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ANALYSIS OF VOLATILE ORGANIC COMPOUNDS (VOCs): AN INNOVATIVE APPROACH TO DISEASE CHARACTERIZATION IN ELDERLY PATIENTS

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CHAPTER 1 GENERAL INTRODUCTION

Non communicable disease (NCD) is an umbrella term which refers to all those diseases of non-infective origin usually characterized by long duration and generally slow progression. They account for about the 63% of the global death, 80% of which can be referred to one of the four hereby clusters: cardiovascular diseases, cancers, chronic pulmonary diseases and diabetes [1]. Even potentially affecting every age of life, NCDs are more prevalent in elderly people [2], because aging associates with increased enthropy at the cellular, organ, and system level, improving the detrimental effect of external and internal stressors (e.g. smoking) [3], and usually co-occur, the so called multimorbidity [4], affecting the functional status, quality of life, and survival much more than single diseases do [5,6]. Therefore, these chronic diseases associate with high costs that are mainly the results of repeated hospital admissions. The development of new, safe, non-invasive, repeatable and reproducible technologies, also performable at home by not experienced physicians, are warranted for manifold reasons:

- More timely and correct diagnosis: it is estimated that about 75% of chronic obstructive pulmonary disease (COPD) patients are not diagnosed [7] and causes are manifold: patients are unable to perform an acceptable and repeatable spirometry [8], trained technicians are not widely available, procedure is time-consuming [7], scarce awareness of the prevalence among general practitioners [7]. Similarly, chronic heart failure (CHF) is underdiagnosed because sometimes the presentation is atypical, especially in older subjects [9], or because the typical symptoms are not specific of the disease and can be ascribed to other diseases.
- More accurate disease severity stratification: severity stratification is an issue still unsolved in COPD. Indeed, the classification has changed repeatedly in the last seven years, moving from an algorithm based on the forced expiratory volume in the first second (FEV1) taken alone to a composite algorithm integrating FEV1 with dyspnea severity and exacerbation occurrence. In CHF New York Heart Association (NYHA)

assessment is the most widely used, but it scarcely reliable [10] and is mostly based on dyspnea severity. The improvement in disease severity stratification will help to tailor the maintenance therapy and to timely refer patients to palliative care programs or to nursing home, with consequent benefits for patients, who receive a more tailored management, and for health care, which allocates more precisely its resources minimizing wastes.

- More accurate prediction of patients' prognosis: both in COPD and CHF prediction of mortality and disability is still inaccurate [11,12]. More efforts to identify new predictors are warranted. Indeed, an accurate prognosis will help to integrate the disease oriented-care with palliative care, relieving symptom burden and improving quality of life especially when treatment is almost ineffective in prolonging survival.

CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Chronic obstructive pulmonary disease (COPD) is a common, preventable and treatable disease characterized by persistent respiratory symptoms and airflow limitation due to airway and/or alveolar abnormalities usually caused by significant exposure to noxious particles or gases [13]. Therefore, a diagnosis of COPD can be established when a subject with risk factors (e.g. smoking) and respiratory symptoms (e.g. chronic dyspnea or cough) present a persistent airflow limitation, defined as a post-bronchodilator FEV1/forced volume capacity (FVC) ratio lower than 0.7 [13]. Spirometry is therefore essential in the diagnosis, but is still underused in subjects at risk to have the disease, either because not prescribed in asymptomatic or poorly symptomatic patients [14] or because of the need of trained technicians [8].Globally, the prevalence of COPD ranges from 9% to 15%, with people older than 60 years old being three times more likely to receive the diagnosis [15], and a prevalence that ranges from 19% to 46%

in subjects aged 70 or more [16]. It accounts for about three million deaths per year worldwide [17]. The disease severity assessment has been recently updated and is based on the integration between spirometry and dyspnea and exacerbation assessment [13]; however it is still scarcely able to predict mortality or other health outcomes (e.g. hospitalization) [11], with consequent ineffective management of patients and high costs for the health care system. Indeed, COPD is complex and it is characterized by a wide pulmonary and extra-pulmonary heterogeneity, not yet fully understood. The persistent airflow limitation might associate with an eosinophilic airways inflammation [18], bronchiectasis, systemic inflammation, body composition abnormalities [19], and the different combination produces different phenotypes of disease, much more than those described by Filley and colleagues [20], which require individualized treatment to maximize the efficacy and minimize side-effects and costs.

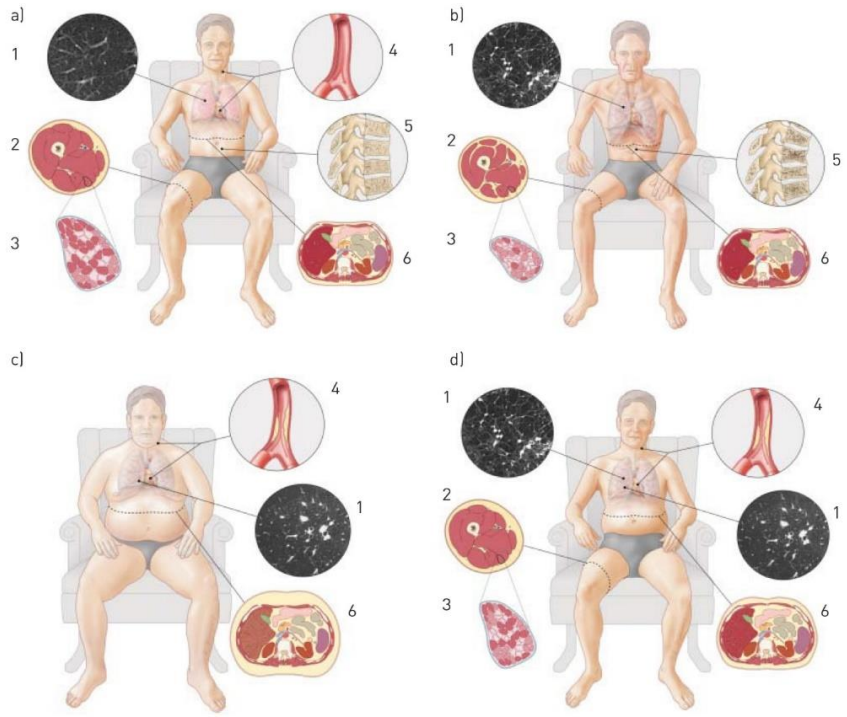

Figure 1 Metabolic phenotypes of COPD and common comorbidities (modified from A.Schols et al., Eur Resp J, 2014).

Since COPD is characterized by a narrowing of peripheral airways and lung parenchyma destruction (emphysema), driven by systemic and local inflammation, with an increased number of macrophages, neutrophils and T-lymphocytes, mainly CD8+ than CD4+ (helper) [21], by oxidative stress [22], inhibiting the elastase inactivators, and by body composition abnormalities [19], it is possible to suppose that breath analysis might represent a valuable tool in the diagnosis, phenotype-definition and prognosis evaluation of COPD.

CONGESTIVE HEART FAILURE

Congestive heart failure (CHF) is a clinical syndrome characterized by symptoms (e.g. breathlessness, ankle swelling and fatigue) and signs (e.g. elevated jugular venous pressure, pulmonary crackles and peripheral oedema) caused by a structural and/or functional cardiac abnormality, resulting in a reduced cardiac output and/ or elevated intracardiac pressures at rest or during stress [23]. Similarly to COPD, CHF prevalence raises with the increasing of age, affecting almost the 10% of subjects aged 75 or more [24], and no incidence reduction has occurred in the last decades because of the ageing of the population, the longer survival of heart failure patients, and the improvements in the treatment of acute coronary syndromes [25]. The diagnosis of HF is confirmed by structural or functional abnormalities at echocardiography, and the patients to refer are those who present a clinical history (e.g. history of coronary artery disease) and physical examination (e.g. bilateral ankle edema) compatible with HF, ECG abnormalities and brain natriuretic peptide (BNP) $\geq$ 35 pg/mL or N-terminal BNP $\geq$ 125 pg/mL [23]. Although useful in young subjects, the algorithm is less effective in elderly people. Indeed, their symptomatic presentation is usually atypical and misleading, with symptoms like confusion or loss of appetite that are rare in young patients affected by CHF [26]. Moreover, natriuretic peptide level is frequently over the fixed threshold in healthy elderly individuals, because blood natriuretic peptide level is affected by several factors, for instance patients' age [27]. Thereby, in clinical practice age-corrected levels of natriuretic peptides should be used instead of fixed values to address this issue [28]. The different caveats have prompted the research to find new biomarkers, but none of the molecules identified in the last decades, such as midregional proadrenomedullin [29], soluble ST2, galectin-3 [30], neutrophil gelatinase-associated lipocalin [31] or chromogranin A [32,33], have yet overcome the limitations of natriuretic peptides in elderly people.

Heart beats about 100,000 times and cycles about 6 Kg of ATP per day, a huge amount of energy obtained by burning fatty acids and glucose [34]. In heart failure heart cells are characterized by decreased energy production from fatty acids and glucose, energy transfer and energy utilization, resulting in abnormal contraction and relaxation, but also decreased expression of mitochondrial transcription factors and mitochondrial proteins in skeletal muscles, ending in a reduced production of ATP [35]. Obtaining a lower amount of ATP per molecule of fatty acid or glucose, the myocardium becomes more demanding of substrates, partially satisfied by the increased lipolysis induced by β-adrenergic stimulation. Furthermore, β-adrenergic stimulation promotes hepatic gluconeogenesis, which increases the levels of acetyl-CoA, that is converted partially in glucose and partially in ketone bodies [36]. This metabolic disarray produces volatile compounds like acetone that are cleared by the lung, therefore exhaled breath analysis might play a role in the diagnosis, but also disease severity stratification and prognosis of patients affected by HF.

VOLATILE ORGANIC COMPOUNDS

Exhaled breath is composed for about 78% by nitrogen, which is inhaled and exhaled without diffusion, 16% by oxygen and 4% by carbon dioxide, one-hundred times the concentration of the inhaled air [37]. However, human breath contains thousands of other molecules of different size, chemical structure and concentration that can potentially carry information on the physiologic and pathologic processes related to respiratory and non-respiratory diseases. These molecules, known as volatile organic compounds (VOCs), are very heterogeneous in nature, but essentially they can be exogenous compounds (inhaled or derived from enteric or skin absorption) and endogenous compounds (derived from cellular metabolism, not limited to lungs – see *Figure 2* –, and, as the most recent evidence suggests, from lung and intestinal microbiomes [38,39]). They present a really low concentration in the exhaled air, with some compounds like acetone, methan and carbon monoxide having concentration of part per million by volume (ppmv), and others like formaldehyde, acetaldehyde, isoprene, pentane, and several hydrocarbons having concentration of part per billion by volume (ppbv) or lower [40], and their combination define the smell of exhaled breath and can be used to achieve information about the state of cellular metabolism. Therefore, clinical application of breath analysis is really promising, even more considering that it is completely safe, non-invasive, repeatable, reproducible, and, from the last decades, also real-time performable.

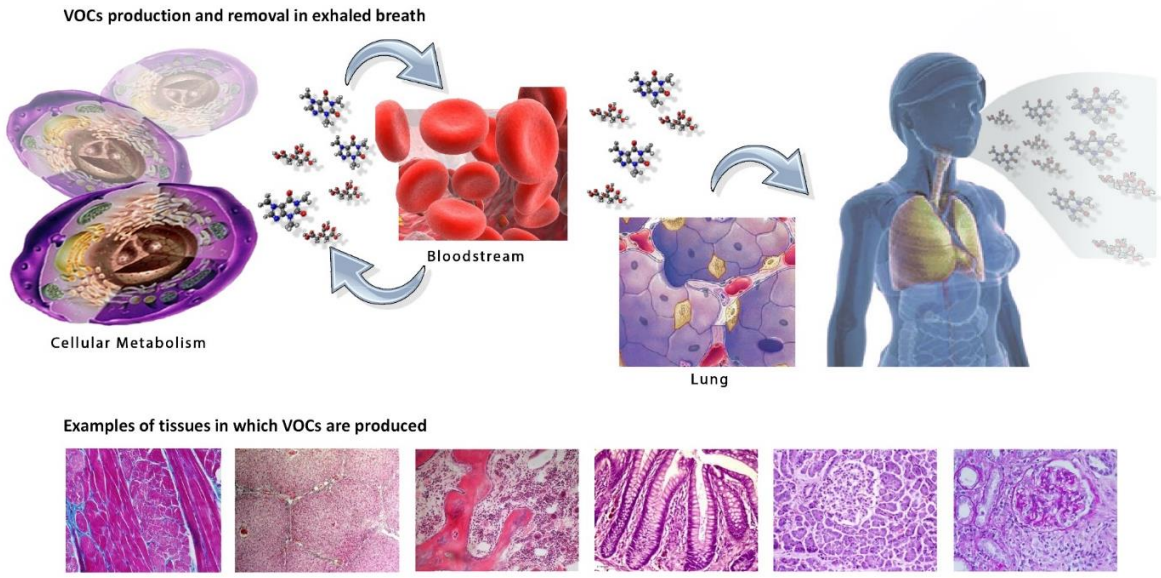

Figure 2 Chain of volatile organic compound (VOC) production and release in the exhaled breath.

In recent years, the interest shown in the clinical application of VOCs has rapidly increased and has fostered the development and validation of exhaled breath sampling methods, devices, and data analysis algorithms for obtaining information from exhaled breath molecules.

VOC COLLECTION

Breath is usually collected and stored before being analyzed, albeit the existence of real time analyzers of exhaled breath. This approach ensures the technology is compact and transportable, being VOC analyzers often bulky and susceptible to mechanical stressors. Commonly used devices are:

1. Collection bags (e.g. Tedlar®, Mylar®): are made of inert material, like polyvinyl fluoride, are inexpensive and efficient. In order to exclude exogenous VOC collection, bags are connected to a non-rebreathing valve with an inspiratory VOC filter. The patient breaths into the mouthpiece connected to the non-rebreathing valve and the

exhaled air fills the bag. To maintain a good temperature, even with a shift in ambient air pressure, and to avoid the bag bursting, it should not be filled until the end, but just the half. Furthermore, collection bags should be empty as soon as possible, and care should be taken during delivery.

2. BIO-VOC™ [41]: is a tube/syringe made of non-emitting plastic and allow the collection of end-tidal air, without contamination or dilution with breath from the bronchial tubes or mouth. The patient breathes through a disposable cardboard mouthpiece into the sampler, which has an open end allowing air to be displaced as exhalation proceeds allowing the collection of the alveolar air. When the collection is complete (100 ml of air), the breath is discharged into a sorbent tube (or a direct read-out instrument) using a screw-in plunger.

Both the above mentioned techniques do not allow to pre-concentrate samples (a limitation potentially affecting the reproducibility of the method) and should be disposable, or at least carefully cleaned when re-used.

3. Other devices (e.g. ReCIVA® [42], Pneumopipe®) allow to collect the exhaled air into cartridges made of adsorbent material (e.g. Breath Biopsy Cartridges, Tenax GR®), which enable adequate pre-concentrations and ensure longer storage of the compounds (especially if frozen), but are prompt to a selective VOC adsorption.

Given these well-known limitations, none of such techniques can be considered the gold standard, yet.

VOC ANALYSIS

At the current state of the art, the main available approaches for VOC analysis after collection are:

1) Analytical techniques (e.g. gas chromatography and mass spectrometry): they can qualitatively and quantitatively identify VOCs present in the mixture. Below a list of the commonly used analytical techniques with their principal characteristics:

Name	Type of technique	Strength/limitation
Selected Ion Flow Tube Mass Spectrometry (SIFT-MS)	Analytical technique	*Strengths*: Real-time assessment; LOD: pptv; high sensitivity and specificity. *Limitations*: expensive; not portable.
Proton Transfer Reaction Mass Spectrometry (PTR-MS)	Analytical technique	*Strengths*: Real-time assessment; LOD: pptv; high sensitivity and specificity. *Limitations*: expensive; not portable.
Gas Chromatography–Mass Spectrometry (GC-MS)	Analytical technique	*Strengths*: LOD: pptv; sensitivity and specificity higher than SIFT-MS and PTR-MS. *Limitations*: Off-line analysis (it requires a pre-concentration and chromatographic separation). Therefore it is relatively slow, has high maintenance requirements, and must be operated by skilled personnel. Not portable.
Ion-mobility Spectrometry (IMS)	Analytical technique	*Strengths*: Real-time analysis; LOD: ppbv; possible association with other techniques to improve discriminative ability. *Limitations*: Lower sensitivity and specificity than GC-MS.

Abbreviations: LOD: limit of detection; ppmv: part per million by volume; ppbv: part per billion by volume; pptv: part per trillion by volume; VOCs: volatile organic compounds.

2) Semi-analytical approaches using electronic noses, which are instruments composed by an array of electronic chemical sensors and an appropriate pattern recognition system [43]. In this case, after breath collection, air is desorbed into the e-nose device, while chemical sensors, variously interacting with VOCs in a way closely dependent on VOC nature and concentration, consequently develop a frequency shift that can be displayed as a sort of graphical print. This, although unable to identify every single component in the breath, provides a comprehensive and often peculiar VOC pattern commonly named 'breath-print' or 'breath-fingerprint'.

The chain of collection and analysis is illustrated in *Figure 3*. Each technique has its strengths and limitations, and the need for method validation, reproducibility and procedure standardization is ever present. Nonetheless, the potential of this approach is so highly perceived that the European Respiratory Society has recently published a consensus paper defining available evidence and paving the way for future research [44].

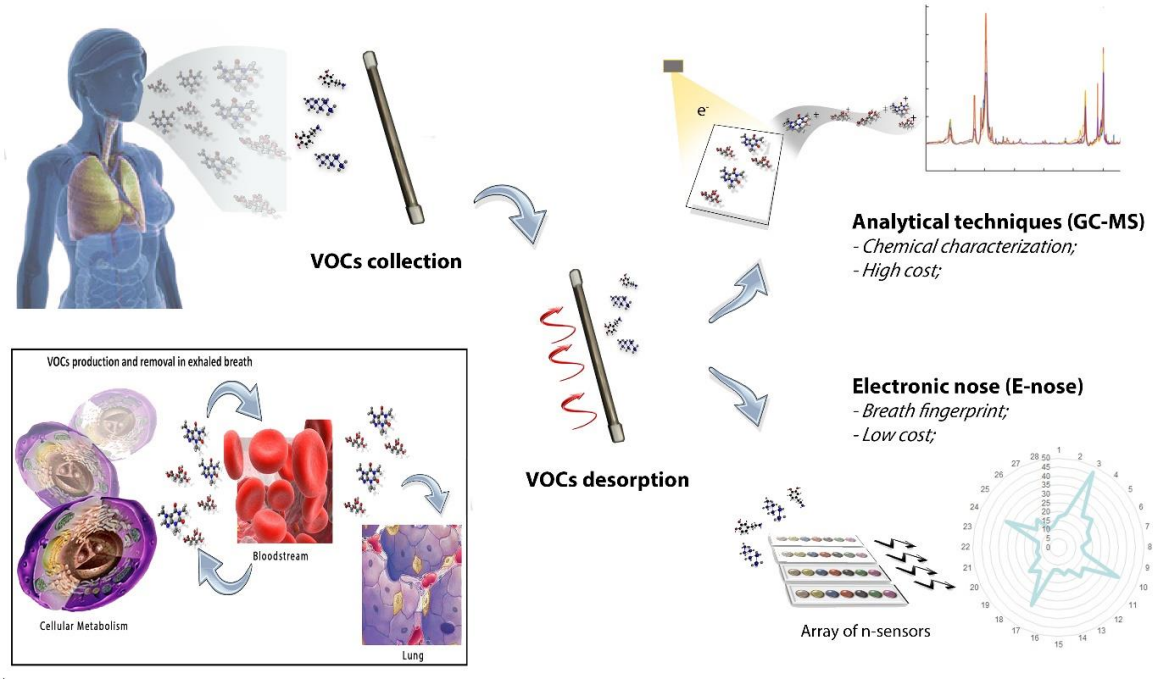

Figure 3 Volatile organic compounds (VOC) collection and types of analysis

FOCUS ON PNEUMOPIPE® AND BIONOTE

The Pneumopipe® (European patent n. 12425057.2, Rome- Italy) is a pipe-shaped device designed by the department of engineering of Campus Bio-Medico University to sample in a non-invasive and inexpensive way the exhaled breath into an adsorbent cartridge [45]. The instrument is built using polyoxymethylene (POM), a stiff, wet-resistant and inert material, which can be sterilized and re-used reducing the cost of the single measurement. It is made up by four components:

- Bottom part (A): it contains the two rebreathing valves that split inhaled from exhaled flow.
- Connection tube (B): it connects the bottom part of the Pneumopipe® with the disposable mouthpiece in which the patient breathes.
- Collection chamber (C): it contains patients' exhaled air and the adsorbent cartridge.
- Lid (D): it contains a no re-breathing valve that allows a flow from the cylinder to outside but not backwards.

