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Analysis of exhaled breath fingerprints and volatile organic compounds in COPD

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Abstract

Background: Exhaled air contains many volatile organic compounds (VOCs) produced during human metabolic processes, in both healthy and pathological conditions. Analysis of breath allows studying the modifications of the profile of the exhaled VOCs due to different disease states, including chronic obstructive pulmonary disease (COPD). The early diagnosis of COPD is complicated and the identification of specific metabolic profiles of exhaled air may provide useful indication to better identify the disease. The aim of our study was to characterize the specific exhaled VOCs by means of the electronic nose and by solid phase micro-extraction associated to gas chromatography–mass spectrometry (SPME GC-MS).

Methods: Exhaled air was collected and measured in 34 subjects, 7 healthy and 27 former smokers affected by COPD (GOLD 1–4).

Results: The signals of the electronic nose sensors were higher in COPD patients with respect to controls, and allowed to accurately classify the studied subjects in healthy or COPD. GC-MS analysis identified 37 VOCs, nine of which were significantly correlated with COPD. In particular the concentration of two of these were positively correlated whereas seven were negatively correlated with COPD. The partial least squares discriminant analysis (PLS-DA) carried out with these nine VOCs produced a significant predictive model of disease.

Conclusions: This study shows that COPD patients exhibit qualitative and quantitative differences in the chemical compositions of exhale. These differences are detectable both by the GC-MS and the six-sensor e-nose. The use of electronic nose may represent a suitable, non-invasive diagnostic tool for characterization of COPD.

Keywords: COPD, Volatile organic compounds (VOCs), Electronic nose, Gas chromatography–mass spectrometry (GC-MS)

Background

Chronic obstructive pulmonary disease (COPD) is an inflammatory disease characterized by a mixture of small airways disease (obstructive bronchiolitis) and parenchymal destruction (emphysema) that causes flow limitation (http://www.goldcopd.org/uploads/users/files/GOLD_Report_2014_Jun11.pdf). A proper and early detection of COPD is essential to improve the prognosis of this progressive and debilitating disease, but although pulmonary functional tests (PFTs) are mandatory in the diagnosis of COPD, none of the derived parameters are so specific or sufficiently sensitive to detect the peripheral damage of the small airways or the different underlined mechanisms [1]. Moreover, it has been observed that the greater decline in lung function often occurs in the moderate stage of disease and an early intervention may reduce the progression of pathology [2, 3].

Volatile organic compounds (VOCs) are a wide class of small organic molecules that are volatile at ambient temperature. In exhaled breath, VOCs include several molecular families such as hydrocarbons, compounds containing nitrogen, oxygen or sulfur. Important components are the byproducts of the oxidation of phospholipids cytoplasmic DNA or membrane protein or can be the result of various pathophysiological processes or formed by bacteria [4, 5]. Under normal conditions, the

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exhaled air contains many VOCs that are both endogenous, produced during the body's metabolic processes, and exogenous, absorbed by the external environment. Exogenous compounds enter into the body through the skin, inhalation or ingestion and then are metabolized, modified by physiological processes such as the absorption in the epithelial lining fluid of airways, and by biochemical alteration such as oxidation processes and exhaled [4, 6]. A recent review indicates some 1840 VOCs in exhaled air of healthy subjects [7].

The presence of inflammation and oxidative stress alters the chemical composition of the exhaled air in various pathologies, both in the lungs and in other organs [4, 8]. In normal conditions, the lung has a very effective anti-oxidant defensive system, but in pathological condition such as COPD, the depletion of anti-oxidant defenses due to the presence of excessive oxidative load [9, 10] leads to a series of chain reactions resulting in uncontrolled tissue destruction [11].

The study of specific compounds associated with inflammation in COPD is an area of vital interest and deserves to be further investigated. COPD has been considered as a confounding comorbidity in lung cancer detection studies with electronic noses [12, 13] but a specific detection method based on the analysis of VOCs for COPD diagnosis is not yet developed. Since the inflammation in COPD is mainly localized at the level of small airways, the identification of inflammatory biomarkers in the exhaled breath may represent a suitable non-invasive procedure to facilitate an early diagnosis and a better characterization of this disease [2, 14].

Nowadays, there are various techniques to study the metabolic profile of exhaled air, and those that are most commonly used are the gas chromatography, the gold standard for air analysis, and the arrays of gas sensors usually called electronic noses [15].

