirmed the increased energy and metabolic requirements for ACO with respect to asthma and COPD. Eicosanoids profiles, obtained with UHPLC-Q-TOF/MS,[74] were also used to discriminate ACO and COPD. In agreement with the fact that the basic levels of inflammatory parameters in ACO are higher than those for asthmatics and COPD, hydroxyeicosatetraenoic acids (HETEs) increased in ACO patients with respect to COPD, together with higher concentrations of hydroperoxyeicosatetraenoic (HPETEs) and hydroperoxyoctadecadienoic (HPODE) acids, which well correlated with FEV_1/FVC. It was concluded that HETEs, produced by lipoxygenase (LOX) from HPETEs, could be possible biomarkers for ACO diagnosis.

Conclusions

In conclusion, a complex chronic disease like COPD cannot be properly classified by using functional parameters alone. COPD can manifest different phenotypes showing different outcomes each induced by a specific metabolic perturbation. Currently, none of the available biomarkers, considered singularly, can represent the complex and multifaceted aspects of COPD, and it is not able to identify subjects exposed at risk of developing the disease and multiple endotypes/phenotypes, or to predict the severity or the evolution and the effects of drug treatment. Only panels of biomarkers can successfully describe homogeneous COPD subclasses related to endotypes/phenotypes that could favor personal and efficacious treatments (pharmacological and rehabilitative) of patients, therefore reducing the clinical costs relating to COPD managing. It can be foreseen that the multiple omics approaches, together with technological and computational advances, will simplify the clinical interpretation of COPD pathophysiology and give rise to patient-oriented therapeutic protocols.

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