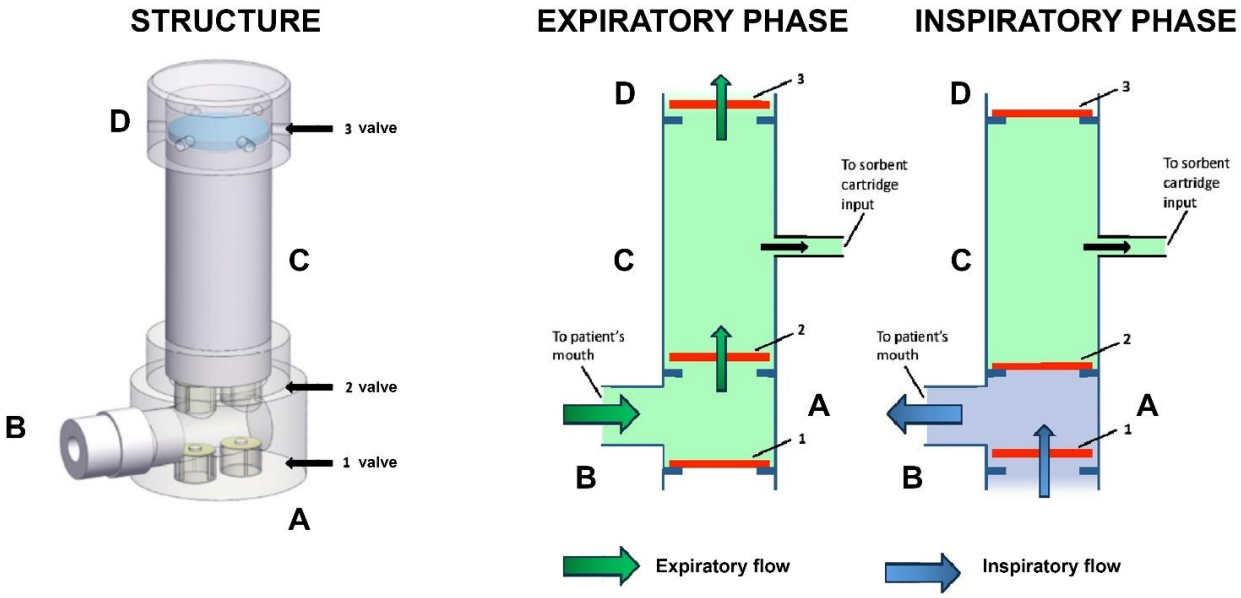

Figura 4 Pneumopipe structure and functioning. Adapted from Sensors and Actuators B 204 (2014) 578–587.

Once the Pneumopipe® has been assembled, the technician connects the patient to the device through a disposable mouthpiece and asks him/her to breathe at his/her tidal volume for three minutes. When patient inhales, valve number 1 opens, but valve number 2 and 3 normally close, thus environmental air passes through the bottom part (A) and the connection tube (B) without reaching the collection chamber (C). When patient exhales, expiratory flow closes valve number 1 and opens valve number 2, filling the collection chamber (C); the increased pressure opens valve number 3 and the exhaled air flows into the environment through the lid

(D). Thanks to its double-chamber structure (see *Figure 4*), the Pneumopipe® permits a continuous sampling of exhaled air, even during an inhalation step. When the patient starts to breath, the technician switches on the pump, which is connected via an inert tube to the adsorbent cartridge, guaranteeing a constant flow of 80 ml/min of patient exhaled air from the Pneumopipe® collection chamber (C) to the cartridge for the three minutes of the exam. After three minutes the pump automatically turns off and the patient is de-connected. No technical skills are required to perform the analysis and the device is susceptible of a portable and compact embodiment, rather useful in clinical practice. The exhalation and inhalation resistance perceived by the patient during the exam is minimal, guaranteeing a comfortable procedure, also suitable for subjects with breathing difficulties.

In this thesis the adsorbent cartridge used to pre-concentrate, preserve and transport the sample was the Tenax GR®. Adsorbent cartridges are thin steel tubes containing a resin able to capture VOCs that pass through. The diameter is around 3-4 mm, while the length is about 10-20 cm. The functioning of the cartridges is based on the concept of *breakthrough volume*, which is the volume of vector gas per unit of resin needed to allow the complete passage of the molecules of one chemical species throughout the entire length of the tube. It is measured in liters per gram (l/g) and is inversely proportional with the environmental temperature. Indeed, during the exam, the low environmental temperature increases the *breakthrough volume*, therefore the gas volume required to allow the complete passage of the compounds is so much to be unfeasible, and this allows the capture of VOCs in the cartridge, preventing their exit. Conversely, heating the cartridge, the *breakthrough volume* reduces, and thus low volumes of vector gas allow the release of the compounds for the analysis. The heating procedure is defined "thermal desorption" and is preparatory for the analysis with the electronic nose. In our study the thermal desorption was carried out using the Breath-stiller device, a muffle-furnace chamber with a hollow cylinder perfectly fitting the Tenax GR® cartridge, in order to grant a uniform and

efficient heating. Cartridges were evenly heated up to four different temperatures: 50°C, 100°C, 150°C and 200°C. Different compounds are released at each different temperature due to their different *retention time* (rt) and analyzed by the electronic nose. *Figure 5* summarizes the compounds released per single temperature. Finally, the cartridge is heated until 300°C and, after five minutes, all the residual compounds are released and the cartridge can be re-used.

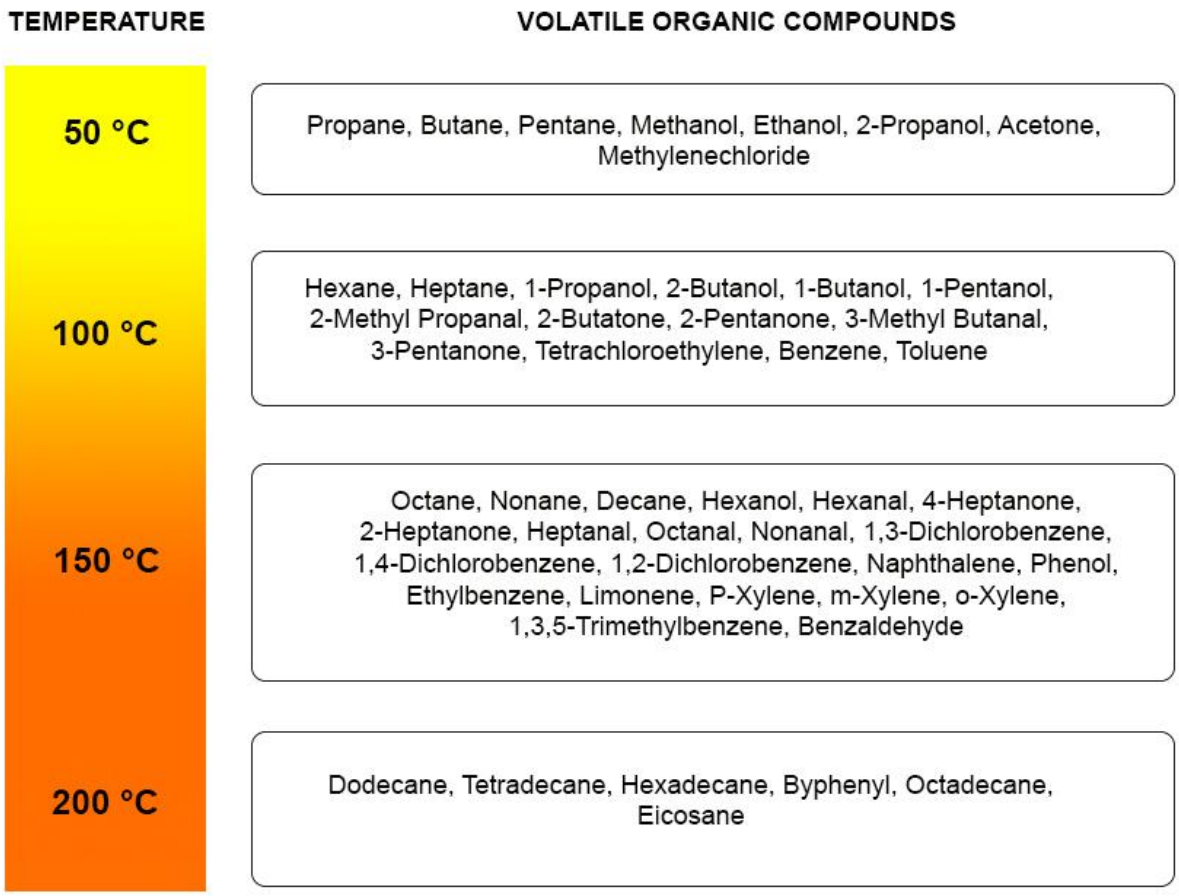

Figura 5 Flowchart with the different volatile organic compounds released for each temperature. Adapted from Sensors and Actuators B 204 (2014) 578–587.

The BIONOTE is an electronic-nose mimicking mammal olfaction [45]. It analyzes the VOCs and eventually provides a breath-fingerprint of the exhaled air. This device is made of an array of 7 quartium microbalances (QMB), with every sensor having a resonant frequency of 20 MHz in the thickest direction and being covered by a different mixture of anthocyanins obtained from three different plants: red rose, red cabbage and blue hydrangea. Sensor 1 is covered with

a drawn of rose 65mM, Sensor 2 with a drawn of blue hydrangea 65 mM, Sensor 3 with a drawn of red cabbage 65 mM, Sensor 4 with a drawn of red rose 65 mM and sucrose 10 mM, Sensor 5 with a drawn of red cabbage 65 mM and sucrose 10 mM, Sensor 6 with a drawn of hydrangea 65 mM and sucrose 10 mM and Sensor 7 with sucrose 10 mM. The covered surface is pivotal in guaranteeing the sensibility of sensors to chemical compounds in the air, while thanks to the piezoelectric effect the sensor transform the mechanical compression produced by the bounding of the VOCs in an electric beam. Having 7 sensors tested at four different temperatures, the final breath-fingerprint provided by the BIONOTE is composed of 28 signals. The overall procedure of sampling and analysis is illustrated in *Figure 6*.

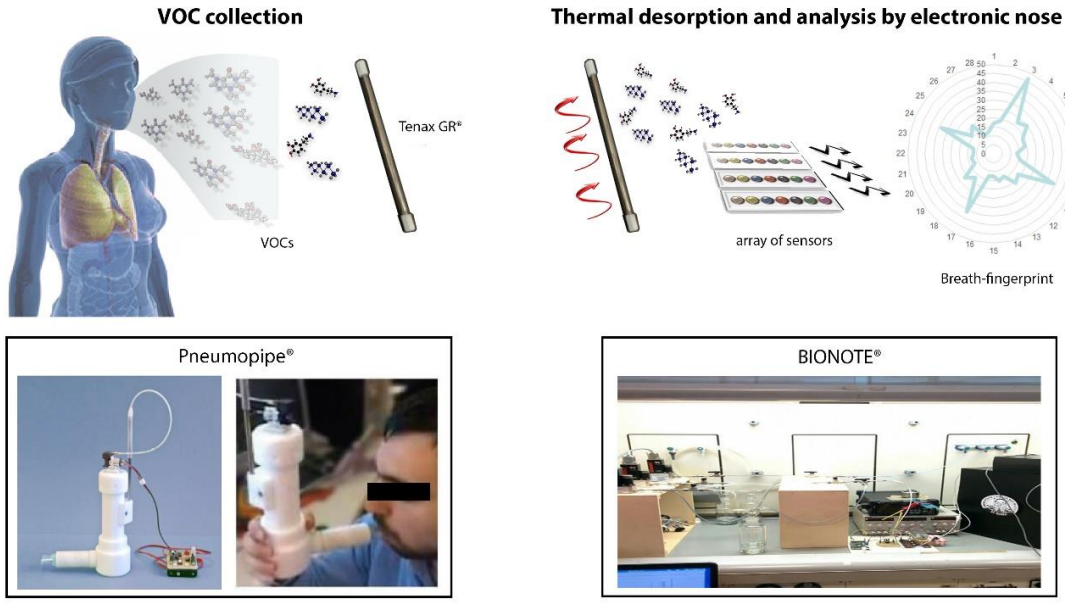

Figure 6 Volatile organic compound (VOC) collection by Penumopipe® and analysis by BIONOTE®.

AIM OF THE THESIS

The aim of the thesis was to understand whether exhaled breath analysis using an electronic nose is able to diagnose CHF and COPD in elderly people, and to which extent it is useful in the definition of the severity of these disorders and in the prediction of patients' prognosis.

We therefore identified primary and secondary end-points:

1) Primary end-point: Evaluate whether breath-fingerprint (BP) discriminates elderly people affected by congestive heart failure (CHF) from those affected by chronic obstructive pulmonary disease (COPD) and from healthy controls (HC);
2) Secondary end-point:
    - Evaluate to which extent BP correlates with the severity of the disorders (CHF and COPD);
    - Evaluate factors influencing BP area and shape;
    - Evaluate to which extent BP predicts patients' prognosis;

OUTLINE OF THE THESIS

Chapter 2 is a review of the peer-reviewed literature, updated as of July 2018, describing the state of art about breath analysis in respiratory and cardiac diseases.

Chapter 3 shows the results of a study that was performed as part of this PhD program with the objective of investigating breath-fingerprint ability to discriminate patients affected by CHF from healthy controls and patients affected by COPD. Furthermore, it investigated breath-fingerprint ability to predict CHF severity, stratified according to NYHA criteria, and recovery.

Chapter 4 shows the results of a study that was performed as part of this PhD program with the objective of investigating whether exist clusters of breath-fingerprint of COPD patients with similar clinical features and disease severity. Furthermore, it investigated whether and how inhaled therapy affect the breath-fingerprint in COPD patients.

Chapter 5 shows the results of a study that was performed as part of this PhD program with the objective of investigating the association between breath-fingerprint and proxies of functional status and prognosis in COPD.

Chapter 6 discusses the findings and future directions for the research and clinical application of breath analysis.

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CHAPTER 2 BREATH ANALYSIS IN RESPIRATORY DISEASES AND HEART FAILURE: STATE OF THE ART

Modified from *Finamore P, Scarlata S, Incalzi RA. Breath analysis in respiratory diseases: state-of-the-art and future perspectives. Expert Rev. Mol. Diagn. 2019; 19: 47–61.*

CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Chronic Obstructive Pulmonary Disease (COPD) is defined as the persistent presence of respiratory symptoms and non-reversible airflow limitation caused by significant exposure to noxious particles and gases, mainly cigarette smoke. According to the World Health Organization (WHO), COPD represents the fourth cause of death worldwide, with a global prevalence of about 12% [1]. However, despite the relatively univocal definition, COPD is a complex and heterogeneous disease with different phenotypic presentations. Patient personalized management is therefore extremely difficult, and requires a multidimensional approach, in addition to standard pulmonary function tests. Moreover, obtaining reliable, acceptable and repeatable spirometric curves, fundamental for a diagnosis of fixed airway obstruction, requires both a trained technician and a compliant patient. Many subjects, mainly children and elderly people, are often unable to correctly perform an informative spirometry, which urges the need for alternative diagnostic tools [2]. Furthermore, it is known that the severity of airflow limitation alone is not sufficient to fully grasp the wide heterogeneity and the multiplicity of pathogenetic mechanisms involved in COPD. The search for novel biomarkers that can predict disease progression, clinical presentation and features, and outcome is currently a hot topic. Breath analysis is a perfect candidate for this, since it is not invasive, it is easily performed (also on subjects unable to perform a spirometry), and has proved to be extremely informative.

Electronic Nose

Exhaled breath analysis via electronic noses has demonstrated a fairly good discriminative capacity between COPD and healthy subjects (accuracy of 78%) [3] as well as between COPD and diseases such as asthma (accuracy of 96%) [4], even in cases of fixed obstruction (accuracy of 88%) [5], pneumonia [6] or heart failure (accuracy of 69%) [7]. Remarkably, this evidence

was confirmed also in elderly people. VOC patterns in COPD patients have demonstrated to be reproducible, showing good within-day and moderate between-day reproducibility [8]. A correlation with expiratory flows [9], and the power to diagnose viral [10] and bacterial exacerbations [10,11], which are one of the main determinant of COPD steeper decline [6], were also proved. Furthermore, great interest has been elicited from the likely power of VOCs to identify 'treatable traits' and phenotypes of COPD [12] and, consequently, to represent a potential tool for improving the clinical management of the disease. Indeed, Fens et al. have demonstrated that it is possible to identify sputum eosinophilia [13], and, more recently, de Vries et al. have demonstrated that different breath-prints associate with systemic neutrophilia and eosinophilia [14]. Remarkably, eosinophilia associates with hospital re-admission in COPD patients [15], who respond to treatment with inhaled corticosteroids, leukotriene receptor antagonists and biological drugs (e.g. anti-IL5) [16], therefore allowing to identify the overlap between asthma and COPD (so-called 'ACOS syndrome'), and clearly showing the potential of breath analysis in predicting clinical outcomes and tailoring therapy.

Moreover, breath-print is associated with COPD patients' functional status and multidimensional assessment, expressed respectively through the six minute walk test and the BODE index, and it seems to allow for better discrimination of COPD patients with accelerated decline compared to the GOLD classification alone [17]. It is probably because breath-print reflects a widespread array of multiple pathologic and extra-pulmonary domains involved in COPD (i.e. systemic inflammation, multiorganic response to hypoxemia, and associated and disease-related comorbidities). In other words, breath-print in COPD is not only the effect of airway remodeling and airflow limitation expressed through pulmonary function tests, but also the representation of multi-dimensional indexes such as the severity of dyspnea or the frequency of acute exacerbations due to systemic pathologic mechanisms. Identifying COPD patients more likely to deteriorate through a breath analysis will help to prioritize their access

to the health care system and to plan for a more comprehensive treatment, also aimed at comorbidities, thus improving patients' survival expectations [18]. However, the identification of disease phenotypes through VOCs is not limited to the definition of the different types of inflammation pathways, but it has also proved successful at characterizing clusters of COPD patients sharing previously uninvestigated clinical features. This would likely help to improve patients' assessment and drive the standard of care towards a more individual approach.

Lastly, a comprehensive knowledge of information contained in the breath-print of COPD subjects could limit the need of more expensive and/or invasive tests [19,20]. Indeed, exhaled breath analysis could also be useful to identify COPD patients affected by alpha1-antitrypsin (AAT) deficiency. The study of Hattesol et al., although preliminary and not yet conclusive, has demonstrated that breath analysis can discriminate among COPD patients with and without the deficiency, and it can also detect VOC pattern changes associated with augmentation therapy [21]. Interestingly, the breath-print also modifies longitudinally according to the type of inhaled medication, with different responses seen in association with corticosteroids/bronchodilators versus bronchodilators alone. This evidence makes e-nose technology potentially suitable for clinical control of patients' adherence and therapy effectiveness [20].

Analytical Techniques

A good level of accuracy in discriminating COPD patients from healthy controls was obtained when single VOCs or a combination of VOCs, identified through analytical techniques (e.g. gas chromatography followed by mass spectrometry, ion mass spectrometry), were used with correct classification rates ranging from 70% to 92% [22–25]. To date, the compounds found in association with COPD are: indole [26], aromatic hydrocarbons, acetic acid, phenol [25],

hexanal, nonanal and decanal [23], and 2-pentanone [27]. However, it is still unclear where these compounds originate from and which mechanisms are responsible for their onset. Further research is then required to have firm conclusions, also because some of the above mentioned compounds might be related to confounders (e.g. smoking, associated comorbidities, still unsolved technical issues) and not to COPD itself [28].

Furthermore, even if VOC analysis proved to be a poor marker of emphysema [29], it still showed good accuracy in identifying COPD patients with sputum eosinophilia ≥2% (accuracy after leave-one-out cross-validation of 88%), moderately and inversely correlated with exhaled concentrations of 1,1'-bipheny l,3-methyl-$C_{13}H_{12}$, and patients with more than one exacerbation per year (accuracy after leave-one-out cross-validation of 83%), moderately and inversely correlated with undecane and 3,7dimethyl-$C_{13}H_{28}$ and 2,2,4,4-Tetramethyloctane-$C_{12}H_{26}$ concentrations [23].

ASTHMA

According to current guidelines, asthma is defined as a chronic disease characterized by airway inflammation leading to airflow obstruction with different degrees of reversibility and airway remodeling [30]. Usually, clinical presentation and history, combined with pulmonary function test, are sufficient to ascertain the diagnosis and address the most appropriate treatment to achieve an acceptable control of symptoms and airway inflammation. However, there are cases where the diagnosis is more challenging and the disease difficult to control. Asthmatic inflammations, in fact, can be sustained by different pathways and produce a variable set of biomarkers. Eosinophil airway inflammation (the most frequent phenotypic expression of the disease) is mainly mediated by a Th2-lymphocyte response, which leads to an increased eosinophil prevalence, *via* the IL-5 stimulus, and to an increased IgE production, by the

activation of B-lymphocytes *via* IL-4 and IL-13, and to an increased phlegm production and smooth muscle cell contraction, *via* IL-13 [31]. Potential biomarkers of this type of inflammation are serum IgE levels, blood and/or sputum eosinophil counts, serum periostin and fractions of exhaled nitric oxide (FeNO) [32].