Electronic noses are versatile instruments typically portable, based on arrays of partially selective gas sensors system. Electronic noses can typically detect a large spectrum of VOCs to provide a discrimination among samples classified according to their chemical composition [16]. Among a manifold of other applications, some electronic noses have been used to differentiate healthy subjects from patients affected by various diseases [15]. In combination with other "omics" platforms, the electronic nose could contribute to exploit the knowledge about biomarkers of lung inflammation, respiratory diseases and to characterize different phenotypes, providing important information to personalize drug treatment and facilitate the development of new drugs [17].

Electronic noses may provide information about the respiratory profile characterized by a set of various VOCs, but they do not provide specific information about the individual molecules. To identify the compounds present in the exhaled air it is necessary to use different techniques based on a detection mechanism able to separate the different compounds in the mixture. GC-MS is certainly the most widely used of these instruments, in particular complemented by solid phase micro-extraction techniques [18].

The main objective of this study was to validate the COPD detection capability of an electronic nose that is successfully used for the detection of lung cancer. The study has been complemented by the GC-MS analysis of the breath samples in order to identify some VOCs that could be specifically connected to COPD.

Methods

Study population

We assessed 7 healthy subjects (control population) and 27 outpatients with diagnosis of COPD (post-bronchodilator $FEV_1/FVC < 70$ %, GOLD stage 1–4) managed at Pulmonary Disease Outpatient Clinic of Tor Vergata Hospital, Rome. The control population was represented by healthy never-smoker subjects with normal respiratory function values, and negative history for respiratory disease, further significant diseases, or allergy. All COPD patients were former-smokers (abstinent for at least 6 months) with a smoke history ≥ 10 pack/year (P/Y), in a stability phase of the disease, as defined by ATS/ERS guidelines [19]. Active smokers, patients with a history of asthma, allergic rhinitis, atopy, a high eosinophil count, or a recent respiratory tract infection were excluded from the study. Patients in regular treatment with inhaled corticosteroids were required to discontinue the drugs for two-weeks before collecting the exhaled air. All the procedures have been performed according to Declaration of Helsinki (http://www.wma.net/en/30publications/10policies/b3/).

Pulmonary function tests

All the studied subjects performed pulmonary function tests (PFTs) such as spirometry by pneumotachograph and static pulmonary volumes measured by body pletismography. Every patient was under regular treatment with long-acting bronchodilators, therefore PFTs were performed after 24 h (therapy with long acting bronchodilators) or 48 h (therapy with once daily bronchodilators) since last inhalation of the drug. PFTs were performed with automated equipment (Master Screen Body PFT Jaeger, Würtzburg, Germany), according to current recommendations of the ATS/ERS Task Force on standardization of pulmonary function tests [20, 21].

All study participants were asked to abstain from food or drink for 2 h before collecting exhaled air, in order to reduce any factors that could interfere with the composition of exhalation profiles of VOCs. All samples were collected in the same two-hour time slot

(2–4 pm), in order to limit the circadian variation in the breath composition.

Exhaled air collection

A simple sampling protocol was developed to collect the first 500 ml of exhaled breath in a disposable Tedlar bag (SKC) [22]. Participants were asked to wear a nose clip, to inspire deeply to total lung capacity (TLC), to hold the breath for 5 s, and then to expire slowly and maximally. The collecting system was composed by a mouthpiece inside which the subject exhale, a T-valve and connectors in Teflon, that allows to direct the first portion of exhaled air (500 ml) inside the gas sampling bag. The sampling bag is filled until the resistance to further loading is sufficient to close the valve. The breath sampler is characterized by a low resistance and the breath can be easily collected even from people with reduced pulmonary functionality.

Analysis of exhaled air

The analysis of exhaled air was carried out by an electronic nose and GC-MS as previously described [12].

The electronic nose was the latest version of the instrument designed and developed by the Sensors Group at the Department of Electronic Engineering of the University of Rome Tor Vergata. It is composed by an array of quartz microbalances (QMB), each coated by a different metalloporphyrin. Each QMB is connected to an electronic oscillator. VOCs are reversibly adsorbed to the metalloporphyrin layers changing the resonant frequency of the QMBs. The shift of frequency between before and during the exposure to the sample is the sensor response. This electronic nose is applied to breath analysis for the diagnosis of respiratory diseases such as lung cancer [23] and asthma [24]. Six sensors were used in the experiments here described. The sensors were coated with the same 5,10,15,20-tetrakis-(4-alkyloxyphenyl) porphyrins but complexed with six different metals: cobalt, zinc, manganese chloride, iron, tin, and chromium.