On the other hand, a variable percentage of asthmatic subjects display a peculiar non-eosinophil pathway triggered by innate and cell mediated immune response that is usually associated with a higher neutrophil count in the sputum (>61%) [33], but which might also be associated with a poor amount of granulocytes ('paucigranulocytic asthma') [31,33]. These differences must be considered when a personalized approach to asthma is performed, especially since new therapies such as anti-interleukin 5 or anti-IgE have been suggested. Furthermore, the current approach to diagnosis and treatment suffers from several pitfalls. In fact, the current approach assumes a cause-and-effect link between airway inflammation and airway obstruction; the asthma diagnosis focuses then on the reversibility of the airway obstruction at spirometry, while treatment is centered on increased steroid inhalation and bronchodilators irrespective of underling inflammatory endotypes [30]. Nowadays, it is clear that airway obstruction and airway inflammation are not necessarily and mutually related, and might occur independently [34], thus they both have to be evaluated in asthmatics, since airway obstruction affects patients' exercise capacity and quality of life the most, while airway inflammation is also a predictor of disease exacerbation [34]. All this considered, breath analysis, by giving rapid and non invasive information about airway inflammation and not being affected by acute changes in airway caliber [35], might serve as a diagnostic and phenotyping tool for clinical purposes.

Electronic Nose

Existing evidence already proved that exhaled breath analysis via electronic nose allows to differentiate patients affected by asthma from both young and old controls (cross-validated accuracy higher than 90%) [36], and also by COPD patients [4], who share an airway obstruction. Although with a lower accuracy, breath analysis can also stratify disease severity, discriminating patients with mild asthma from patients with severe asthma [36]. Moreover, the power to identify asthma endotypes was observed: breath-print discriminates with moderate accuracy eosinophilic, neutrophilic and paucigranulocytic sputum of asthmatic patients [37] and also correlates with bronchoalveolar lavage (BAL) fluid eosinophil counts [38]. Considering VOCs power to identify asthma phenotypes and exacerbation, it is conceivable that their use might improve predictions on patients' responsiveness to steroid therapies. Indeed, van der Schee et al. did not only confirm this hypothesis, but also demonstrated that VOCs predictive power is higher than that of sputum eosinophils or that of exhaled nitric oxide [39].

Analytical Techniques

A good discriminative capacity, higher than 85%, has also been reached using gas-chromatography and mass spectrometry techniques in adult subjects. Several compounds have been associated with the disease, but fewer are shared, such as dodecane or benzene [40,41]. Interestingly, breath analysis has also demonstrated a good discrimination of very young children affected by asthma from transient wheezing children [42,43], where spirometry is difficult to be performed due to the lack of children's compliance. Moreover, it can also identify asthma endotypes, with an area under ROC curve (AUROC) of 98% for sputum eosinophilia ≥2%, and an AUROC of 90% for sputum neutrophilia [40]. The potential of this analysis is not just limited to diagnosis and identification of phenotypes of asthma patients, but also extends

to exacerbation risk assessment and the monitoring of inhaled therapy. In fact, VOCs produced during the exacerbation are different from those produced during the steady state and from those produced during recovery [44], and this explains why selective VOCs can predict with a good level of accuracy asthmatic patients who will undergo an exacerbation over the follow-up period from those who will keep stable [45]. However, this prediction power is good only when the exacerbation occurs within two weeks from the exhaled breath collection, as suggested by van Vliet et al. [46], hence further studies are required to improve our knowledge on this issue.

LUNG CANCER

Among potential applications of exhaled breath analysis in respiratory diseases, the early detection of lung cancer is by far the most appealing. Nowadays, screening programs using low-dose computerized tomography (LDCT) are widely encouraged [47], given the evidence suggesting that these may help early diagnosis and reduce lung cancer mortality [48]. However, no definite consensus exists in terms of real benefits deriving from early detection of lung cancer among the general population, nor on the criteria to select subjects at higher risk. Concern was raised about the appropriateness of chest CT scan-based screenings in terms of safety (chronic and prolonged exposure to radiation), timing and cost-effectiveness [49]. Indeed, although capable of identifying a few millimeter large pulmonary nodules, LDCT cannot provide any information on their nature. It is clear how the frequent identification of a low-risk or overtly benign pulmonary nodule during a screening program often exposes the patient to repeated CT scans and unnecessary radiation, as well as useless and potentially harmful invasive diagnostic approaches. On the other hand, a window period exists for people with lung cancer at a very early stage, still not detectable because under the traceability power

of existing diagnostic tools (i.e. about 30-35% of cancers are in advanced stages when identified [48,50]).

Electronic Nose

Exhaled breath analysis via electronic nose has demonstrated a good level of accuracy in discriminating lung cancer patients from healthy controls (sensitivity of 71.4% and specificity of 91.9% in the validation population) [51], particularly in early stages, and it is not affected by patients' comorbidities [52]. Interestingly, accuracy in detecting lung cancer within a population of subjects at risk, like COPD patients, is still high [53]. Given these premises, the potential benefit of using exhaled breath analysis via e-nose in lung cancer screenings is straightforward, since it could allow, for example, to dramatically reduce the number of false-positive diagnoses during screening programs [54]. Breath analysis has in fact shown an accuracy level of 88% in discriminating benign from malignant pulmonary nodules [55]. Moreover, VOC analysis could also be helpful in defining cancer histotypes: in fact, preliminary research has demonstrated the power of breath print analysis to discriminate NSCLC from SCLC, adenocarcinoma from squamous cell carcinoma [56,57], and to distinguish subjects with EGFR or KRAS mutations [58,59] from wild types to be addressed to a specific personalized therapeutic approach.

Analytical Techniques

The use of a combination of VOCs in discriminating lung cancer patients from healthy controls has also obtained a good accuracy level, with correct classification rates higher than 80% [60–62]. Several compounds seem to be associated with lung cancer, among them: n-octane [55,60], n-nonane and 2,3-butanedione [60], CHN, methanol, CH_3CN, isoprene, 1-propanol [62].

However, the nature of identified VOCs widely differs from one study to the other, and, as in COPD, it is still unclear where these molecules originate from and whether they depend on the sampling technique or on the environment (e.g. smoking, plastic materials) [63–65]. Interestingly, although small differences in composition and concentration were found in the air of the two lungs, a selective analysis of the alveolar air coming from the lung not affected by the tumor can still identify tumor-associated compounds [66]. The reason for this lies probably in the systemic spill-over of inflammatory and tumor biomarkers and mediators exhaled by the not affected lung after reaching pulmonary circulation. Furthermore, it is interesting to highlight the documented power of exhaled breath analysis to distinguish among cancer histotypes and mutations (e.g. the EGFR mutation is associated with higher n-dodecane [67]) and to detect the persistence of the disease after radical surgery or chemotherapy. Broza et al. demonstrated a change in the VOC profile after surgical treatment of lung cancer patients, particularly regarding 2-methyl-1-pentene, 2-hexanone, 3-heptanone, styrene and 2,2,4-trimethyl-hexane concentrations [68,69], with a loss of cross-validated accuracy of their nanomaterial-based breath test in discriminating between lung cancer patients and controls from 94% to 63%, while Kischkel et al. described a reduction in butane or pentane after surgery [70]. Moreover, Nardi-Agmon et al. showed that VOC analysis can discriminate patients with partially responding/stable disease from patients with disease progression, with three involved VOCs: α-phellandrene, styrene, and dodecane-4-methyl [71].

OBSTRUCTIVE SLEEP APNEA SYNDROME

Obstructive sleep apnea syndrome (OSAS) is a high prevalent sleep disorder having detrimental multi-organ effects, such as the increased risk of cardiovascular, neurological and metabolic events as well as motor vehicle accidents [72,73]. These side effects can be largely

prevented or reversed thanks to the application of continuous positive airway pressure (C-PAP). The gold-standard diagnostic test is polysomnography that, given the huge prevalence of OSAS among the general population, and the need of devoted personnel and dedicated setting, represents an underused, cost and time-consuming tool. For this reason, the number of tests that can be performed on regular bases is by far sufficient to satisfy the demand, also because any attempt in selecting high risk subjects at pre-polysomnographic screening using dedicated questionnaires or clinical assessments, lacks in sensitivity and specificity: only 60% of patients undergoing polysomnography after screening questionnaires is effectively affected by OSAS [74,75]. The need of new screening tools fostered the research on the application of exhaled breath analysis in discriminating OSAS patients from controls. Several studies have been conducted by using electronic noses (Cyranose320® and BIONOTE) and demonstrated a good accuracy level in discriminating OSAS patients from healthy weight and overweight controls and COPD patients, but only a moderate capacity in distinguishing them from healthy obese [76–78] as well as a moderate capacity in distinguishing children with OSAS from those with habitual snoring [79]. At the moment, evidence is then too weak to recommend the use of exhaled breath analysis as a screening tool for OSAS. Furthermore, a relevant circadian variability, likely due to intermittent nocturnal inflammation and oxidative stress, exists in the breath-print profile of OSAS patients and this may affect diagnostic accuracy [80], confirming the need for further research so to identify modifiers of the VOC patterns during the day and their related diagnostic potential. However, breath-print changes after apneas are reverted with a C-PAP therapy. A single night of C-PAP therapy is enough to provide early changes in inflammatory and clinical profile of OSAS patients [81], and these modifications were also seen in their breath-print [82]. Interestingly, comorbidities seem to play a confounding role, interfering with the e-nose power to correctly identify OSAS from other high risk populations [82]. In summary, exhaled breath analysis reveals promising to complement existing

instruments for widespread screening and early diagnosis of OSAS patients, as well as for monitoring their adherence and compliance to C-PAP ventilation.

PNEUMONIA AND INFECTIVE RESPIRATORY DISEASES

Respiratory infections represent a major issue in respiratory medicine since, despite very high incidence rates, many clinical aspects are far from being solved and many clinical decisions are still undertaken through probabilistic and empiric approaches. Moreover, the unwise and extensive use of antibiotics in the last decades has favored the onset of several multidrug- and/or extensively drug-resistant bacteria, with overwhelming clinical, social and public health consequences. Improving the diagnosis, by allowing early detection of microorganism colonization of the airways, and orienting a precocious etiologic treatment tailoring a more precise assessment tool, is therefore a main aim. More than ever, there is the need for early and correct identification of pathogen microorganisms and antibiotic resistance, particularly in hospital-acquired pneumonia (HAP), where a higher prevalence of resistant microorganisms is often catastrophic. Excluding *in vitro* studies, which focus on the headspace of cultures using gas chromatography and mass spectrometry, exhaled breath analysis was generally performed in an intensive care setting (ICU) on patients usually developing ventilator-associated pneumonia (VAP). This was a complex diagnosis, made combining clinical features and bronchoalveolar lavage pathogen isolation or biomarkers detection. In this context, the diagnostic reliability of the approach is often unsatisfactory. For this reason, exhaled breath analysis, being performed at bedside and providing results within a short time-frame, might represent a useful tool. In a study by Humphreys and colleagues, breath analysis via electronic nose allowed to discriminate among several macro-families of pathogens ('gram+' vs 'gram-' vs fungi vs no microbiologic growth) [83]. If confirmed and replicable, these results might

enable a quick, cheap, and useful tool for respiratory infection management, treatment decision making and antibiotic regimen definition. However, to date, *in vivo* studies are limited, sample sizes are too small and diagnostic accuracy is not fair enough to discriminate patients affected by VAP, due to any cause, from controls [84–87]. Better results, providing a good discriminative accuracy level, even though in a small study sample, were obtained using an electronic nose assessing *Aspergillus fumigatus* colonization in patients with cystic fibrosis [88], or invasive pulmonary aspergillosis in patients with neutropenia due to chemotherapy [89]. Indeed, while there are data in literature regarding the application of breath analysis in the HAP, there is a lack of evidence on diagnostic accuracy of the analysis in community-acquired pneumonia (CAP). Nevertheless, CAP are frequent, often undiagnosed and underestimated, so studies evaluating breath analysis usefulness are to be carried out and might provide an insight on quicker diagnostic and cost saving methods in respiratory infections.

Another promising application of the e-nose relates to *Mycobacterium tuberculosis complex* diagnosis. The gold standard for diagnosing tuberculosis is still the culture, however this requires several weeks before a conclusive response can be given. Real-time polymerase chain reaction (rt-PCR) highlights the presence of genes deriving from the mycobacterium and is useful to diagnose and assess TB drug-resistant strains. Unfortunately, it is expensive, it requires a specific technologic setting and does provide under optimal sensitivity and specificity levels. Furthermore, it is not widely available in developing countries where the infection is more common. Exhaled breath analysis through electronic nose has demonstrated to discriminate TBC patients from healthy controls with a good accuracy level [90–92], active from latent TBC with 85% of accuracy [93] and, as demonstrated by Zetola et al., can aid to monitor the effects of antibiotic therapies, since it produces a reduction of the sensors' response [90]. Thereby, further evaluation is needed to confirm the addition of the technique to those already available.

CYSTIC FIBROSIS

Cystic fibrosis (CF) is an inherited autosomal recessive disorder caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR), with detrimental effects on several organs, like lung and sinuses, liver, pancreas and intestine or endocrine system. Thanks to screening programs, CF is usually diagnosed in newborns, even though the diagnosis can be made later, and, although it is not yet possible to correct the CFTR protein dysfunction, early and multidrug treatment led to an increase in life expectancy, mostly in high-income countries [94]. However, new challenges are arising, particularly regarding early detection of infections and the definition of drug-resistant strains (e.g. methicillin-resistant S. aureus – MRSA –), which might further improve patients' outcome.

Exhaled breath analysis was successfully applied to the diagnosis of CF, with a moderate to good discriminating power in children affected by CF from healthy controls, either using an e-nose [95] or using a combination of VOCs [96], and the definition of colonized patients. When using a combination of VOCs, it is possible to discriminate with a good accuracy level CF patients with *Pseudomonas aeruginosa* colonization from those without [96,97], and to similarly identify patients infected by *Staphylococcus aureus* [98], and, using an electronic nose, CF patients colonized by *Aspergillus fumigatus* [88]. Although the reliability of existing screening and diagnostic tests limits its development as a screening or diagnostic tool, its use in the detection of infected patients is really promising, considering that sputum samples are often difficult to obtain and induced-sputum is unpleasant for children. While a moderate diagnostic accuracy of the e-nose was confirmed by Joensen et al. [99], further research is still needed, particularly in order to confirm the promising, but preliminary results of analytic techniques and to definitely shed light on VOCs associated with the diagnosis of CF and the presence of infections. Indeed, while Paredi et al. suggested an elevated exhaled ethane level [100], Barker et al., correcting for ambient ethane, did not find any difference, while reporting

a lower concentration of dymethil sulphide [101], which is not present in the study of Kamboures et al., even though they found significant differences, particularly in the concentration of another sulfide [102]. Other incongruences were encountered in the analysis of isoprene, while Španěl et al. found a higher concentration during *Pseudomonas aeruginosa* infection [97], McGrath et al. found a lower level of this marker during the exacerbation stage, concluding that it should not be considered a reliable marker of oxidative stress [103].

OTHER RESPIRATORY DISEASES

Further respiratory diseases with a distinguishable VOC production are: sarcoidosis, where breath-print discriminates untreated (but not treated) patients from healthy controls [104]; mesothelioma, where breath-print is distinguishable from that of patients exposed to asbestos without mesothelioma and healthy controls, with an accuracy level higher than 80% [105,106]; pulmonary embolism (PE), where breath-print identifies patients with PE among patients with a high suspicion of disease and without comorbidities influencing VOC production [107]; idiopathic pulmonary fibrosis, where p-cymene, acetoin, isoprene, ethylbenzene and an unknown compound show a significantly different concentration compared to healthy controls [108]; gastroesophageal reflux disease (GERD) [109]. Given the very low number of studies, the low numerosity of the sample size and, in some cases, the poor methodologic approach weakening the level of evidence and related conclusions, however intriguing and promising, these data still require further evaluation before they can be considered more than simply anecdotal.

LUNG MICROBIOME

Recently, new evidence has changed the background of the respiratory system physiology and respiratory disorder pathophysiology and, likely in forthcoming years our knowledge on the mechanisms underpinning these conditions will be deepened. Among recent breakthrough acquisitions, the existence of a lung microbiome, making the lungs non sterile as previously believed, but colonized by communities of microorganisms of different types (i.e. bacteria, virus and fungi), is one of the most revolutionary [110]. The lung microbiome is organized in niches of different microorganisms, from the nostrils to the alveoli [111], and it seems to play a key role in maintaining the lungs healthy, and, consequently, in maintaining the whole organism homeostasis. There are clues suggesting that microbiomes bear the development of local immune response in the respiratory tract. Remarkably, this seems to change both in quality and quantity during chronic respiratory diseases such as COPD [112] or asthma [113]. Although these finding are still preliminary, it is likely that in the near future, the assessment of lung dysbiosis could represent an important subject for both diagnostic and therapeutic purposes. Assuming the role of the lung microbiome to be similar to the role of the gastrointestinal microbiome, determining microbacterial composition across ages would be pivotal for explaining important adaptive, immunologic, and homeostatic mechanisms regulating mucosal physiology [111,114].

HEART FAILURE

Although less investigated than respiratory disorders, exhaled breath analysis in heart failure represents a promising approach. Heart failure is a complex syndrome dramatically reducing subject's quality of life and survival and it affects more than 8% of the population worldwide [115]; considering the aging of population, it accounts to reach epidemic proportions, hence

the availability of inexpensive, rapid and non-invasive biomarker/s for diagnosing, monitoring and guiding therapy are warranted. Analysing exhaled breath by gas-chromatography, in 1995 Kupari et al. demonstrated that levels of exhaled breath acetone are elevated in subjects with chronic heart failure than patients with cardiac problems without heart failure and healthy controls [116], result confirmed in following studies which also demonstrated that acute decompensated heart failure has higher levels than stable chronic heart failure, that exhaled breath acetone level increases with severity of heart failure following New York Heart failure classification and, finally, that exists a positive correlation between exhaled breath acetone and BNP [117]. Recently, D. Biagini et al. demonstrated that breath acetone level decreases with improved clinical condition suggesting a possible role also in monitoring [118]. Among other molecules increased in the exhaled breath of heart failure patients there are ion products (e g $H3O^+$, $O2^+$ and NO^+) and pentane [119,120], that seem to be related with inflammation and lipid peroxidation since their level linearly depend on scavenger dose [121].

Albeit the analytical approach, mainly based on gas-chromatography and mass spectrometry, is carrier of qualitative and quantitative information about exhaled breath volatile organic compounds (VOCs) of heart failure patients, another suitable approach for VOCs analysis in heart failure is that using electronic nose. The device does not provide information about single components, but produces a fingerprint of the mixture of VOCs useful for clinical purposes. In 2012 Voss et al. firstly used a metal oxide gas sensor electronic nose to diagnose heart failure. Authors analysed sweat volatile gases from the skin surface of patients with decompensated heart failure, compensated heart failure and healthy controls and obtained a good discriminative accuracy, ranging from 76% between decompensated heart failure and healthy controls to 87% between decompensated and compensated heart failure [122]. In 2013 the same group conducted the analysis, with the same electronic nose, on the exhaled breath of decompensated

and compensated heart failure patients and of healthy controls, improving the discriminative accuracy to more than 90% [123].

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CHAPTER 3 ANALYSIS OF VOLATILE ORGANIC COMPOUNDS: AN INNOVATIVE APPROACH TO HEART FAILURE CHARACTERIZATION IN OLDER PATIENTS

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ABSTRACT

Background: Analysis of exhaled volatile organic compounds (VOCs) may be applied for diagnostic purposes in some chronic diseases, but there are no data on their role for discriminating people with congestive heart failure (CHF), particularly in older patients where natriuretic peptides have lower accuracy.

We evaluated whether VOCs analysis can discriminate patients with or without CHF, stratify CHF severity and predict the response to therapy of decompensated CHF.

Methods and Results: We recruited 89 subjects admitted to an acute care ward with acutely decompensated CHF, 117 healthy controls and 103 chronic obstructive pulmonary disease (COPD) controls. CHF patients performed echocardiography. VOCs were collected using the Pneumopipe® and analyzed with the BIONOTE electronic nose. Partial least square (PLS) analysis was used to evaluate discriminative capacity of VOCs.

Accuracy in discrimination of CHF vs. healthy and COPD controls was 81% and 69%, respectively; accuracy did not decrease in a sensitivity analysis excluding subjects younger than 65 and older than 80 years. In CHF patients VOCs pattern could predict with fair precision ejection fraction and systolic pulmonary arterial pressure, but not changes in weight due to therapy.

Conclusions: VOCs pattern is able to discriminate older CHF patients from healthy people and COPD patients and correlates with cardiac function markers.

INTRODUCTION

Congestive heart failure (CHF) is the leading cause of disability and death in the elderly as well as the first cause of hospital admission and readmission in this age group [1], and aging of the population accounts for CHF progressively reaching epidemic proportions. CHF is associated with several conditions such as coronary atherosclerosis, diabetes mellitus, chronic obstructive pulmonary disease (COPD) and chronic kidney disease (CKD), either because of a causal relationship or by sharing of risk factors such as age and smoking [2]. These comorbidities may frequently make a clear-cut diagnosis of CHF problematic [3], therefore a biomarker of CHF with adequate diagnostic, classification and prognostic properties might improve both diagnosis and monitoring of CHF in these patients. Many efforts have been made to reach this goal, but so far only the Brain Natriuretic Peptide (BNP) has been shown to be an useful multipurpose biomarker [4].

However, considering the limitations of natriuretic peptides, particularly in elderly patients [5], their sizeable cost and that their measure requires a blood collection, the search of a non invasive, inexpensive biomarker able to improve natriuretic peptides performance, also in patients with comorbidities, is warranted.

CHF has important metabolic implications, being characterized by a reduction in mitochondria energy production, in energy transfer and in energy utilisation, particularly in muscles, as well as by an inflammatory state [6–9], that may be reflected in the production of volatile organic compounds (VOCs) [10]. We hypothesized that exhaled VOCs may be able to discriminate patients with CHF from controls, and also to classify CHF severity. Indeed, exhaled VOCs assessed through the electronic nose have been reported to distinguish patients with lung cancer or with selected other cancers from controls [11–13]. Furthermore, different exhaled VOCs patterns have been found in patients with obstructive sleep apnea with or without selected comorbidity [14]. Also COPD, asthma, liver failure and selected psychiatric conditions seem

to be associated with a distinctive VOCs pattern [15–17]. To-date, we only know that CHF is characterized by an increase in exhaled acetone and pentane [18], but exhaled VOCs pattern in CHF has not been yet investigated using a quartz microbalance (QMB) sensors electronic nose.

The objective of this exploratory study is to verify whether exists a specific pattern of exhaled VOCs discriminating CHF from control groups and if it has classification properties in elderly and multimorbid CHF patients. Furthermore, we aimed at evaluating whether distinctive VOCs patterns characterize patients with exacerbated CHF who will improve more in response to CHF therapy.

MATERIALS AND METHODS

Study participants and design

We enrolled a convenient sample of 89 subjects consecutively recruited among those admitted with a diagnosis of decompensated CHF to the geriatric acute care ward of the University Hospital "Campus Bio-Medico" in Rome (Italy). Diagnosis of decompensated CHF was based on symptoms and signs of CHF in patients with history of heart diseases and it was confirmed on the basis of clinical assessment, NT-proBNP value, and echocardiography [2].

Exclusion criteria were the inability to perform VOCs collection and comorbidities with a demonstrated effect on the VOCs production: respiratory infections, COPD, asthma, pulmonary fibrosis, obstructive sleep apnea, estimated Glomerular Filtration Rate (eGFR) < 30 mL/min/1.73 m^2, liver failure, cancer. Comorbid conditions were identified based on patients' documentation, history, physical examination, routine blood analysis, chest X-ray and ECG.

To study the capacity of the VOCs to discriminate patients with and without CHF, we also enrolled two control groups. The first one (N: 117) was made up by people who, considering clinical history and medical reports, symptoms, signs and home therapy, were free from chronic cardiac, respiratory, or renal diseases and without active neoplastic disease and attend the University Hospital "Campus Bio-Medico" for routine blood tests. In this group, dubbed healthy controls (HC), we only collected information about height, weight, and smoking status. The second control group was made up by 103 patients affected by COPD that were enrolled among people attending the pulmonary medicine outpatient clinic of the University Hospital "Campus Bio-Medico". Diagnosis of COPD was based on FEV1/FVC < 0.7 not completely reversible after administration of 400 mcg of salbutamol [19]. In these patients we collected information about height, weight, smoking status and comorbidities; we excluded COPD patients with history or clinical signs of CHF, an exacerbation in the previous three months,

obstructive sleep apnea, pulmonary fibrosis, liver failure, eGFR < 30 mL/min/1.73 m^2, and diagnosis of cancer. We did, basing on our sample, a post-hoc formal calculation of the power for the area under our receiver operating curve (AUC) finding it to be 100% with an error I rate set at 5%.

The investigation conforms with the principles outlined in the Declaration of Helsinki. All the study participants provided written informed consent. The study protocol was approved by the local Ethical Committee (protocol number: 21.16 TS ComEt CBM).

At admission, participants in the CHF group underwent multidimensional assessment that included: clinical history (smoking), medication taken prior to admission, physical examination (anthropometric measures, New York Heart Association symptom severity), laboratory analysis (NT-proBNP, blood cell count, myoglobin, creatine kinase MB Isoenzyme – CK-MB –, Troponin I, urea, creatinine, uric acid, aspartate aminotransferase – AST –, alanine aminotransferase – ALT –, gamma-glutamyltransferase – γ-GT –, alkaline phosphatase –ALP –, total bilirubin, erythrocyte sedimentation rate, c-reactive protein – CRP –, D-dimer, fibrinogen, total cholesterol, HDL, LDL, triglycerides, glycemic control and protein electrophoresis), and instrumental evaluations (ECG, haemogasanalysis, chest X-ray and echocardiogram). The first 39 patients had weight assessed only at baseline because the analysis of the correlation between VOCs pattern and changes of weight in response to the therapy was planned after the beginning of the study. Comorbid conditions were identified based on patients' documentation, history, physical examination, routine blood analysis, chest X-ray and ECG. CHF patients received standard treatment according to current guidelines and tailored to the individual clinical characteristics [2].

Echocardiography

Echocardiography was performed in 86 out of 89 subject included in the study [2]. We systematically calculated left ventricular ejection fraction (EF) using the Simpson method. We studied left ventricular diastolic function using E/A ratio, and we estimated the left ventricular filling pressure through E/E' average ratio. The systolic pulmonary arterial pressure (sPAP) was estimated using tricuspid gradient and inferior cava vein dimension and collapsibility. sPAP was not estimated in patients without a tricuspid regurgitation.

Collection and analysis of VOCs

In the control groups exhaled breath was collected during outpatient visit, while in the CHF group it was collected the day after admission in the geriatric ward. Exhaled breath of both CHF and controls was collected in the morning, immediately after awakening, with subjects fasting and smoking free for at least 12 hours. Similarly, they were not allowed to receive medications for at least 12 hours. Exhaled breath collection was performed using the Pneumopipe® (European patent n. 12425057.2, Rome- Italy), which allows a non invasive collection of exhaled breath into an adsorbent Tenax GR cartridge (Supelco/Sigma-Aldrich, Bellefonte, PA, USA), with very low water-binding capacity [20], by an individual normally breathing into it for three minutes [21]. Cartridge content is then thermally desorbed into BIONOTE's sensor cell, a gas sensor array developed and fabricated by the Lab of Electronics for Sensor Systems at the University Campus Bio-Medico of Rome. The array used for this study is composed of seven QMB sensors covered with anthocyanins extracted from three different plant tissues: red rose, red cabbage, blue hortensia and used as chemical interactive materials. The collected VOCs, when desorbed into the sensor cell, interact with the chemicals covering the sensor's surface and they are adsorbed via weak bounding forces. Sensors responses to VOCs result in seven frequency shifts of each of the QMB respect to their typical

resonance frequency [22]: it is like a balance measuring the molecules burdening on each crystal. The desorption of the exhaled breath collected into the cartridge is controlled by a temperature program with four temperatures (50, 100, 150, 200°C). The achieved twenty-eight responses (seven QMB tested per four different temperatures) form the VOCs pattern, a sort of fingerprint of the patient's exhaled breath dubbed breath-print (BP) [21].

Statistical analysis

We reported the characteristics of our sample using descriptive statistics (mean and standard deviations for continuous variables, proportion for categorical variables). To evaluate the discriminative capacity (diagnosis and severity of CHF) and the ability of VOCs to differentiate patients who did improve their clinical status, expressed by reduced weight compared to those who remained stable or worsened, we used a Partial Least Square Discriminant Analysis (PLS-DA), a supervised learning machine algorithm [20]. PLS-DA was used on a random subset of patients including 66% of our sample (training set) and tested in the remaining 33% of the sample (test set). To avoid overfitting we used a "repeated k-fold cross validation". The overall effectiveness of VOCs testing was expressed as diagnostic accuracy, which is the proportion of subjects correctly classified by the PLS among all subjects. To evaluate if age impacts on the diagnostic accuracy of VOCs, we performed a sensitivity analysis on a sample of patients with CHF and control groups aged from 65 to 80 years old. Further sensitivity analyses were performed to evaluate impacts of gender (male CHF: 43, HC: 73, COPD: 68 and female CHF: 46, HC: 44, COPD: 35), diabetes (subjects without diabetes CHF: 54, HC: 86, COPD: 64), renal disease (subjects with creatinine $\leq$ 1.2 mg/dL CHF: 59, COPD: 51) and smoking (non smoking population CHF: 82, HC: 79, COPD: 63), if any. Within each subgroup age and gender had the same distribution as the complete sample (data not shown).

We used the Root-Mean-Square-Error Cross-Validation (RMSECV) to aggregate in a single measure of predictive power the magnitudes of the machine errors in prediction of continuous variables (EF and sPAP). All the analyses were performed using R version 3.3.0 (The R Foundation for Statistical Computing, Wien, Austria, 2016).

RESULTS

Of the 89 enrolled CHF subjects, 16 (17%) were in NYHA II, 43 (48%) in NYHA III, and 30 (33%) in NYHA IV class. Mean age was 81 (SD: 9) years in participants with CHF, 64 years (SD: 9.1) in controls without chronic diseases and 71 years (SD: 7.9) in controls with stable COPD. Men were 62%, 48% and 66% in the CHF, healthy control (HC) and COPD group, respectively. In the CHF group mean NT-proBNP was 8938.2 pg/mL (SD: 9524.2), mean Hb was 11.7 g/dL (SD: 1.8), mean sPAP was 45.2 mmHg (SD: 12.3) and mean EF was 44.8 % (SD: 14.7). Of the 86 patients with measured EF, 56 (65%) had a EF<50% (reduced – EF <40% – or mid-range ejection fraction – 40%<EF<50% –) and 30 (35%) had a EF>50% with diastolic impairment (preserved ejection fraction) [2].

CHF and HC had significative differences in anthropometric characteristics and smoking habitus. Comparing CHF with COPD patients, there were significant differences in height, weight, creatinine, smoking and comorbidities. Characteristics of HC, CHF and COPD subjects, creatinine and echocardiography of the CHF group are summarized in *Table 1*.

Figure 1 shows comparison of mean BP of CHF, COPD and HC. From a qualitative point of view, CHF BP area is wider than the others and its shape is characterized by 4 peaks given by the third QMB tested at the four temperatures (3, 10, 17 and 24). CHF BP differs from HC BP because the latter has a smaller area and a different response on radii 5, 6 and 7. In contrast, the BP of CHF and COPD are mostly similar, although a difference is evident in peaks given by the seventh QMB tested at the four temperatures (7, 14, 21 and 28) in COPD.

Table 2 shows the confusion matrix for the classification of CHF status comparing CHF group with HC and COPD controls. Comparing CHF with HC, of the 69 subjects in the testing sample, 56 were correctly classified (81% of accuracy, 95% CI 69%-89%), 6 were false/negative (80% of sensitivity) and 7 were false/positive (82% of specificity). Accuracy

was only marginally modified in sensitivity analyses evaluating the impact of gender, diabetes and smoking habitus (see *Appendix*). Comparing CHF with COPD controls, of the 65 subjects in the testing sample, 45 were correctly classified (69% of accuracy, 95% CI 57%-80%), 11 were false/negative (74% of sensitivity) and 9 were false/positive (63% of specificity). Still marginal changes in accuracy resulted by sensitivity analyses evaluating impact of gender, diabetes, renal disease and smoking habitus (see *Appendix*).

To assess if difference in age may affect the model's accuracy, we excluded subjects < 65 and > 80 years old from CHF and HC obtaining a new population of 60 subjects, 23 CHF with a mean age of 75 years and 37 controls with a mean age of 71 years. We divided the population in a training group (N: 41) and in a testing group (N: 19) and reassessed the prediction of the health status by multivariate VOCs analysis. The accuracy is similar (79% versus 81%), with a sensitivity of 85% and a specificity of 75%. The same procedure has been applied comparing CHF and COPD controls, obtaining a population of 57 subjects, 23 CHF with a mean age of 74.7 and 34 COPD with a mean age of 72.2. The accuracy is similar (68% versus 65%), with a sensitivity of 78% and a specificity of 55%. (*Table 3*).

The PLS-DA could not discriminate the NYHA class of CHF patients nor NT-proBNP values, but can predict EF and sPAP (*Figure 2*).

Among the 86 CHF subjects with measured EF, we obtained a training group of 59 subjects and a testing group of 27 subjects. The RMSECV between observed and predicted EF in the testing group was 14.2. The same procedure was performed on the 80 CHF subjects with measured sPAP, with a training group of 55 subjects and a testing group of 25 subjects; the RMSECV between observed and predicted sPAP in the testing group was 14.1.

In the 61 patients for whom we collected weight change between admission and discharge, PLS-DA was not able to discriminate patients who lost from those who gained weight.

DISCUSSION

Results from this proof of concept study show that exhaled VOCs analysis is able to discriminate patients with decompensated CHF from both controls and COPD patients. Furthermore, it predicts cardiac function markers, such as ejection fraction and systolic pulmonary arterial pressure, but it is not able to identify responders to CHF therapy.

Previous studies assessing the role of exhaled VOCs in CHF has also obtained a good discriminative capacity compared to healthy controls. We are not able to compare our results with those of Samara et al [18], because VOCs were analysed using a selected ion-flow tube mass-spectrometry (SIFT-MS), while we used an electronic nose, furthermore no data are available on the age of the population of that study. Our data, however, can be compared to those of Witt et al. [23], albeit they used an electronic nose based on a metal oxide gas sensor versus our based on QMB sensors. Witt et al. found a discrimination rate between controls and patients with heart failure of 91% compared to 81% in our study, however that study had a smaller sample size (13 patients with decompensated heart failure and 13 controls) and did not use a train/test approach.

In our study, CHF patients were older than controls subjects, but a sensitivity analysis limited to subjects aged from 65 to 80 years showed that age did not affect the discriminating performance of exhaled VOCs pattern. Similarly, discriminating performance was only marginally affected by gender, diabetes, kidney disease or smoking. Since comorbidities characterized CHF patients, but not, by definition, HC, we performed a further analysis using a group of subjects affected by COPD with a profile of comorbidity comparable to that of CHF group. CHF and COPD are diseases both characterized by inflammation, but previous studies analyzing the composition of exhaled air have demonstrated a different pattern of molecules [18, 24]. The accuracy of the discrimination between CHF and COPD group was 69%, lower than the accuracy of the discrimination between CHF and HC

It is likely that inflammation and the metabolic effects of tissue hypoperfusion in CHF and hypoxia in COPD account for analogies in BP between CHF and COPD. Indeed, while intrinsically different, hypoperfusion and hypoxia have similar metabolic effects by impairing the oxidative metabolism and promoting the anaerobe one. Furthermore, both are characterized by increased oxidative stress [25,26] as well as by a variable impairment of glucose tolerance. On the other hand, differences, such as those perceived by the seventh QMD might reflect the disease specificities in bronchial and pulmonary metabolism which are expected on the bases of the very different pulmonary pathology in CHF and COPD. It should also be considered that CHF, but not COPD patients, were in unstable conditions, and this might have widened the differences in BP.

We showed that models based on exhaled VOCs are not able to predict NT-proBNP, while having fair classification capacity with respect to ejection fraction and systolic pulmonary arterial pressure, probably because these parameters have an effect on circulation and cellular metabolism [6]. Exhaled VOCs based model is not able to classify subjects in different NYHA classes and this discrepancy is probably due to the poor reliability of the NYHA classification [27], that may have caused a misclassification and hence a reduction of the discriminative capacity of VOCs. This is especially true for acutely decompensated CHF, where NYHA class is not representative of the usual health status impact of CHF. On the other hand, depressed ejection fraction and systolic blood pressure are key cause of tissue hypoperfusion and, then, previously cited metabolic changes. Thus, the metabolic changes of tissue hypoperfusion qualify as the most likely determinants of BP in CHF. Dedicated and qualitative research is needed to test this hypothesis. Simply, comparing changes in BP and in hemodynamic with the resolution of the CHF decompensation might provide a first insight in this hypothesis.

This study has some limitations. First, the control groups were younger compared to the CHF group, and this may influence the differences in VOCs pattern and hence their discriminative

capacity. Although it has been show, albeit in a younger population, that age does not influence the VOCs pattern [28], we tried to minimize this potential bias using a sensitivity analysis that substantially confirmed the results. Other clinically relevant differences among groups were in gender, in creatinine, percentage of diabetes and percentage of current, former and never smokers, but dedicated sensitivity analyses, even if in smaller samples, suggest that BP still has CHF discriminative capacity. No sensitivity analysis was performed for weight because it is influenced by oedema in CHF patients and then it is not reliable; however, we cannot exclude an influence of weight in the accuracy of discrimination. Second, the relatively small sample size prevented us from analyzing the relationship between etiology, subtypes (reduced, mid-range and preserved ejection fraction) or treatment of CHF and breath-print. Third, we did not measured NT-proBNP in control groups and we were unable to compare the accuracy of VOCs pattern with the accuracy of NT-proBNP in the diagnosis of CHF. Fourth, VOCs pattern may be influenced by room conditions, drugs and food: potential bias of drugs and food was minimized collecting exhaled breath in subjects fasting and without therapy for at least 12 hours. Finally, we recorded VOCs pattern, but we could not identify their components by dedicated analyses. However, we planned to develop and test a simple and inexpensive metabolic marker, whereas methods such as HPLC or spectrophotometry are costly and not suitable for routine clinical use.

This study has also important strengths. It is the first study which analyzed the potentiality of breath-print in CHF subjects in a representative sample of patients. Second, our CHF sample has a mean age of 81 years and this reflects the real CHF population. Third, all subjects have been well characterized by laboratory and echocardiographic assessment. Finally, the method developed to collect and store the exhaled breath does not suffer from technical problems affecting the commonly used storage in plastic bags [21], is non invasive and has a very low

cost (around 10 €) [12] This makes this analysis promising in the diagnosis and management of CHF, not only in elderly people, either alone or combined with clinical or laboratory tests.

CONCLUSION

In this proof-of-concept study we found that VOCs pattern is able to discriminate older CHF patients from HC and COPD subjects and is associated with cardiac function markers. However, VOCs is not able to identify patients more likely to respond to the therapy of an acute decompensation. Thus, in a close future exhaled VOCs may be used to improve the accuracy of the diagnosis of CHF in this population, in which the standard methods may be difficult to implement or less effective than in the younger population. Furthermore assessing whether factors like changes in total body water, oxygen consumption and activation of anaerobic metabolism contribute to characterize patients with a distinctive VOCs pattern will help to understand the meaning of VOCs patterns and, possibly, will shed light on the heterogeneous conditions grouped under the overarching definition of CHF.

Tables

Table 1: Characteristics of CHF (89 subjects) and control groups (healthy controls: 117 subjects; stable COPD controls: 103 subjects).

	CHF* (N = 89)	HC** (N = 117)	COPD*** (N = 103)	Overall ANOVA P-value
Age; mean (SD)	81.2 (9)	64 (9.1)	70.7 (7.9)	<0.001
Males; %	43 (48)	73 (62)	68 (66)	0.03
Height; mean (SD)	161.9 (9.5)	170 (0.1)	167.1 (9.7)	<0.001
Weight; mean (SD)	74,9 (17.5)	72.2 (13.2)	79.1 (17.5)	0.03
Days of inpatient; mean (SD)	6 (3.9)			
Smoking status; N (%)				
Never	49 (56)	42 (36)	11 (11)	<0.01
Smokers	7 (7)	38 (33)	40 (39)	<0.01
Former	33 (38)	37 (32)	52 (50)	<0.01
Diabetes mellitus; N (%)	35 (39)	8 (7)	39 (38)	<0.01
Liver disorders; N (%)	7 (8)	0 (0)	6 (10)	0.87
COPD; N (%)	0 (0)	0 (0)	103 (100)	<0.01
Creatinine; mean (SD)	1.1 (0.3)		1 (0.3)	0.005
Diuretics;	89 (100)	0 (0)	13 (13)	
β-blockers	44 (49)	0 (0)	32 (31)	
ACEi/ARBs	55 (62)	44 (38)	35 (34)	
LABA / LAMA / ICS	0 (0)	0 (0)	103(100)	

**CHF: Congestive heart failure; **HC: Healthy controls; ***COPD: Chronic obstructive pulmonary disease*

Table 2: Discrimination obtained in the testing sample (33% of the whole population) applying the model achieved using PLS-DA on the training sample (66% of the whole population). Upper panel: CHF (N: 30) vs. controls free from chronic diseases (N: 39). Bottom panel: CHF (N:30) vs. COPD controls (N: 35).