Within 10 min of completing the collection of expired air, the sampling bag was connected to the electronic nose. To mitigate both environmental interferences and memory effects, the sensors were exposed to short time pulses of the sample according to a methodology described elsewhere [25].

The GC-MS used in this experiment was a Shimadzu QP 2010. The sample for the GC-MS analysis was obtained with the solid phase microextraction (SPME) technique. A DVB/CAR/PDMS (Divinylbenzene/Carboxen/Polydimethylsiloxane) 50/30 μg triphasic fiber from Supelco was used. The SPME was kept 2 h in contact with the sampled breath. The analytes adsorbed in the fiber were thermically desorbed in the injection port of the GC at an inlet temperature of 250 °C. The GC setup began with an initial oven temperature of 40 °C for 2 min. The temperature was then ramped at 10 °C/min until it reached 300 °C, and it was held at 300 °C for 2 min. Mass spectra was obtained by electron ionization, and the reconstructed chromatograms were acquired in the full scan mode in the mass range m/z 40–250. The identification of the peak was carried out with the NIST127 and NIST147 libraries.

Statistical analysis

Thirty-four subjects (27 COPD patients and 7 controls) were studied, for a total of 34 measurements with the electronic nose and 34 measurements with the GC-MS. If not different indicated, all the values are expressed as mean and 95 % interval confidence (IC).

The measurements collected with electronic nose were used to create a classification model by using partial least squares discriminant analysis (PLS-DA) algorithm aimed at identifying the breath of COPD affected individuals from the healthy control group. The PLS-DA model was validated by leave-one-out cross-validation (LOOCV) [26].

The correlation between the dependent variables (healthy or sick) and predictors (VOCs identified with the GC-MS) was indicated by the Pearson correlation coefficient r. For all the statistical analysis a P value < 0.05 was considered significant.

GraphPad Prism (CA, USA), SPSS (Chicago, IL, USA) and Matlab (MathWorks Inc. Natick, Massachusetts, USA) software were used for the statistical analysis.

Results

The measurements were carried out on a population composed by 7 healthy controls and 27 patients affected by COPD (GOLD obstruction stage 1–4). The anthropometric characteristics of the studied population are reported in Table 1, the average BMI of COPD subjects was 28.4 Kg/m^2 (26.2–30.5), just a patient had a BMI lower than 21. COPD population was formed by former smokers, with a mean smoke history of 41 (33–49) P/Y. The mean number of exacerbations during the previous year was 1.18 (0.84–1.53), and 8 out of the 27 patients were frequent exacerbators (≥2 exacerbations in the

Table 1 Anthropometric characteristics of studied subjects

	Healthy	COPD
Sex	F4 M3	F3 M24
Age (yrs)	27 (25–29)	72 (69–74)
Weight (Kg)	80 (71–89)	79 (72–86)
Height (cm)	173 (166–179)	167 (164–171)
BMI (Kg/m^2)	27.5 (23.6–31.4)	28.4 (26.2–30.5)

All the values are expressed as mean and 95 % confidence interval

previous year) (29.62 %). In Table 2 are listed the values of the respiratory function of the COPD patients.

Electronic nose

The magnitude of the frequency shift (Hz) of the signal of each sensor significantly correlated with the presence of COPD (Pearson correlation; sensor 1: 0.41, $P = 0.01$, sensor 2: 0.58, $P < 0.001$, sensor 3: 0.51, $P < 0.001$, sensor 4: 0.54; $P = 0.001$, sensor 5: 0.53, $P = 0.001$, sensor 6: 0.534, $P = 0.001$).

Figure 1 shows the average and the standard deviation of the sensors signals for healthy and COPD patients. Among COPD patients, the sensors signals do not correlate with FEV_1, RV and TLC.

A cross-validated PLS-DA model aimed at classifying the breath according to the COPD was calculated from the electronic nose data. The cross-validation error was minimized by a model with three latent variables. In Fig. 2 the first two latent variables are plotted to provide a simple visualization of the electronic nose capability to separate healthy and COPD group.