	Reference	
Prediction	**CHF***	**HC****
CHF	24	7
Controls	6	32

	Reference	
Prediction	**CHF***	**COPD*****
CHF	19	9
COPD	11	26

**CHF: Congestive heart failure; **HC: Healthy controls; ***COPD: Chronic obstructive pulmonary disease*

Table 3: Sensitivity analysis with the discrimination obtained in the testing sample (33% of the whole population), applying the model achieved using PLS-DA on the training sample (66% of the whole population). Upper panel: CHF (N: 7) vs. controls free from chronic diseases aged from 65 to 80 years old (N: 12). Bottom panel: CHF (N: 7) vs. COPD controls aged from 65 to 80 years old (N: 11).

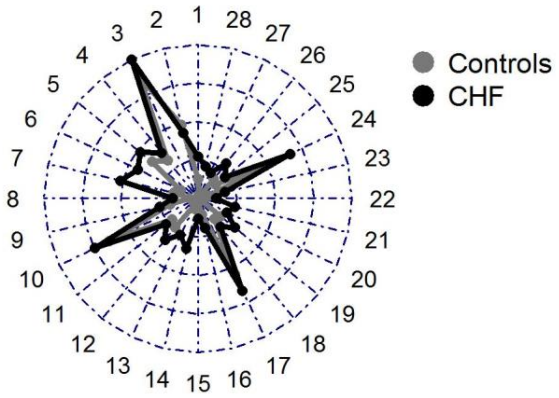
	Reference	
Prediction	**CHF***	**HC****
CHF	6	3
Controls	1	9

	Reference	
Prediction	**CHF***	**COPD*****
CHF	6	2
COPD	1	9

**CHF: Congestive heart failure; **HC: Healthy controls; ***COPD: Chronic obstructive pulmonary disease*

Figures

Figure 1: Patient's breath-print (BP) is composed of the 28 BIONOTE QMB responses (numbers from 1 to 28 on the figure are the seven BIONOTE QMB sensors tested per four different desorption temperatures). Mean CHF BP was compared with mean healthy control BP (upper panel) and mean COPD control BP (bottom panel). Values on radii are frequency shifts (Hz) of each of the QMB respect to their typical resonance frequency. The CHF and COPD breath-prints share frequency shifts on radii 3, 10, 17 and 24, whereas the COPD breath-print is characterized by lower area and distinctive frequency shifts along the radii 7, 14, 21 and 28; these shifts correspond to clusters of VOCs qualifying as the hallmark of COPD. The breath-print of controls free from chronic diseases is characterized by lower frequency shifts along all radii, particularly on radius 7.



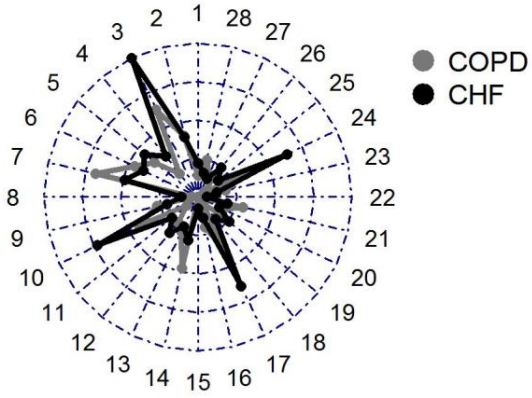
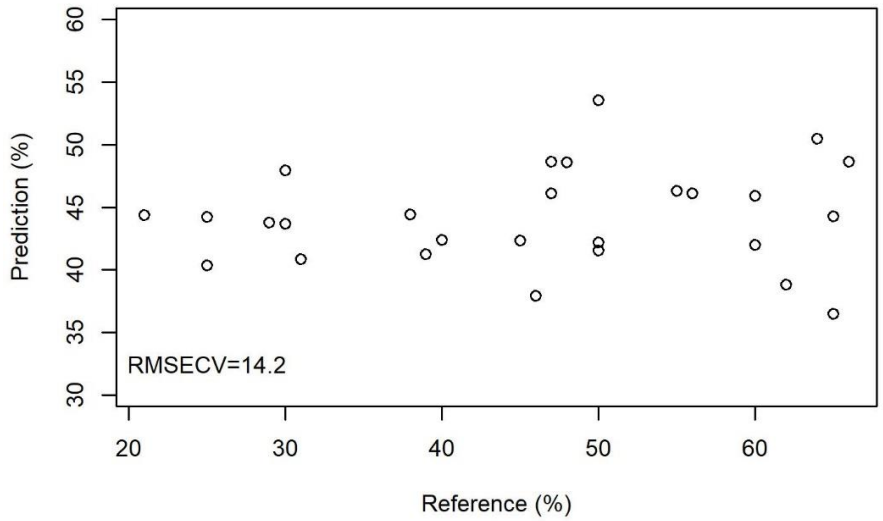
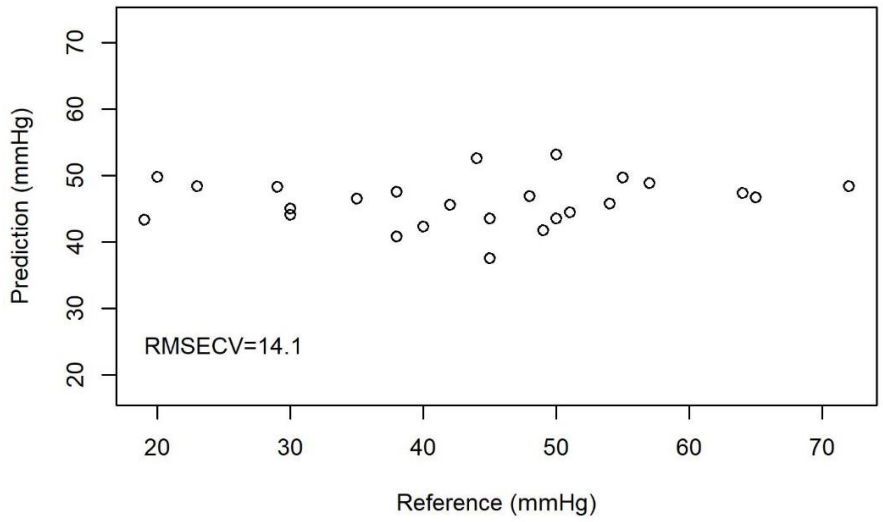


Figure 2: Prediction of EF (N: 27, upper panel) and sPAP (N: 25, bottom panel). We split population in a training sample (66% of the whole population) and we used a PLS-DA algorithm to create a machine model of prediction. Model was cross-validated using "repeated k-fold cross validation" and subsequently tested in a testing sample (33% of the whole population). Error in model prediction has been summarized as RMSE in cross validation.





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APPENDIX

Table S1: Sensitivity analysis in the population without diabetes (CHF: 54, HC: 86, COPD: 64) with the discrimination obtained in the testing sample (33% of the whole population), applying the model achieved using PLS-DA on the training sample (66% of the whole population). Upper panel: CHF (N: 18) vs. HC (N: 29). Bottom panel: CHF (N: 18) vs. COPD controls (N: 21).

Accuracy 85%, CI 95% 72%-94%

	Reference	
Prediction	**CHF***	**HC****
CHF	15	4
Controls	3	25

**CHF: Congestive heart failure; **HC: Healthy controls*

Accuracy 62%, IC 95% 45%-77%

	Reference	
Prediction	**CHF***	**COPD****
CHF	12	9
COPD	6	12

**CHF: Congestive heart failure; **COPD: Chronic obstructive pulmonary disease*

Table S2: Sensitivity analysis in the male population (CHF: 43, HC: 73, COPD: 68) and in the female population (CHF: 46, HC: 44, COPD: 35) with the discrimination obtained in the testing sample (33% of the whole population), applying the model achieved using PLS-DA on the training sample (66% of the whole population). Upper panel: male CHF (N: 14) vs. male HC (N: 24) and female CHF (N: 15) vs. female HC (N: 14). Bottom panel: male CHF (N: 18) vs. male COPD controls (N: 23) and female CHF (N: 18) vs. female COPD controls (N: 11).

Male accuracy 82%, CI 95% 66%-92% **Female accuracy 76%, CI 95% 56%-90%**

	Reference	
Prediction	**Male CHF***	**Male HC****
Male CHF	10	3
Male Controls	4	21

	Reference	
Prediction	**Female CHF***	**Female HC****
Female CHF	11	3
Female Controls	4	11

**CHF: Congestive heart failure; **HC: Healthy controls;*

Male accuracy 68%, CI 95% 50%-82% **Female accuracy 73%, CI 95% 52%-88%**

	Reference	
Prediction	**Male CHF***	**Male COPD****
Male CHF	9	7
Male COPD	5	16

	Reference	
Prediction	**Female CHF***	**Female COPD****
Female CHF	11	3
Female COPD	4	8

**CHF: Congestive heart failure; **COPD: Chronic obstructive pulmonary disease*

Table S3: Sensitivity analysis in non smoking population (CHF: 82, HC: 79, COPD: 63), defined as the sum of never smokers and former smokers, with the discrimination obtained in the testing sample (33% of the whole population), applying the model achieved using PLS-DA on the training sample (66% of the whole population). Upper panel: CHF (N: 27) vs. HC (N: 21). Bottom panel: CHF (N: 27) vs. COPD controls (N: 23).

Accuracy 79%, CI 95% 65%-89%

	Reference	
Prediction	**CHF***	**HC****
CHF	20	3
Controls	7	18

**CHF: Congestive heart failure; **HC: Healthy controls;*

Accuracy 76%, IC 95% 62%-87%

	Reference	
Prediction	**CHF***	**COPD****
CHF	20	5
COPD	7	18

**CHF: Congestive heart failure; **COPD: Chronic obstructive pulmonary disease*

Table S4: Sensitivity analysis in the population with creatinine ≤ 1.2 mg/dL (CHF: 59, COPD: 51) with the discrimination obtained in the testing sample (33% of the whole population), applying the model achieved using PLS-DA on the training sample (66% of the whole population).

Accuracy 70%, CI 95% 53%-84%

	Reference	
Prediction	**CHF***	**COPD****
CHF	15	4
COPD	3	25

**CHF: Congestive heart failure; **COPD: Chronic obstructive pulmonary disease*

CHAPTER 4 CLUSTER ANALYSIS ON BREATH-PRINT OF NEWLY DIAGNOSED COPD PATIENTS: EFFECTS OF THERAPY

Simone Scarlata, Panaiotis Finamore, Simona Santangelo, Gilda Giannunzio, Giorgio Pennazza, Simone Grasso, Marco Santonico, Raffaele Antonelli Incalzi, J Breath Res 2018; 12: 036022.

ABSTRACT

Background: Chronic obstructive pulmonary disease (COPD) is a highly heterogeneous disease and airflow limitation and symptoms only partially capture such heterogeneity. Since COPD is known to affect the production of volatile organic compounds (VOCs), we aimed at verifying to which extent exhaled VOCs can characterize newly diagnosed COPD patients and changes in response to inhaled therapy.

Materials and methods: Fifty newly diagnosed COPD patients were consecutively recruited among those attending the pulmonary medicine outpatient clinic at "Campus Bio-Medico" University Hospital. VOCs were collected using the Pneumopipe® and analysed by the BIONOTE electronic nose both at baseline and after 12 weeks of inhaled therapy. Patients were grouped using K-mean cluster analysis on BIONOTE responses and the obtained clusters compared *via* non parametric tests.

Results: We identified three clusters of subjects: a) without remarkable comorbidities; b) with air trapping and higher BODE index score (mean 1.2); c) without air trapping and with a lower BODE index. Inhaled bronchodilators caused a quantitative reduction in VOCs amount, while inhaled steroids provided a qualitative modification of the breath profile.

Conclusion: VOCs patterns categorize newly diagnosed COPD subjects. VOCs production declines after bronchodilators administration and changes in quality after topic steroid treatment.

INTRODUCTION

Defining Chronic Obstructive Pulmonary Disease (COPD) through airflow obstruction alone does not reflect the heterogeneous nature of the disease. Pulmonary function tests, originally leading to COPD severity stratification, weakly correlated with patients' symptoms and response to the inhaled therapy. Thus, the 2017 GOLD guidelines have recommended to use also clinical variables such as patients' dyspnoea and annual number of exacerbations to stage COPD severity [1]. Nonetheless, a deeper knowledge of clinical and bio-metabolic markers of disease phenotyping is currently considered a pivotal aim. For these reasons, the research of newer biomarkers and the development of innovative technologies to phenotype COPD is crucial.

In COPD, pulmonary and systemic inflammation, hypoxemia and cellular oxidative stress contribute to shape the pattern of exhaled volatile compounds (VOCs) [2]. The electronic nose (e-nose) technology is able to provide a finger print of patients' exhaled breath and, for such a reason, is currently used for research purposes. Indeed, the e-nose has been proved to identify breath patterns of COPD patients and to differentiate them from healthy controls, asthmatics and heart failure patients [3–5]. In people with COPD, VOCs pattern is characterized by fair intra- and between-day repeatability [6]. VOCs pattern proved to discriminate patients with COPD from healthy non smokers controls and asthmatics [4] and it has been shown to correlate with severity of bronchial obstruction and patients' functional status [7,8]. Furthermore, the finding of an association between VOC pattern and inflammatory markers suggests that VOCs analysis might help to phenotype patients with COPD [9].

VOCs pattern is reproducible in healthy subjects and hypoxemic COPD patients and it also correlates with expiratory flows of the small airways, site of important inflammation in COPD [10], and, to a lesser extent, with indexes of air trapping and DLCO [7]. Furthermore, small

airways extensively contribute to bronchial obstruction and correlate with patient centred outcomes in COPD [11].

Thus, it would be of interest to explore the effect of topic treatment on the VOCs pattern, dubbed breath-print (BP), of COPD patients, since inhaled therapy has been shown to decrease the bronchial inflammation, both in central and peripheral airways [11]. At the current state of the art, in fact, it is unknown whether the e-nose can detect changes in VOCs determined by inhaled therapy.

We aimed first at assessing BPs of newly diagnosed COPD patients and characterizing clusters of patients defined only on the basis of BP. Our latter objective was to reassess BPs after a 12 week course of topical bronchodilator or anti-inflammatory therapy and to verify whether and to which extent VOCs pattern and the baseline clustering vary.

MATERIALS AND METHODS

Fifty newly diagnosed, stable and untreated COPD patients were consecutively enrolled among those attending the pulmonary outpatient clinic of the University Hospital "Campus Bio-Medico". Diagnosis of COPD was based on a post bronchodilator FEV1/FVC ratio < 0·7 [12]. COPD was considered stable if the levels of physical activity and dyspnoea, rated respectively through the PASE questionnaire and the mMRC score, and either volume or quality of the sputum were considered the usual ones by the patient [13,14]. Patients receiving oral corticosteroids for any cause, and those with a diagnosis of cancer, asthma, or any other known respiratory condition potentially affecting VOCs' pattern, ascertained by medical records and medical history, were excluded [15]. Due to the potential effects of hypoxaemia on the breathprint [16], oxygen dependent subjects were also excluded. The investigation conforms to the principles outlined in the Declaration of Helsinki. All the study participants provided written informed consent. The study protocol was approved by the local Ethical Committee (protocol number: 4711 CBM).

Design of the study

The study design is resumed in Figure 1.

At baseline, patients underwent a multidimensional assessment including: clinical and anthropometric features, main comorbidities ascertained by medical charts and clinical examination, pulmonary function tests, blood gas analysis and six-minute walking test. Exhaled breath was collected, in the same room, both at baseline and after a 12 week therapeutic trial of topical bronchodilators (Long Acting Muscarinic Agents –LAMA- or Long Acting B_2-adrenergic Agents -LABA) and/or Inhaled Corticosteroids (ICS) prescribed according to

current GOLD guidelines [1]. During this period, any concomitant, other than respiratory, pharmacological therapy was left unchanged with regard to baseline.

The choice of the medication regimen was oriented according to GOLD international recommendation, but also taking into account the presence of comorbidities and/or the patient's attitude to correctly use a specific inhaler's device.

Pulmonary function tests

Respiratory function tests, six-minute walk test, and blood gas analysis were performed at morning smoking free for at least 12 hours. Forced expiratory volumes were measured using a water-sealed bell spirometer (Biomedin, Padua, Italy) following the acceptability and reproducibility criteria proposed by the American Thoracic Society and the European Respiratory Society (ATS/ERS) [17]. The manoeuver was repeated after inhalation of salbutamol, and post-bronchodilator data were used to characterize COPD patients [12]. Peripheral, intermediate and proximal airflows were expressed by MEF25, MEF50 and MEF 75 (the maximum expiratory flow over the last 25%, 50% and 75% of the forced vital capacity, respectively). Total Lung Capacity (TLC) and Residual Volume (RV) were obtained using the Helium-rebreathing technique [18]. Values were expressed as a percentage of the predicted value calculated using standardized reference equations. Diffusing capacity of the lung for carbon monoxide corrected for alveolar volume (Krogh's coefficient – KCO –), expressed as [ml/(min*mmHg)], was measured according to current ATS/ERS guidelines [19]. It was obtained by a single-breath method using a dedicated gas-chromatography system (Biomedin, Padua, Italy). A five-minute resting period was maintained before the measurement. Finally, fractional exhaled nitric oxide (FeNO) determination was obtained in all patients to characterize the eosinophilic component using NIOX VERO® device, following 2011 ATS guidelines [20].

Breath sampling

Exhaled breath was collected in the morning, with patients fasting and smoking free for at least 12 hours (8 hours in diabetics subjects to avoid ketogenesis), and close after rinsing the mouth to minimize the influence of VOCs produced by oral cavity bacteria. Neither alcoholic beverages nor medications, others than inhaled therapy, were allowed within 12 hours before the collection. Patients breathe for three minutes, at tidal volume, into a dedicated storage device for direct sampling of exhaled breath on adsorbing cartridge (Pneumopipe®, European patent n. 12425057.2, Rome- Italy). The adsorbent cartridge used in this work is the Tenax GR, by Supelco. Exhaled breath collection on cartridges of adsorbent materials offers numerous advantages over bag use. In fact, it enables to pre-concentrate the sample and guarantees high sample storage stability and great transport practicality [21,22]. The mouthpieces used for the sampling apparatus were disposable.

Breath delivery and sensors

Following breath collection, the cartridge was thermally desorpted into the sensors chamber of the electronic nose using a device able to uniformly heat the tube at four different temperatures (50°C, 100°C, 150°C and 200°C), and finally cleaning the cartridge holding the temperature at 300°C for five minutes. According to the internal protocol developed for VOCs analysis, all cartridges were stored at 4°C and desorbed within 4 hours from sampling [21].

The electronic nose used in the study, named BIONOTE, is composed of seven quartz microbalance (QMB) sensors covered with metalloporphyrins [23] as chemical interactive materials. This technology has been previously validated in calibration experiments with gases

vapours [24]. Supplementary information, including a detailed list of validation studies, is available elsewhere [21].

The fingerprint of the exhaled breath (BP) is made up by twenty-eight responses, which is the result of the seven sensors tested at the four different temperatures of thermal desorption.

Statistical method

We reported the characteristics of our sample using descriptive statistics (mean and standard deviations for continuous variables, proportion for categorical variables). In order to assess the optimum number of factors to take into account, a scree plot was performed and three clusters were found to be optimal. To avoid sub-optimal partitions, we set fifty random starting points. To distribute the population within three clusters we applied the unsupervised *K-means* clustering method on BIONOTE sensors responses obtained at baseline. Once population has been split out into the three clusters, groups were compared with regard of respiratory function, anthropometric and clinical characteristics using a non-parametric Kruskal-Wallis test, for continuous variables, and Fisher exact test, for dichotomous variables. In order to test clusters reliability, we have used a partial least square discriminant analysis to investigate the clustering input parameters, to verify whether it significantly discriminated membership between clusters. The 94% agreement we found demonstrates that the discriminant function model was very accurate and therefore reliable for predicting case membership to the clusters.

BPs have been represented both with radar-plot and with box-plot. The first is composed of twenty-eight equi-angular radii, representing the twenty-eight BIONOTE sensor responses, and the BP is the result of the connection of the magnitude of each radius length. The shape of the radar plot provides a qualitative evaluation of the breath print whilst a semi-quantitative interpretation of the sampled VOCs can be obtained through the determination of the area under

the BP profile (AUBP). Further details about the AUBP calculation can be found elsewhere [25].

All the analyses were performed using R version 3.3.0 (The R Foundation for Statistical Computing, Wien, Austria, 2016).

RESULTS

The fifty subjects enrolled – mean age: 68 (SD:8) years, 70% males – resulted to be mainly affected by moderate airway obstruction – mean FEV1 74% (SD: 16·9%) of predicted and mean FVC 98% (SD: 16·9%) of predicted – with preserved 6 minute walking distance. Only thirteen out of 50 had important symptoms or/and more than two episodes of acute exacerbations *per year* (GOLD A: n=37; GOLD B: n=4; GOLD C: n=6; GOLD D: n=3).