The cross validated model provided the correct classification of 26 of 27 COPD patients and 5 of 7 control subjects. Thus, the sensibility of the test resulted of 96 %, the specificity of 71 %, and negative predictive value (NPV) of 83 %, corresponding to 1 false negative, while the positive predictive value (PPV) was 93 %, corresponding to 2 false positives. The diagnostic accuracy of the test was 91 %. A similar classifier model aimed at separating the breath of frequent exacerbators (≥2 events) from infrequent exacerbators (≤1 event) did not provide satisfactory results.

Gas chromatography – mass spectrometry (GC-MS)

The analysis of the GC-MS data provided the identification of 37 VOCs from the chromatograms of the exhaled breath of the studied population. Overall, nine VOCs were significantly correlated with COPD: two of these were positively correlated with COPD (Pearson Correlation: 0.35 ± 0.01; $P < 0.05$), whereas seven VOCs were negatively correlated with COPD (Pearson Correlation: −0.43 ± 0.01; $P < 0.01$) Table 3. The compounds correlated with COPD are reported in Fig. 3. In COPD subjects, the decane and 6-ethyl-2-methyl-decane were found at larger abundance in the frequent exacerbators in comparison to infrequent exacerbators, although this variation is not statistically significant (Fig. 4). VOCs did not correlate with FEV1.

Table 2 Respiratory function values of COPD patients

	Healthy	COPD
FEV_1 %	98 (93–103)	55 (49–61)
FVC %	99 (96–103)	83 (77–88)
FEV_1/FVC %	75 (73–77)	51 (46–56)
RV %	103 (99–107)	149 (133–166)
TLC %	104 (99–108)	108 (101–116)

All the values are expressed as mean and 95 % confidence interval

Table 3 Volatile organic compounds (VOCs) positively and negatively correlated with COPD

Compounds negatively correlated with COPD	Compounds positively correlated with COPD
Benzene, 1,3,5-tri-tert-butyl-	Decane
Butylated hydroxytoluene	Decane, 6-ethyl-2-methyl-
Hexane, 3-ethyl-4-methyl-	
Hexyl ethylphosphonofluoridate	
Limonene	
1-Pentene, 2,4,4-trimethyl-	
2-Propanol	

Discussion

This study provides evidences that the differences in the breath of control and COPD groups are sufficiently different to be captured by the Tor Vergata electronic nose. Furthermore, the GC-MS identified 9 VOCs whose relationship with the COPD is worth of further investigations.

In this study two different groups were included: a clinically relevant group and an asymptomatic one. According to the current guidelines for evaluating diagnostic accuracy, the first step in the assessment of a novel test has to evaluate the discriminative ability between a priori defined, gold standard diseased and non diseased subjects [27–29]. As can be seen from Table 1, controls and COPD subjects differs in age and smoking history. Although this difference does not allow extending our results to a general population of smokers and non-smokers of a similar age, definitely it gives two clear indications for further analysis.

Our findings are consistent with the results obtained by Incalzi et al. although the different e-noses used (a six-

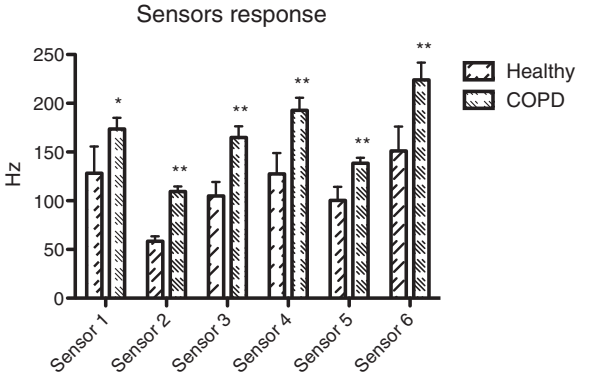

Fig. 1 Sensors response in healthy and COPD patients. Values are represented as mean and 95 % IC. *$p. < 0.05$; **$p. < 0.01$