Three clusters were identified in the study cohort, with distinct clinical, functional and blood gas profiles (*Table 1 and Table 2*). Cluster number one (n=24) was characterized by the least severe obstruction of both central and peripheral airways and least impairment of indexes of gas exchange, as expressed by higher KCO. On the opposite, cluster number three (n=19) displayed the most severe airways obstruction and KCO impairment; furthermore, this subset of patients also displayed a significantly higher BODE score. Finally, cluster number two showed intermediate impairment of distal airways indexes but, interestingly, it was characterized by the absence of remarkable comorbidities. *Figure 2* represents the radar-plots of the three clusters at baseline. Noteworthy, clusters one and three share the same shape, but present different areas (Cluster one AUBP: 2487.9, 95%CI 2120-2855.8 vs Cluster three AUBP: 1183.5, 95%CI 965.4-1401.7), indicating similar qualitative compounds profile, but quantitative differences. On the other hand, the cluster number two presented a different shape, indicating a cluster specific qualitative profile. *Figure 2* also displays the sensor-by-sensor responses between the three clusters, suggesting that VOCs pattern differences are caught mainly by selected sensors (i.e. #3[Zn-TPP]; #4 [Mn-TPP], #6 [Sn-TPP] and #7 [Ru-TPP]).

Figure 3 shows the changes in the BPs of the three groups after a 12 week trial with inhaled therapy. Mean post treatment functional indexes showed an overall improvement in terms of FEV1, MEF25, RV and KCO. Interestingly, BPs of clusters number one and three tended to overlap AUBP cluster one radar plot: 1858.3, 95%CI 1146.6-2569.7 vs Cluster three AUBP:

1508.5, 95%CI 1045.3-1971.8), whilst that of cluster number two maintained its shape, but mildly increased its area (Cluster two AUBP before treatment: 1398.6, 95%CI 960-1838 vs Cluster two AUBP after treatment: 2466, 95%CI 1337.2-3596.5). When we compared the sensor-by-sensor response after treatment we observed that inhaled therapy modifies VOCs, equalizing the responses of the QMB sensors. Boxplots of *Figure 3*, in fact, clearly show that the differences between groups observed at baseline are significantly less evident in the BPs obtained during inhaled therapy.

At baseline, thirty patients received inhaled bronchodilators (LAMA, LABA or the combination of the two), and twenty patients received inhaled corticosteroids in adjunction to inhaled bronchodilators. *Figure 4* represents boxplots and radar-plots of patients treated with inhaled bronchodilators. Sensor-by-sensor responses suggest that VOCs pattern differences after 12 weeks of treatment are caught mainly by the sensor #1 [Cu-TPP] (radii 1, 8, 15, 22) and sensor #4 [Mn-TPP] (radii 4, 19, 25) almost at all four different temperatures, although there are statistically significant differences also on radii 10, 17, 18, 24 and 26. Noteworthy, radar-plots share the same shape and only present a quantitative reduction (AUBP before treatment: 1978.6, 95%CI 1632.2-2325 vs AUBP after treatment: 1521.2, 95%CI 973.4-2069). Comparing the effect of inhaled corticosteroids on radar-plot with that of inhaled bronchodilators used alone, we observed a change in the BP profile (qualitative change) and not in its area (AUBP before treatment: 1842.7, 95%CI 1410.2-2275.2 vs AUBP after treatment: 1857.9, 95%CI 1181-2534.8): VOCs changes are caught mainly by sensor #7 [Ru-TPP] (radii 7, 14, 21 and 28) at all four temperatures (*Figure 5*).

DISCUSSION

This study shows that well distinguished BPs may be recognized in the naïve COPD populations and these patterns change in distinctive ways depending upon whether the patient has been prescribed LAMA and/or LABA alone or any combinations of inhaled drugs including ICS.

Indeed, that BPs could identify potential phenotypes within a COPD cohort of patients has already been suggested by Basanta and colleagues by showing that sputum eosinophilia and exacerbation frequency correlated with different BPs in a principal component analysis and discriminant function analysis with leave-one out cross validation [2]. Similarly, Fens has successfully demonstrated the role of VOCs in characterizing COPD patients with and without eosinophilic inflammation using e-nose technology [9]. In these studies, however, patients were regularly receiving an inhaled therapy before the study recruitment and baseline evaluation and, importantly, factors known to affect the BP were not taken into account. Both current and former smoking, in fact, as well as the number and the nature of comorbidities, were associated with relevant changes in BP [26]. Furthermore, our cluster analysis showed that a the BODE index, a multidimensional scoring system and capacity index, severity of airflow limitation and gas exchange impairment, represented by MEF 75% and KCO, respectively, are associated with distinctive changes in BP. The relationship between BP and airways disease could be explained by the important inflammation characterizing the airways in COPD [27]. Indeed, exhaled breath profiles obtained with an eNose resulted to be significantly associated with pulmonary neutrophilia in patients with COPD in a principal component analysis [9]. Interestingly, in that study, the second principal component of analysis was the one effectively correlated with inflammatory cell counts. Accordingly, the information on the inflammatory subtype might be carried by some part of the exhaled breath differing from that accounting for the discrimination between diseased and non-diseased subjects. The evidence that a not

invasive, safe, cost and time saving approach, as the one provided by breath analysis, is able to catch these COPD-specific pathophysiologic aspects opens the way to many potential applications.

We found the cluster number three to display a higher BODE index. Indeed, considering that clusters did not differ amongst each other in term of BMI, degree of obstruction and 6-minute walking distance, we can conclude that the main determinant of this significant difference may be a higher perception of dyspnoea by more airflow-limited subjects.

Data from this study show that comorbidities affect the BP and contribute to cluster determination in COPD. The impact of comorbidities on both metabolic and clinical dimensions of COPD patients is well known, with several conditions being highly prevalent among COPD patients, especially the oldest [28,29]. Evidence also suggests that systemic inflammation may be the link between COPD and comorbidities; however, this issue is still debated [30]. In COPD, chronic comorbidities proved to affect survival, and important outcomes, like dyspnoea, exercise capacity, quality of life, healthcare utilization, and exacerbation risk [31]. The potential of detecting comorbidity-related changes in the BP has already been reported in an obstructive sleep apnoea syndrome population [26], where heavier comorbidity identified a distinctive breath pattern. The fact that this finding was replicated in COPD points at the need of a really comprehensive approach to COPD patients when trying to categorize this broad diseased population. (See *Table 1*).

To our knowledge, this is the first study evaluating changes in BP due to inhaled therapy. We observed a different behaviour of BP with regards to the different inhalation regimen adopted: bronchodilators alone did not affect the shape of the BP, and hence the quality of exhaled VOCs, but only were associated with a mild decrease in sensors responses leading to a reduction, even not statistically significant, of the BP area; on the other hand, inhaled steroids

significantly modified the shape of the radar plot profile, which is consistent with a qualitative change in VOCs production. ICS are currently indicated for the treatment of airway inflammation and, potentially, to prevent airway remodelling in patients with chronic airway diseases. However, the indications to ICS are controversial [32]. In a proof-of-concept study, van der Schee et al showed that breath analysis was superior to sputum eosinophil and fraction of exhaled nitric oxide measurement in predicting oral steroid responsiveness in patients with mild-to-moderate asthma who were previously withdrawn from steroids [33]. The BPs also showed a strong correlation with eosinophil counts in sputum, thus strengthening the pathophysiologic link between exhaled BP and inflammatory status within the lung. A similar mechanism, likely involving the neutrophilic inflammation featuring COPD, might be responsible for the observed differences in our study.

This study has some limitations. First, the small sample size and its proof-of-concept design, make present data preliminary in nature. Second, we recorded VOCs and, then, BP, but we could not identify individual gases by dedicated analyses (i.e. Gas Chromatography or Mass Spectrometry). Furthermore, lack of information on selected indexes of airways inflammation prevented us from exploring the biological bases of differences in BPs among clusters and after inhaled therapy. Similarly, we were not able to perform any correlation between breath print and markers of systemic inflammation such as the C-reactive protein. Indeed, we were able to measure the nitric oxide (NiOX) levels in the breath finger print and found no differences in the mean values of this marked amongst the three clusters, excluding that the observed differences in the BP could be attributed to changes in the NiOX inflammatory pathway induced by the inhaled therapy.

This study also has notable strengths. Indeed, it relies upon a highly standardized diagnostic method and deals with a well characterized population. Furthermore, patients at baseline were

naïve to therapy, and this allowed assess the true BP of the disease. Finally, the systematic recording of comorbidities allowed assess their role in characterizing VOCs based clusters.

CONCLUSION

The present study allows hypothesize that VOCs analysis via e-nose technology might help to phenotype COPD patients and to characterize the response to inhaled therapy. If this hypothesis will be confirmed, VOCs analysis would promote our knowledge of disease progression and therapeutic response. For instance, changes in BP after a period of treatment might be a marker of patients' compliance to inhaled therapy and inhaler devices. Further research is needed to confirm these preliminary findings and to translate them in easily measurable diagnostic tools.

Table 1. Clinical characteristics of the three clusters of naive COPD patients.

	Cluster 1 (N:24)	Cluster 2 (N:7)	Cluster 3 (N:19)	p-value
Age; mean (SD)	65.9 (8.3)	70.7 (6.1)	70.6 (6.2)	0.11
Male; N (%)	19 (79)	5 (71)	11 (58)	0.32
BMI; mean (SD)	25.5 (3.3)	26.1 (3.8)	24.7 (3.2)	0.45
Smoke				
Former smokers; N (%)	7 (29)	2 (29)	7 (37)	0.17
Smokers; N (%)	14 (61)	2 (29)	11 (58)	0.17
GOLD stage				
GOLD A	18 (75)	6 (86)	13 (68)	0.89
GOLD B	1 (4)	1 (14)	2 (11)	0.89
GOLD C	3 (12)	0 (0)	3 (16)	0.89
GOLD D	2 (8)	0 (0)	1 (5)	0.89
Exacerbation/year				
0-1	20 (83)	7 (100)	17 (89)	0.6
>1	4 (17)	0 (0)	2 (11)	0.6
BODE index	0.5 (1.4)	0.3 (0.5)	1.2 (1.3)	<0.01
Comorbidities				
Hypertension; N (%)	11 (48)	2 (29)	9 (47)	0.99
Diabetes; N (%)	2 (9)	0 (0)	2 (11)	0.79
CHF; N (%)	0 (0)	0 (0)	3 (17)	0.09
CKD; N (%)	1 (4)	0 (0)	1 (6)	0.87
CVD; N (%)	6 (26)	0 (0)	5 (28)	0.49
Mean n° of comorbidities	0.83 (0.7)	0.29 (0.4)	1.05 (0.6)	<0.001
Inhaled Therapy				
LABA; N (%)	8 (33)	0 (0)	4 (21)	0.54
LAMA; N (%)	6 (25)	3 (43)	6 (32)	0.54
LABA/LAMA; N (%)	1 (4)	0 (0)	2 (10)	0.54
LABA/ICS; N (%)	9 (38)	4 (57)	7 (37)	0.54

CHF: Congestive heart failure; CKD: Chronic kidney disease; CVD: Chronic vascular disease; LABA: Long-acting β_2-agonist; LAMA: Long-acting muscarinic antagonist; ICS: inhaled corticosteroid.

Table 2: Respiratory characteristics of the three clusters of naive COPD patients.

	Cluster 1 (N:24)	Cluster 2 (N:7)	Cluster 3 (N:19)	p-value
Spirometry				
FEV1cc; mean (SD)	2.2 (0.5)	2 (0.6)	1.8 (0.7)	0.18
FEV1pct; mean (SD)	76.9 (16.5)	74.1 (10)	71.6 (19.5)	0.41
TLC cc; mean (SD)	6.2 (1.6)	6.8 (1.6)	6.2 (1.7)	0.62
TLC pct; mean (SD)	103.5 (11.5)	107.4 (17.4)	105.2 (16.5)	0.84
FEF25-75cc; mean (SD)	1 (0.4)	1 (0.4)	0.8 (0.5)	0.09
FEF25-75pct; mean (SD)	32.9 (9.9)	34.6 (11.2)	29 (13.5)	0.27
MEF75cc; mean (SD)	3.8 (1.5)	3.4 (1.9)	2.6 (1.3)	0.02
MEF75pct; mean (SD)	58.6 (20.5)	50.9 (22.2)	42.8 (18.5)	0.03
MEF50; mean (SD)	1.3 (0.5)	1.4 (0.5)	1.1 (0.7)	0.14
MEF50pct; mean (SD)	32.6 (10.8)	34.3 (9.8)	29.2 (16.8)	0.18
MEF25cc; mean (SD)	0.4 (0.2)	0.3 (0.1)	0.3 (0.1)	0.17
MEF25pct; mean (SD)	27.2 (9.9)	25.6 (5.4)	26.3 (11.5)	0.79
RV cc; mean (SD)	2.9 (0.7)	3.4 (0.9)	3.1 (0.9)	0.44
RV pct; mean (SD)	124.3 (29.7)	141.1 (36.7)	130.6 (38.1)	0.53
RV/TLC pct; mean (SD)	110.9 (23.3)	123 (18.5)	121.1 (23.2)	0.27
KCO pct; mean (SD)	50.5 (14.6)	46.9 (17.6)	38.3 (10.6)	0.02
Haemogas-analysis				
pH; mean (SD)	7.43 (0.03)	7.41 (0.02)	7.42 (0.02)	0.02
pO2; mean (SD)	81.5 (10.9)	80.6 (6.8)	76.9 (10.8)	0.49
pCO2; mean (SD)	36.9 (4.1)	41.9 (3.2)	39.2 (4.5)	0.02
$HCO3^-$; mean (SD)	25.0 (1.8)	25.8 (1.9)	25.0 (1.9)	0.56
FeNO ppb; mean (SD)	22.6 (22.9)	19 (12.7)	23.3 (14.3)	0.94
6' minute walking test				
Meters; mean (SD)	448.5 (58.5)	431.7 (58.9)	417.6 (61.1)	0.11
Meters pct; mean (SD)	90.7 (15)	86.0 (14.2)	88.6 (12.8)	0.62
Reversibility test – Salbutamol –				
Partial response; N (%)	12 (50)	5 (71)	8 (42)	0.42
Absence of response; N (%)	12 (50)	2 (29)	11 (58)	0.42

FEV1: forced expiratory volume in the first second; TLC: total lung capacity; FEF25-75: forced expiratory flow 25-75%; MEF75: maximum expiratory flow 75% of the vital capacity; MEF50: maximum expiratory flow 50% of the vital capacity; MEF25: maximum expiratory flow 25% of the vital capacity; RV: residual volume; KCO: carbon monoxide transfer coefficient (Krogh's coefficient).

Figure 1*:* Study design.

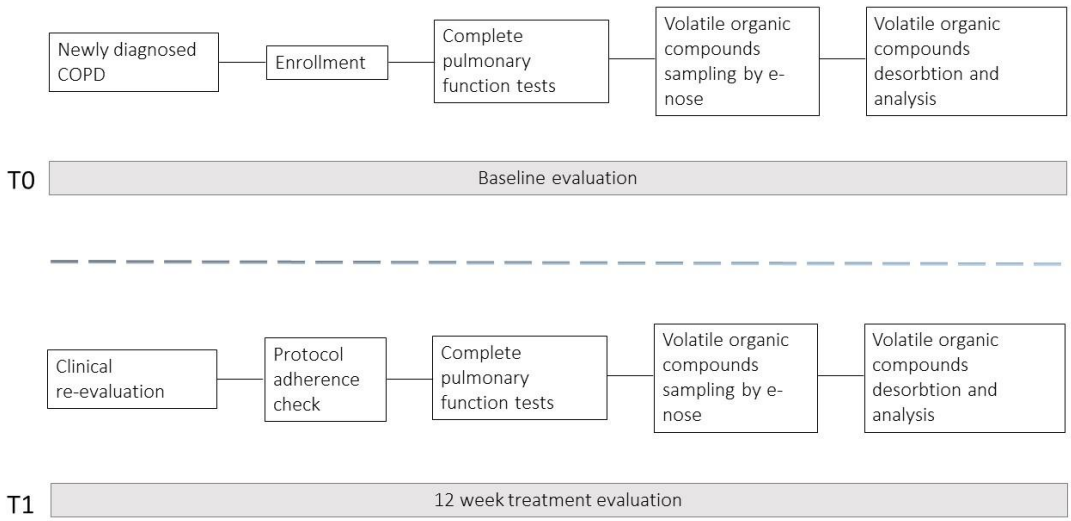


Figure 2: At the centre, radar plots comparing the mean breath print (BP) of the three clusters of COPD patients at baseline. Numbers outside the circle represent the twenty-eight BIONOTE responses (seven sensors tested at four different temperatures) and the BP is obtained connecting the values on the radii, which represent each QMB shift (in Hertz) from its baseline frequency. Cluster 1 and Cluster 3 have a similar QMB sensors activation pattern although Cluster 3 is characterized by lower frequency shifts and lower AUBP. Cluster 2 differs completely from the others, with distinctive frequency shifts along the radii 8, 16, 24 and 28. Box plots at the corners compare frequency shifts (Hz) of each of the QMB respect to their typical resonance frequency in the three clusters at baseline. QMB sensors are reported on *x* axis, while frequency shift (Hz) on *y* axis. Clusters differ particularly on #3 [Zn-TPP], #4 [Mn-TPP], #6 [Sn-TPP] and #7 [Ru-TPP] at four temperatures.

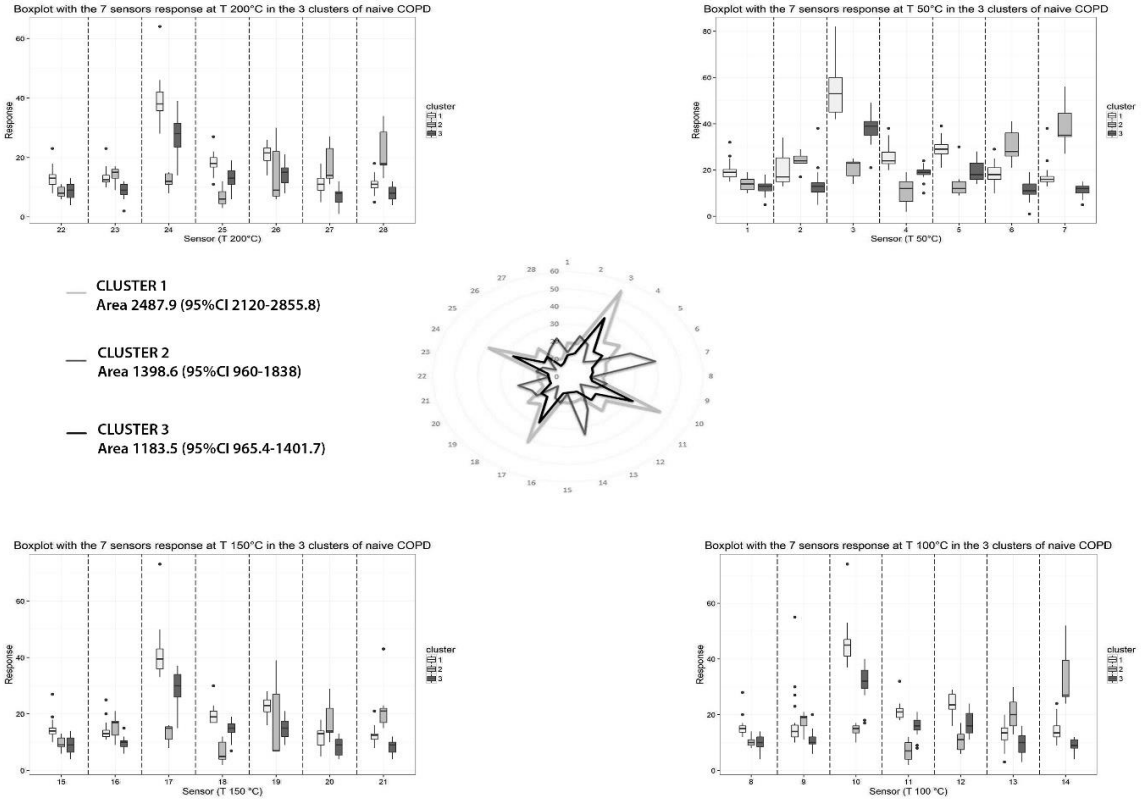


Figure 3: At the centre, radar plot comparing the mean breath print (BP) of the three clusters of COPD patients after 12 weeks of inhaled therapy. See legend to Figure 2 for the description of BP graphical representation. Compared with baseline, all clusters frequency shifts tend to equalize, while the shape is preserved. Box plots at the corners compare frequency shifts (Hertz) of each of the QMB with respect of their typical resonance frequency in the three clusters after 12 weeks of inhaled therapy. QMB sensors are reported on *x* axis, while frequency shift (Hz) on *y* axis. Differences among clusters on #3 [Zn-TPP], #4 [Mn-TPP]; #6 [Sn-TPP] and #7 [Ru-TPP] sensor are reduced at all four temperatures, particularly those between Cluster 1 and Cluster 3.