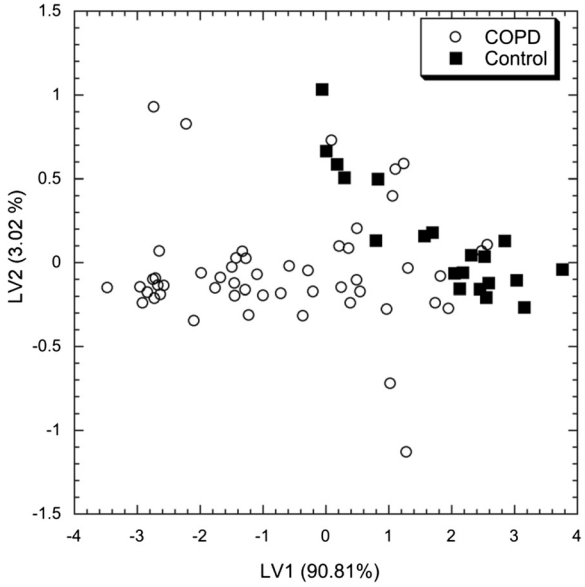

Fig. 2 Plot of the first two latent variables (LV) of the PLS-DA classifier. Each point is one measured sample. Even if the complete cross-validated model contains 3 latent variables the plot of the first two variables provides a simple visualization of the electronic nose capability to separate healthy and COPD group. LV = latent variable

sensor system in our study instead of the seven-sensor system used by the other Authors) [30]. In both study the VOC patterns were different between healthy and COPD subjects. The authors have showed that VOC pattern was highly reproducible in healthy subjects and in more severe hypoxemic COPD population (GOLD IV), but less in mild – moderate COPD patients. They explained this different reproducibility to a greater variability of the less severe COPD population in comparison to severe COPD [30]. In this study the reproducibility of the e-nose was not evaluated, but differently from the Incalzi RA et al., we have integrated the VOC pattern with the analysis of exhaled gases. With both methods (e-nose and GC-MS) was possible to differentiate health subjects from COPD. Moreover, the difference VOCs concentration between populations as detected by the GC-SM is the possible cause of a different VOCs pattern found with the e-nose in COPD subjects.

In this study GC-MS allowed to identify 7 compounds negatively correlated with COPD and 2 positively correlated with this disease. Decane and the 6-ethyl-2-methyl decane, the compounds that positively correlated with COPD, are alkanes that belong to the broader class of hydrocarbons. In previous studies, the analysis of exhaled air with the GC-MS has identified increased levels of decane [31] and its derivatives [32] in subjects with

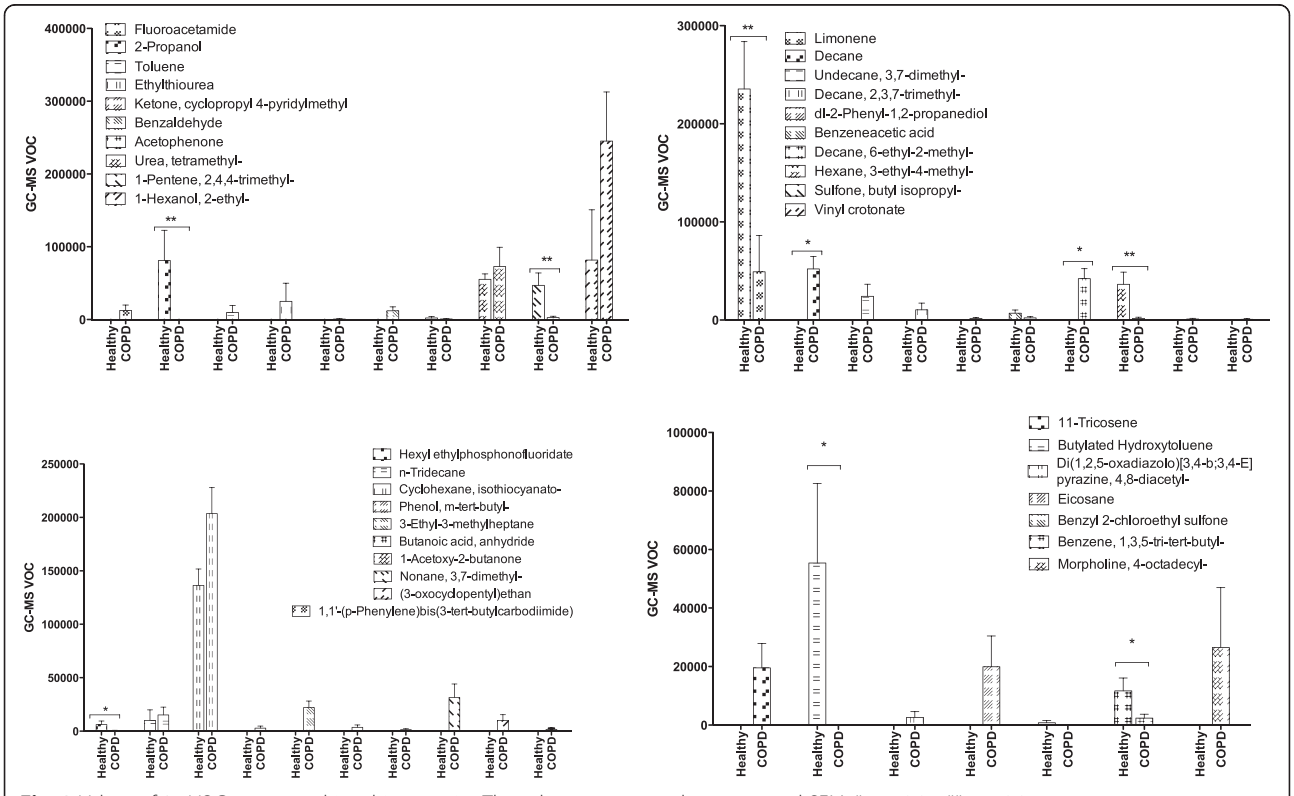

Fig. 3 Values of 37 VOCs, measured in arbitrary units. The values are reported as mean and SEM. *$p. < 0.05$; **$p. < 0.01$

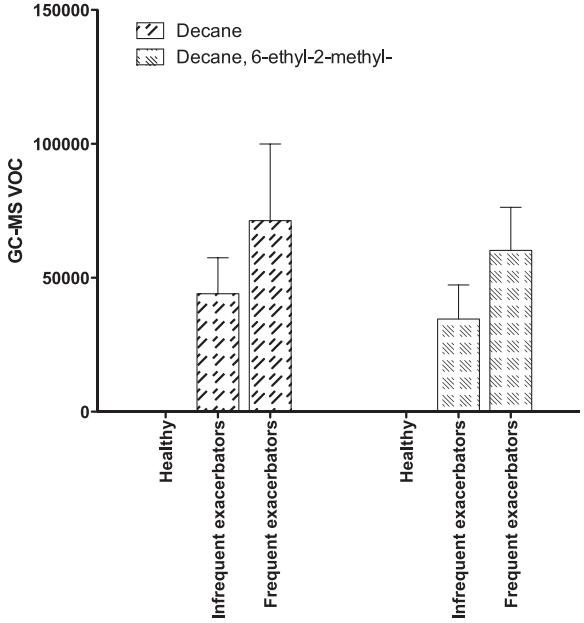

Fig. 4 Decane and decane, 6-ethyl-2-methyl- values in healthy, infrequent exacerbators and frequent exacerbators

COPD [33] and lung cancer [34], compared with healthy subjects. In our patients, the levels of decane and 6-ethyl-2-methyl decane were increased in frequent exacerbators COPD [35, 36] compared to non-frequent exacerbators, and in the latter group compared to healthy subjects. However, these increases were not statistically significant, likely because of the small number of the enrolled patients, especially in the group of frequent exacerbators.

Seven compounds were significantly increased in healthy: limonene; butylated hydroxytoluene (BHT); 2-propanol; benzene, 1,3,5-tri-tert-butyl-; hexane, 3-ethyl-4-methyl-; hexyl ethylphosphonofluoridate; and 1-pentene, 2,4,4-trimethyl-.

Limonene is a cyclic monoterpene. It is an essential oil that is mostly found in citrus fruits and in high concentrations in fruit juices and is also used as a food additive, and in a variety of products for personal care such as perfume and creams [37]. This compound is frequently detected in exhaled air of healthy subjects, [38] and increased levels have been detected in exhaled breath of patients with chronic liver disease [37]. Food is indicated as a possible source of limonene in the airways [37]. Since limonene is an antioxidant, its low levels found in patients with COPD could be explained by its consumption in presence of oxidative stress. However, a previous work did not report statistically significant differences between the levels of limonene in exhaled breath of healthy subjects and patients with cystic fibrosis [39].