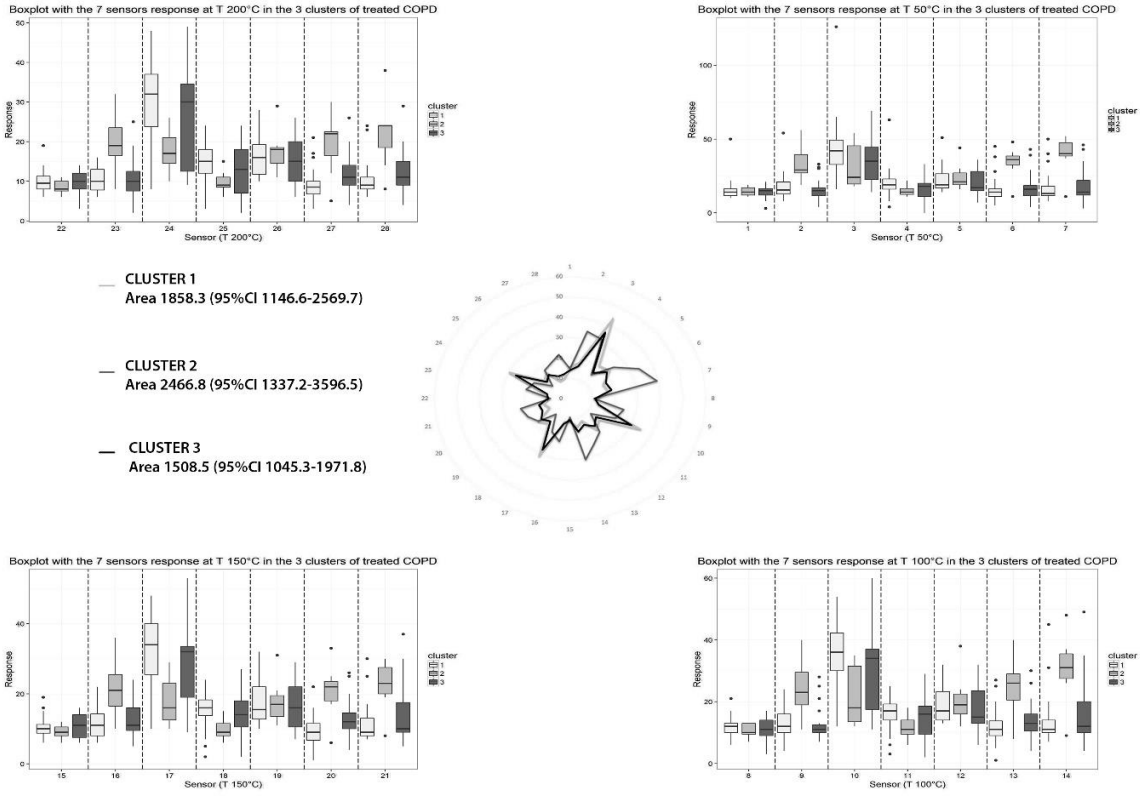


Figure 4: At the centre, radar plot comparing the mean breath print (BP) of the three clusters before and after treatment with inhaled bronchodilators (LAMA, LABA or their combination). See legend to Figure 2 for the description of BP graphical representation. BPs have a similar QMB sensors activation even if BP after treatment is characterized by lower frequency shifts, particularly on radii 1, 4, 8, 10, 15, 17, 18, 19, 22, 24, 25 and 26, and a lower mean area**.** Box plots at the corners compare frequency shifts (in Hertz) of each of the QMB with respect of their typical resonance frequency before and after bronchodilator treatment. QMB sensors are reported on *x* axis, while frequency shift (Hertz) on *y*-axis. BPs differ particularly on #1 [Zn-TPP] and #4 [Mn-TPP] at all four temperatures.

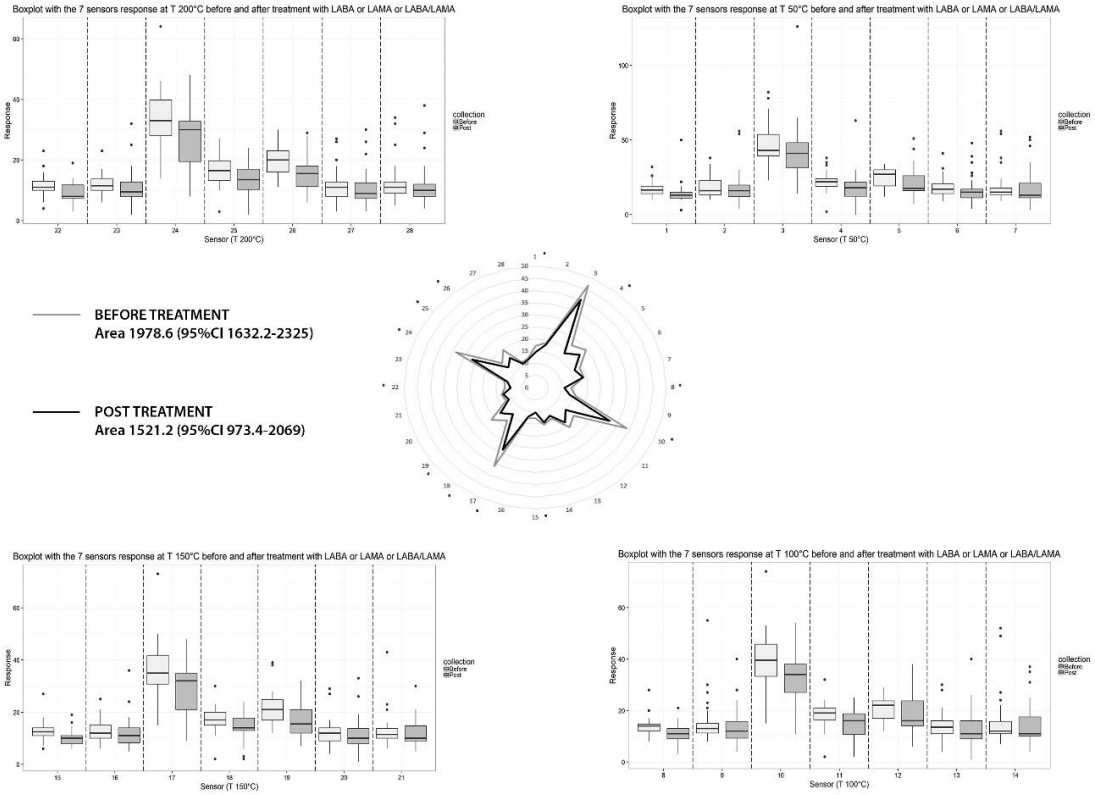
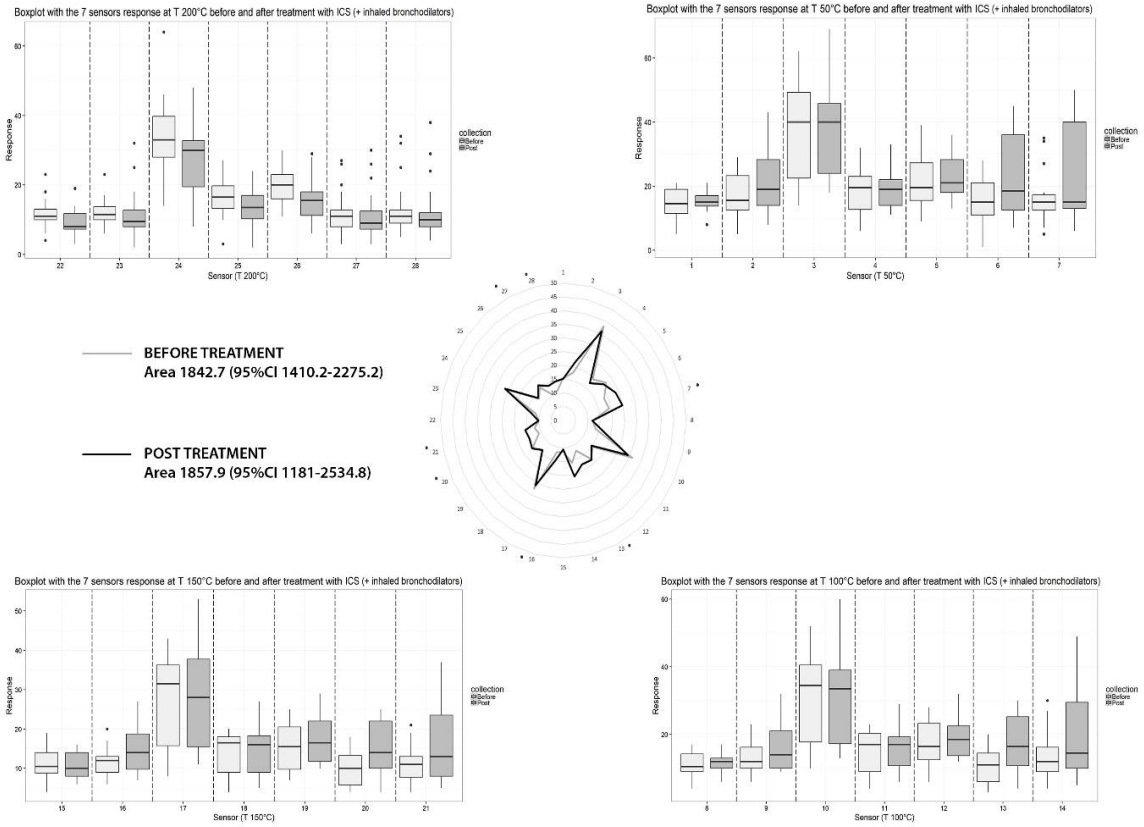


Figure 5: At the centre, radar plot comparing the mean breath print (BP) before and after treatment with inhaled corticosteroids (ICS) in adjunction to inhaled bronchodilators. See legend to Figure 2 for the description of BP graphical representation. BP after treatment is characterized by different frequency shifts, particularly on radii 7, 13, 16, 20, 21, 27 and 28, and a different shape. Box plots at the corners compare frequency shifts (Hertz) of each of the QMB respect to their typical resonance frequency before and after corticosteroid treatment. QMB sensors are reported on *x* axis, while frequency shift (Hertz) on *y*-axis. BPs differ particularly on sensor #7 [Ru-TPP] at all four temperatures.



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CHAPTER 5 VALIDATION OF EXHALED VOLATILE ORGANIC COMPOUNDS ANALYSIS USING E-NOSE AS INDEX OF COPD SEVERITY

Panaiotis Finamore, Claudio Pedone, Simone Scarlata, Alessandra Di Paolo, Simone Grasso, Marco Santonico, Giorgio Pennazza, Raffaele Antonelli Incalzi, Int J Chron Obstruct Pulmon Dis 2018; 13: 1441–1448.

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ABSTRACT

Background: Six minute walking test distance (6MWD) and BMI, Obstruction, Dyspnea, Exercise (BODE) index are measures of functional status in COPD patients, but require space, time and patient's compliance. Exhaled volatile organic compounds (VOCs) analysis via electronic nose is a quick and easily performed method that has already been used to discriminate COPD phenotypes. Aim of this study is to evaluate whether VOCs analysis can predict functional status and its variation over time.

Methods: Monocentric prospective study with 1 year of follow-up. All patients performed pulmonary function tests, arterial gas analysis, bioimpedance analysis, six minute walking test and VOCs collection. Exhaled breath was collected with Pneumopipe® and analyzed using BIONOTE electronic nose. Outcomes prediction was performed by k-fold cross-validated partial least square discriminant analysis: accuracy, sensibility and specificity as well as Cohen's kappa for agreement were calculated.

Results: We enrolled 63 patients, 60.3% men, mean age of 71 (SD:8) years, median BODE of 1 (IQR 0-3) and mean 6MWD normalized by squared height (n6MWD) of 133.5 (SD: 42) meters/m^2. The BIONOTE predicted baseline BODE (dichotomized as BODE score < 3 or ≥ 3) with accuracy of 86% and quartiles of n6MWD with accuracy of 79%. n6MWD decline more than the median value after 1 year was predicted with accuracy of 86% by BIONOTE, 52% by GOLD class and 78% by combined BIONOTE and GOLD class.

Conclusion: Exhaled VOCs analysis identifies classes of BODE and n6MWD quartiles, and outperforms GOLD classification in predicting n6MWD variation.

INTRODUCTION

In 2010 chronic obstructive pulmonary disease (COPD) has become the third leading cause of death, accounting for 2.8 million deaths worldwide.[1] To identify patients with a steeper progression towards disability and death is warranted in order to implement monitoring and therapy.

Different systems have been proposed to categorize COPD severity and rate of functional status decline. The Global Initiative for Chronic Obstructive Lung Disease (GOLD) proposed a severity classification based on reduction of FEV1 expressed as percent of predicted value,[2] and then updated the risk stratification adding clinical parameters [3] to take into account the burden of symptoms. Nonetheless, neither the first nor the second GOLD classification have demonstrated sufficient discrimination ability in stratifying COPD functional impairment severity.[4–6] Compared with GOLD classifications, the 6' minute walking test have a better association with clinical outcomes, such as mortality, [7–9] either alone or used together with other variables in the BMI, Obstruction, Dyspnea, Exercise (BODE) index.[10] The 6' minute walking test should be performed in a corridor of at least 30 meters of length, that may not be available in some setting, and requires patient's compliance to obtain reliable measures,[11] thereby new approaches less demanding in terms of space and patient's compliance may be helpful to rate functional impairment.

Exhaled volatile organic compounds (VOCs) analysis trough electronic nose is a promising approach in the study of lung disease. This technique provides a "fingerprint" of the exhaled breath (dubbed "breathprint"), obtained by the interaction occurring between VOCs and a sensor array, which has been shown to discriminate COPD patients from healthy controls, patients with asthma [12] and heart failure,[13] and seems promising in COPD phenotyping.[14,15] Considering that factors affecting COPD patients' functional status (ie systemic inflammation and hypoxemia[16]) are also modifiers of VOCs production,[17] this proof-of-concept study aims

to evaluate whether exhaled fingerprint predicts with COPD patients functional status and whether and to which extent it might improve the GOLD discrimination ability. Therefore, objectives of the study are: 1) to evaluate whether VOCs analysis of COPD patients using electronic nose discriminates quartiles of six minute walking test distance normalized by squared height (n6MWD) and classes of BODE index, also in comparison with GOLD classification; 2) to evaluate whether VOCs analysis predicts changes in the n6MWD after one year.

MATERIALS AND METHODS

Study design

This is a 1 year monocentric and observational prospective study carried out at "Campus Bio-Medico" Hospital in Rome (Italy); present data have been collected from September 2015 to September 2016. The study protocol was approved by the Ethical Committee of Campus Bio-Medico university (protocol number: 30/15 PAR CMB).

Inclusion criteria were the diagnosis of COPD defined as the evidence at spirometry of persistent airflow limitation, defined as FEV1/FVC<0.7 after administration of 400 mcg of salbutamol, [18] and the absence of exacerbations and of changes in pharmacological therapy in the previous 3 months.

Exclusion criteria were the inability to perform an acceptable spirometry, following ATS/ERS guidelines, [18] the diagnosis of pulmonary cancer or pulmonary fibrosis, severe cognitive impairment, as indicated by a mini-mental state examination (MMSE) score < 10, inability to perform the six minute walking test due to mobility limitations, heart failure in NYHA class III or IV and, finally, refusal to provide informed consent.

Clinical assessment

The study involved 63 stable COPD patients consecutively enrolled. Demographic and physiological characteristics, level of dyspnea (using the modified Medical Research Council – mMRC – dyspnea scale), number of exacerbations, pulmonary function tests (post-bronchodilator spirometry and lung volumes), 6' minute walking test , arterial gas and bioimpedance analysis, multidimensional assessment and comorbidities, identified based on patients' documentation, medical history, physical examination, and routine blood analysis, were recorded during the visit, both at baseline and after 1 year.

Forced expiratory volumes were measured using a water-sealed bell spirometer (Biomedin, Padua, Italy) following the acceptability and reproducibility criteria proposed by the American Thoracic Society and the European Respiratory Society (ATS/ERS) [18]. Total Lung Capacity (TLC) and Residual Volume (RV) were obtained using the Helium-rebreathing technique. [19] Multidimensional assessment was made up by: functional ability, estimating using the Katz' activities of daily living (ADL) and the Lawton and Brody activities of instrumental of daily living (IADL), [20,21] cognitive function evaluation, using MMSE, [22] and depressive symptoms, estimating using 15-item Italian version of the Geriatric depression scale (GDS). [23]

Collection and analysis of VOCs

Exhaled breath was collected only at baseline, in the morning, early after awakening, with patients fasting, smoking free and refrained from medication assumption for at least 8 hours. All patients performed breath collection in the same room and with the same procedure.

Patients were asked to breath tidally for three minutes into a mouthpiece connected with Pneumopipe® (European patent n. 12425057.2, Rome- Italy), a device ensuring in a fixed time a non invasive collection of mixed expiratory sample into an adsorbent Tenax GR cartridge (Supelco/Sigma-Aldrich, Bellefonte, PA, USA). [24] Cartridge content was then thermally desorpted into the sensor chamber of our electronic nose, named BIONOTE, by a device uniformly heating the cartridge at four different temperatures: 50 °C, 100 °C, 150°C and 200°C. BIONOTE is a seven quartz microbalance (QMB) sensor array developed and fabricated by the Lab of Electronics for Sensor Systems at the University Campus Bio-Medico of Rome. Sensors are covered with anthocyanins extracted from three different plant tissues and used as chemical interactive materials. Desorbed VOCs interact with the chemicals covering the sensor's surface via weak bounding forces and interaction results in seven frequency shifts of

each of the QMB respect to their typical resonance frequency for each desorption temperature, so we finally achieve twenty-eight responses (seven QMB tested per four different temperatures) used in the analysis. This technology has been validated and its performance has been evaluated in gases and vapors calibration experiments. [25]

Six minute walking test and BODE index

Six minute walking test is a clinical exercise test measuring the distance that a patient can quickly walk in a period of 6 minutes. It provides a global information on the organs contributing to exercise capacity, but it is not able to disentangle the contribution of each system. The test was performed in the morning, after exhaled breath collection, according to ATS guidelines. [11] In summary, participants were asked to walk in a flat, straight and 30 meters hallway for six minutes as quickly they could, under the supervision of a trained technician. SpO_2 was measured throughout the test using a pulse oximeter with a finger probe. Dyspnea was rated at the end of the test using Borg scale. The 6MWD, basal SpO_2, nadir SpO_2 and Borg score were recorded. Since anthropometric features affect 6MWD,[26–28] we normalized 6MWD per patients' squared height (n6MWD) to avoid their impact on the treaded distance as has been suggested for FEV1[29].

The BMI, Obstruction, Dyspnea, Exercise (BODE) Index is a scoring system used to predict all-cause mortality in patients with stable COPD and it is based on four variables: body mass index, FEV1 (% of predicted), dyspnea, rated using COPD Assessment Tool (CAT) or modified Medical Research Council (mMRC), and 6MWD. Total score ranges from 0 to 10 and the higher is the score the lower is the estimated survival. BODE score higher than 3 is associated with poorer diagnosis. [10]

Statistical analysis

We reported the characteristics of our sample using descriptive statistics. Participants were divided into quartiles of 6MWD normalized per squared height (n6MWD), that were compared using ANOVA or Kruskall-Wallis test, for normally and non-normally distributed continuous variables, respectively, and χ^2-test or Fisher exact test for categorical variables, as appropriated.

Partial Least Square Discriminant Analysis (PLS-DA), applied on patients' BIONOTE sensor responses, previously centered and scaled, was used to predict quartiles of n6MWD, to take in account individual's anthropometric features affecting 6MWD. The same method was used to predict BODE score, classified as "Good prognosis" (BODE score < 3), and "Poor prognosis" (BODE score ≥ 3) [30] that represented the "gold standard" for this analysis. To avoid overfitting we used a "repeated k-fold cross validation". The overall effectiveness of classification was expressed as diagnostic accuracy, which is the proportion of subjects correctly classified by the PLS-DA among all subjects, sensibility and specificity. Furthermore, we measured agreement between the model based on BIONOTE responses and reference n6MWD quartiles and BODE classes using Cohen's kappa.[31] Then, we compared quartiles of n6MWD (meters/m^2) and classes of BODE score with 2014 GOLD classes, measuring overall effectiveness and agreement with the same procedure as done before.

Finally, we evaluated whether VOC analysis could predict changes in the n6MWD over time. For this analysis, the "gold standard" was represented by the variation of walked distance was dichotomized using the median (-7.9 meters/m^2) as the cut-off value into "Stable/improved" or "Worsened". For this analysis, we compared predictions based on VOC only with predictions made on GOLD classes only and on the combination of the previous two parameters. Accuracy, sensibility, specificity, as well as Cohen's kappa, were calculated.

All the analyses were performed using R version 3.3.0 (The R Foundation for Statistical Computing, Wien, Austria, 2016).

RESULTS

Characteristics of participants at baseline are summarized in Table 1. Mean age of participants was 71(SD: 8) years, 38 (60.3%) were men, mean body mass index (BMI) was 29.1 (SD: 5.9) kg/m^2, mean FEV1% was 69.6 (SD: 22.3), mean pack/year was 35.1 (SD: 26.6) and 25 (39.7%) were current smokers, while 32 (50.8%) were former smokers. Of the 63 participants 35 (55.6%) were in GOLD A class, 9 (14.3%) in GOLD B class, 8 (12.3%) in GOLD C class and 11 (17.5%) in GOLD D class. Mean 6MWD was 366.8 (SD: 111.5) meters and mean n6MWD was 133.5 (SD: 42) meters/m^2. Patients in the first quartile of n6MWD treaded from 165 meters/m^2 to 215 meters/m^2, those in the second from 145 meters/m^2 to 165 meters/m^2, those in the third from 111 meters/m^2 to 145 meters/m^2 and those in the fourth quartile from 22 meters/m^2 to 111meters/m^2. After 1 year of follow-up 35 participants (55.6%) were still in GOLD A class, 10 (15.8%) were in GOLD B class, 9 (14.3%) in GOLD C class and 9 (14.3%) in GOLD D class; mean 6MWD was 342.8 (SD: 119) meters and mean n6MWD was 123.9 (SD: 42) meters/m^2. Median decline in the 6MWD over the year was -23.8 meters and median decline in the n6MWD was -7.9 meters/m^2.