BHT is an alkylated phenol and an exogenous antioxidant. An *in vitro* study has shown that both BHT and N-acetylcysteine attenuated the secretion of tumor necrosis factor-α (TNF-α) and mRNA expression of TNF-α in the lungs of lung transplant recipients [40]. BHT is an exogenous antioxidant and for its properties is used as a food additive in the cosmetics industry, in the pharmaceutical industry [41], in petroleum products, in the rubber and plastics industry, and is also detected in the air of indoor environments [42, 43]. Since it is not endogenously produced, it is a "contaminant" of the airways. We detected BHT in the airways of healthy subjects and it was absent in all patients with COPD. This finding may indicate that the high levels of oxidative stress present in COPD patients have resulted in a depletion of BHT or rather its catabolization into another compound. In literature, the only work in which was measured BHT regards the determination in subjects suffering from lung cancer, but it was not reported if the difference with healthy was significant [44]. As for limonene, the levels of exhaled BHT could be influenced by nutritional habits.

The 2-propanol (or isopropyl alcohol) is widely used in industrial and consumer products. It is used as a disinfectant, as a solvent in the manufacture of products for hair and skin, such as antifreeze agent in the carburetors, and it is present in the windshield wipers of cars and in cleaners for contact lenses. The main metabolite of 2-propanol is acetone that is produced through the oxidation process by liver alcohol dehydrogenase (ADH). Acetone is removed from the body through the kidneys and the exhaled air. Acetone can be further metabolized to acetate, formate, and finally to carbon dioxide [45]. In a study 2-propanol was detected in the breath of all 39 healthy subjects evaluated [38]. Increased levels of 2-propanol, respect to healthy were reported in lung cancer [46] and in liver disease [47]. Since the 2-propanol can be oxidized with the formation of acetone, there is a close relationship between the levels of the two compounds in exhaled air [48]. The GC-MS method here adopted did not allow for the detection of acetone and therefore it is not possible to ascertain whether a greater amount of 2-propanol was converted into acetone in COPD subjects.

To the best of our knowledge, no data are reported in the literature concerning the other four compounds whose abundance we found larger in healthy subjects: benzene, 1,3,5-tri-tert-butyl-; hexane, 3-ethyl-4-methyl-; hexyl ethylphosphonofluoridate; and 1-pentene, 2,4,4-trimethyl-.

Further researches are needed to clarify the metabolic pathways of formation and the role played by these VOCs. However, although the mechanisms of generation and elimination of some VOCs are not yet fully understood, and none of the individual compounds was specific for COPD, the overall analysis of VOCs adds a valuable contribution to diagnose and characterize the disease.

A limit of our study is represented by the difference in age between the two studied populations, since the healthy subjects were younger than COPD patients. However, it has been shown that VOCs pattern in exhaled air is not different between healthy young and healthy elderly [49]. However, we cannot exclude the possibility that age-related differences may alter the profile of the exhaled air in presence of a disease [50]. Although this study found a peculiar fingerprint in COPD in comparison to healthy subjects and some VOCs were correlated to the disease, more studies and a larger population are needed to translate these results into clinical practice. Moreover, subjects were restrained for food, but it was not standardised the carbohydrate intake. Another limit of our study could be that the analysis of VOCs has been done from the first 500 ml of exhaled air. Normally, the first 125–150 ml of exhaled air derive from dead anatomical space. The next volumes of air derive from peripheral airways and alveolar compartments. Our objective was to analyze the air from small airways and alveoli, therefore from the site of inflammation in COPD. More laborious methods to collect exhaled air might include the elimination of the breath portion in contact with upper airways, while in this paper we preferred to use a more simple and reproducible method.

Conclusions

This study indicates that COPD patients exhibit qualitative and quantitative differences in the chemical compositions of exhale. These differences are detectable both by the GC-MS and the six-sensors e-nose. The use of electronic nose may represent a suitable, non-invasive diagnostic tool for characterization of COPD, although further studies are needed.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors played a role in the content and writing of the manuscript. In addition: MC, PR, JO e CC conceived of the study, and participated in its design and coordination and helped to draft the manuscript, AS, RC e AB had input into the study design and conduct of study; EM e LC participated in the design of the study and performed the statistical analysis, RP, CDN e AD collected the data; performed data analysis and prepared it for presentation. All authors read and approved the final manuscript.

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