Analyzing baseline quartiles of n6MWD, we observed a progressive reduction in the mean FEV1% (from 77.7% (SD:9) to 57.9% (SD:22), ANOVA P-value: 0.02) and in the mean pO_2 (from 84 mmHg (SD: 8.5) to 74.4 mmHg (SD: 11.4), ANOVA P-value: 0.04), as well as a reduction in the mean FFM%, from the quartile with the highest treaded distance to that with the lowest, and an increase in mMRC score, BODE index and BMI. The PLS-DA based on e-nose sensors was able to discriminate baseline quartiles of n6MWD, correctly allocating 50 out of 63 patients with an overall accuracy of 0.79 (95% CI 0.67-0.88, P-value:<0.001) and a Cohen's kappa of 0.72 (95% CI 0.59-0.85, P-value: <0.001). Moreover, 54 patients out of the 63 participants were correctly classified into the baseline classes based on BODE index ("Good prognosis" vs. "Poor prognosis"), with an accuracy of 0.86 (95% CI 0.75-0.93, P-value:

<0.001), a sensitivity of 0.71, a specificity of 0.93 and a Cohen's kappa of 0.67 (95% CI 0.47-0.87, P-value: <0.001) (see Table 2).

GOLD classes did not identify quartiles of n6MWD (meters/m^2), and we did not find a linear reduction of the walked distance across GOLD classes, obtaining an accuracy in predicting quartiles of walked meters/m^2 of 0.35 (95% CI 0.23-0.48, P-value: 0.05), with a poor agreement: Cohen's kappa of 0.14 (95% CI -0.02-0.29, P-value: 0.03) (see Table 3).

Prediction of the two classes ("Stable/improved" vs "Worsened") of n6MWD variation based on the electronic nose sensors responses correctly classified 54 out of the 63 COPD patients, with an accuracy of 0.86 (95% CI 0.75-0.93, P-value:<0.001), sensibility of 0.84 and specificity of 0.88, while the accuracy obtained using GOLD classes was 0.52 (95% CI 0.39-0.65, P-value: 0.45). Applying PLS-DA on GOLD classes and electronic nose responses together, 49 out 63 patients were correctly classified with an accuracy of 0.79 (95% CI 0.67-0.88, P-value: <0.001), a sensibility 0.81 and a specificity of 0.78. Cohen's kappa was 0.71 (95% CI 0.54-0.88, P-value: <0.001) for BIONOTE-based prediction, 0.05 (95% CI -0.20-0.29, P-value: 0.35) for GOLD-based prediction and 0.59 (95%CI -0.39-0.79, p-value: <0.001) for the model based on their combination. Data are summarized in Table 4.

DISCUSSION

Exhaled VOCs analysis using electronic nose discriminates baseline BODE classes and quartiles of n6MWD, and is able to predict n6MWD variation over 1 year of follow up, better than the GOLD classes.

Our data show that inclusion of dyspnea and exacerbation rate notwithstanding, the problem of GOLD classification may be that it remains mainly focused on respiratory parameters, ignoring that COPD is a disease with important systemic impact. Indeed, risk factors of COPD, ie cigarette smoking, induce systemic inflammation and oxidative stress, [32] as well as changes in endothelial function favoring a pro-thrombotic state,[33] resulting in a higher prevalence of chronic comorbidities (eg heart failure, pulmonary vascular diseases, etc).[34] Thus, COPD is seldom an isolate disorder and usually comorbidity impact health outcomes, particularly survival, in COPD patients. Indeed, COPD exacerbation leading to respiratory failure account only for 35% of COPD patients' death. [35] This evidence fosters the use of VOCs analysis. VOCs are low weight molecules produced by cellular metabolism, thereby bacterial infections, metabolic abnormalities, toxic ingestion, chronic diseases (eg heart failure, liver cirrhosis) and cancer produce a qualitative and quantitative change in their production.[17] Hence, although COPD induces its particular change in exhaled VOCs, useful in the diagnosis of the disorder, patient's breathprint is the end result of the overall cellular metabolism disarray, also caused by comorbidities.[36] Indeed, analyzing characteristics of patients divided per quartiles of n6MWD, we observed that VOCs are not just influenced by airflow limitation and dyspnea, also captured by GOLD classification, but besides they take into account body composition, as described by a statistically significant increase in BMI associated with a reduction in FFM%, a well demonstrated factor affecting COPD functional status.[37] Furthermore, they are not affected by the number of exacerbation per year, that, although improving ability to predict hospital admission due to COPD exacerbation,[4] do not seem to be correlated with functional status. This

is a possible explanation of why the model based on VOCs was able to discriminate better than GOLD classes both 6MWD and BODE as well as to identify, in the longitudinal analysis, a worsening of patients' functional status.

Moreover, our results confirm the known limitations of the 2014 GOLD classification in the identification of COPD functional status. This new classification, despite considering dyspnea and exacerbation , did not outperform the 2007 GOLD classification based only on airflow limitation,[4,6] and it is characterized by a poor ability in COPD functional status stratification: subgroup B has poorer survival and a higher rate of hospital admission than subgroup C [4,5] and is characterized by a lower 6MWD and a higher BODE index than subgroup C.[38] In our analysis, GOLD classification showed a poor agreement both with baseline quartiles of n6MWD and classes of BODE and with longitudinal variation of n6MWD, as demonstrated by Cohen's kappa, significantly lower than that obtained by VOCs analysis. Furthermore, we observed that while GOLD A is clearly composed by subjects with a good functional status and GOLD D by subjects with the worst, GOLD B and GOLD C have intermediate impairment and are rather similar, with GOLD B slightly worse, hence there is not a linear progression of impairment confirming reports of previous studies.[38]

Strengths and limitations

This study has some limitations. Firstly, using an electronic nose technology we could not identify single components of exhaled breath, as is possible with other techniques (eg gas chromatography-mass spectrometry). However, the purpose of this study was to develop a simple and inexpensive metabolic marker: electronic nose analysis costs are about 10€, whereas analytic methods of breath samples are costly and not suitable for routine clinical use, and comparable, or even cheaper, than that of 6MWT (about 50€). Secondly, VOCs may be

influenced by room conditions, smoke, drugs and food, but we minimized the potential bias of these factors avoiding patients to smoke, to eat, to drink and to assume medication for at least 8 hours and performing collection ever in the same room. Furthermore, to date does not exist an electronic nose considered the "gold standard" in VOCs analysis, thus BIONOTE has never been compared with other e-nose devices. Finally, we acknowledge that the choice to normalize 6MWD per patients' squared height to minimize the impact of anthropometric features is arbitrary, even conventionally used.

The study also has some strengths. We performed a multidimensional assessment of the whole population, not limited to respiratory parameters. Furthermore, the longitudinal design of the study allowed us analyze the correlation between exhaled VOCs analysis and the variation of the 6MWD.

CONCLUSION

Exhaled VOCs analysis using electronic nose may be used for a better characterization of COPD patients' functional status. Considering that exhaled breath collection and analysis has a very low cost, does not require particular space and is suitable for all patients, even those who are unable to perform a 6MWT or a spirometry, it represents a promising technique in the COPD functional status assessment.

Table 1: Baseline characteristics of the population (N=63) and quartiles of 6MWD normalized per squared height (meters/m^2).

	Population n=63	22-111m/m^2 n=16	111-145 m/m^2 n=16	145-165 m/m^2 n=16	165-215 m/m^2 n=15	P anova
Age	71 (8)	73.9 (6.9)	71.6 (8.6)	70.2 (6.8)	68.2 (8.5)	0.22
Sex (M)	38 (60.3)	12 (75)	11 (69)	9 (56)	6 (40)	0.21
Body Mass Index (Kg/m^2)	28.1 (5.9)	30.2 (7.1)	31.1 (6.3)	25.2 (2.8)	25.8 (4.5)	0.004
mMRC						
0-1	43 (68)	6 (37)	13 (81)	12 (75)	12 (80)	0.01
≥2	20 (32)	10 (63)	3 (19)	4 (25)	3 (20)	0.01
Exacerabation/year						
0-1	48 (76)	13 (81)	10 (63)	12 (75)	13 (87)	0.46
≥2	15 (24)	3 (19)	6 (37)	4 (25)	2 (13)	0.46
Smoking habit						
Current smokers	25 (39.7)	5 (31)	7 (44)	8 (50)	5 (33)	0.6
Former smokers	32 (50.8)	9 (56)	9 (56)	7 (43)	7 (47)	0.6
Pack/year	35.1 (26.6)	41.2 (30)	40.9 (26.7)	26.4 (18)	31.7 (28)	0.32
GOLD classification 2014						
GOLD A	35 (55.6)	4 (25)	9 (56)	11 (69)	11 (73)	0.01
GOLD B	9 (14.5)	5 (31)	0 (0)	2 (13)	2 (13)	0.01
GOLD C	8 (12.4)	1 (6)	3 (19)	2 (13)	2 (13)	0.01
GOLD D	11 (17.5)	6 (38)	4 (25)	1 (6)	0 (0)	0.01
BODE index	1 (0-3)	3 (2-5)	1 (0-3)	0 (0-1)	0 (0-1)	<0.001
Spirometry						
FEV1/FVC	63.2 (9.6)	61.5 (12.6)	61.5 (9.2)	65 (8.4)	65 (7.3)	0.55
FEV1cc	1.7 (0.7)	1.5 (0.6)	1.7 (0.5)	2 (1)	1.8 (0.5)	0.2
FEV1%	69.6 (22.3)	57.9 (22)	64.6 (20.7)	78.6 (27)	77.7 (9.4)	0.02
FVCcc	2.8 (1)	2.5 (1.1)	2.7 (0.6)	3.1 (1.4)	2.8 (0.9)	0.45
FVC%	86.3 (22.6)	72.4 (21.5)	82.3 (23.1)	94.7 (25.2)	96.3 (9.8)	0.006
TLCcc	5.9 (1.6)	5.9 (1.5)	5.8 (1.2)	6.6 (2.1)	5.3 (1.4)	0.17
TLC%	100.9 (24.7)	97.2 (40.7)	92.4 (13.7)	114.1 (15)	100.2 (17)	0.1
RV/TLCcc	52.5 (11.5)	57.5 (10.9)	49.4 (15.4)	52 (9.5)	51.5 (8.5)	0.29
RV/TLC%	126.5 (26.5)	132.4 (29.8)	124.7 (31)	125.4 (22)	123.9 (23)	0.84
Arterial gas analysis						
pH	7.41 (0.03)	7.41 (0.02)	7.42 (0.03)	7.42 (0.03)	7.42 (0.04)	0.13
pO2	77.6 (10.7)	74.4 (11.4)	74.5 (9.9)	77.8 (11)	84 (8.5)	0.04
pCO2	39.3 (5)	41 (6.3)	40.2 (3)	39.2 (5.5)	36.5 (4)	0.08
SO2	95.1 (1.7)	94.5 (1.7)	94.7 (1.7)	95.2 (1.8)	96.1 (0.9)	0.04
Multidimensional assessment						
CIRS comorbidity index	0 (0-1)	0 (0-1)	1 (0-2)	0 (0-1)	0 (0-1	0.2
CIRS severity index	0.7 (0.4)	0.7 (0.4)	0.8 (0.3)	0.5 (0.2)	0.7 (0.4)	0.16
MMSE	27.9 (2.9)	27.5 (2.4)	28.9 (1.7)	27.4 (3.5)	27.7 (3.7)	0.4
Clock test	11 (2.8)	11.2 (2.8)	11.2 (2)	10.8 (3.5)	10.9 (2.9)	0.95
ADL	5.8 (0.5)	5.8 (0.4)	5.6 (0.8)	5.9 (0.2)	5.8 (0.4)	0.4
IADL	7.7 (1)	7.4 (1.5)	7.6 (1)	7.9 (0.5)	7.9 (0.3)	0.34
GDS	1.8 (2.7)	2 (3.6)	2.6 (3.2)	1 (1.7)	1.7 (1.6)	0.4
Blood analysis						
WBC	7.1 (1.7)	7.5 (1.5)	6.8 (1.7)	6.9 (1.9)	7 (1.5)	0.68
Hb	14.2 (1.4)	13.9 (1.9)	14.4 (1.4)	14.3 (1)	14.3 (1.5)	0.81
ESR	25.9 (23.7)	35.6 (36.1)	22.8 (15.9)	20.9 (18.7)	25.9 (20.9)	0.42
CRP	3.2 (5.1)	3.7 (4)	1.4 (2.4)	4.9 (7.9)	2.3 (3.2)	0.32
Creatinine	1 (0.3)	1.1 (0.3)	0.9 (0.3)	0.8 (0.2)	0.9 (0.3)	0.06
eGFR(CKD-EPI)	74.2 (19.5)	64.2 (18)	76.3 (21)	80.6 (18.3)	74.4 (18.9)	0.15
Vitamin D	20.9 (10.6)	23.9 (12.3)	20.5 (8.1)	18.8 (7.8)	20.5 (14)	0.67
Vitamin B12	368.1 (204.7)	338.8 (122)	272.5 (122)	449.1 (304)	383.7 (150)	0.2
Folic acid	7.5 (7.2)	5.5 (3.1)	5.7 (2.5)	9.9 (11.1)	8.3 (6.7)	0.38
Bioimpedance analysis						
Total body water %	52.1 (6)	51.4 (6.7)	50.9 (5.4)	54.5 (6.4)	51.7 (5.3)	0.31
Fat mass %	30.1 (8.4)	32.1 (9.3)	32.9 (6.4)	25.8 (8.8)	29.7 (7.3)	0.07
Fat free mass %	69.9 (8.4)	67.9 (9.3)	67.1 (6.4)	74.2 (8.8)	70.3 (7.3)	0.06

Table 2: Cross-validated partial least square discriminant analysis prediction of quartiles of n6MWD (upper) and classes of BODE (bottom).

	Reference n6MWD			
Prediction n6MWD	**22-111 m/m²**	**112-145 m/m²**	**146-165 m/m²**	**165-215 m/m²**
22-111 m/m²	15	0	1	1
112-145 m/m²	0	13	1	3
146-165 m/m²	0	2	12	1
165-215 m/m²	1	1	2	10

Accuracy: 0.79 (95%CI 0.67-0.88, p-value:<0.001) **Cohen's kappa: 0.72 (95%CI 0.59-0.85, p-value: <0.001)**

***** n6MWD: six minute walking distance/(height)²

	Reference BODE classes	
Prediction BODE classes	**Good prognosis (BODE<3)**	**Poor prognosis (BODE≥3)**
Good prognosis	39	6
Poor prognosis	3	15

Accuracy: 0.86 (95%CI 0.75-0.93, p-value:<0.001) **Sensibility: 0.71** **Specificity: 0.93**

Cohen's kappa: 0.67 (95%CI 0.47-0.87, p-value:<0.001)

* BODE: BMI, Obstruction, Dyspnoea, Exercise index

Table 3: Confusion matrix with the distribution per GOLD classes of the n6MWD (upper) and classes of BODE (bottom).

	Reference n6MWD			
GOLD classes	**22-111 m/m²**	**111-145 m/m²**	**145-165 m/m²**	**165-215 m/m²**
A	4	9	11	11
B	5	0	2	2
C	1	3	2	2
D	6	4	1	0

Accuracy: 0.35 (95%CI 0.23-0.48, p-value: 0.05) **Cohen's kappa: 0.14 (95%CI -0.02-0.29, p-value:0.03)**

***** n6MWD: six minute walking distance/(height)^2

	Reference BODE classes	
GOLD classes	**Good prognosis (BODE<3)**	**Poor prognosis (BODE≥3)**
A	34	1
B	3	6
C	4	4
D	1	10

* BODE: BMI, Obstruction, Dyspnoea, Exercise index

Table 4: Cross-validated partial least square discriminant analysis prediction of the n6MWD variation classes over the year of follow up based only on BIONOTE (upper), only on GOLD classification (centre) and on BIONOTE+GOLD classification (bottom).

	Reference Δn6MWD classes	
BIONOTE based prediction of Δn6MWD classes	**Worsened**	**Stable/improved**
Worsened	28	5
Stable/improved	4	26

Accuracy: 0.86 (95%CI 0.5-0.93, p-value:<0.001) Sensibility: 0.84 Specificity: 0.88

Cohen's kappa: 0.71 (95%CI 0.54-0.88, p-value: <0.001)

***** n6MWD: six minute walking distance/(height)^2

	Reference Δn6MWD classes	
GOLD based prediction of Δn6MWD classes	**Worsened**	**Stable/improved**
Worsened	15	13
Stable/improved	17	18

Accuracy: 0.52 (95%CI0.39-0.65, p-value:0.45) Sensibility: 0.58 Specificity: 0.47

Cohen's kappa: 0.05 (95%CI -0.20-0.29, p-value: 0.35)

***** n6MWD: six minute walking distance/(height)^2

	Reference Δn6MWD classes	
BIONOTE+GOLD based prediction of Δn6MWD classes	**Worsened**	**Stable/improved**
Worsened	25	6
Stable/improved	7	25

Accuracy: 0.79 (95%CI 0.67-0.88, p-value:<0.001) Sensibility: 0.81 Specificity: 0.78

Cohen's kappa: 0.59 (95%CI -0.39-0.79, p-value: <0.001)

***** n6MWD: six minute walking distance/(height)^2

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CHAPTER 6 GENERAL DISCUSSION

GENERAL DISCUSSION

This thesis investigated the role that VOC analysis using an electronic-nose (BIONOTE®) may have in the diagnosis, disease severity stratification and prognosis of COPD and CHF, two of the most common non-communicable diseases that affect elderly people worldwide, and which impact in terms of disability and death is expected to raise in the next future.

The primary end-point was to evaluate the diagnostic ability of breath analysis. Indeed, albeit reliable and validated tests to perform for the diagnosis of these conditions already exist, their applicability in elderly people is limited. Guidelines for the diagnosis of heart failure suggest the use of natriuretic peptides (NP) as a first step to perform in people at risk of having the disease and suggest the use of a fixed cut-off of 35 pg/mL for BNP and 125 pg/mL for NT-proBNP [1], disregarding that age itself is a factor increasing NP blood level [2] and that ageing is associated with multimorbidity [3], which might further boost the increase. On the other side, pulmonary function tests are pivotal for the diagnosis of COPD, however they involve an active participation of the patient not always possible in elderly people [4]. Being non-invasive and easy to perform, breath analysis represents a promising candidate. As described in *Chapter 2*, it is known that breath analysis is able to discriminate COPD patients from healthy controls and people with other chronic respiratory disorders [5–8] and CHF from healthy controls [9,10], however all these studies were carried out in a younger population, with a smaller sample size and, except the study of Fens and colleagues [7], without external validation. Thanks to the larger sample size, almost 7 times the sample of decompensated CHF patients of Witt and colleagues [9]) and 2,5 times the sample of COPD patients of the largest previously conducted study, we were able to investigate the diagnostic ability of breath analysis using a robust approach, testing the model in a different sample from the training set. Results are not just confirmatory of VOC analysis ability to discriminate CHF from healthy controls, but they also show that discriminative capacity is still present in elderly people and

that breath-fingerprint of CHF patients differ from that of COPD patients, enabling a differential diagnosis between these two conditions.

Furthermore, although both the diseases can be diagnosed by VOCs analysis, they present a different behavior in terms of disease severity stratification. Indeed, as described in *Chapter 3*, breath analysis is unable to identify CHF severity stratified according to the New York Heart Association (NYHA) grading, and shows a poor correlation with the ejection fraction and the systolic pulmonary arterial pressure of these patients. Conversely, in COPD breath-fingerprint correlates with expiratory flows and identifies phenotypes of the disease (see *Chapter 4*).

Given the simplicity of the scale, NYHA classification is definitely the most common assessment of disease severity in CHF patients and for such a reason has been used, together with echocardiography, in our study. The lack of association between the breath-fingerprint and the classes of NYHA, as well as the poor association with the ejection fraction and the systolic pulmonary arterial pressure, are at a first glance disappointing. However, some considerations are warranted before concluding that VOCs do not associate with CHF severity. The first is that NYHA scoring is mainly based on the severity of dyspnea, thereby is focused on the hemodynamic impairment following the heart pump failure rather than considering the metabolic disarray and the inflammatory status of patients. This hypothesis is also supported by the evidence that breath analysis is unable to predict the plasma level of NT-proBNP, which increases with the stretching of myocardial fibers produced by the hemodynamic overload of left ventricle [11], while predicts, even poorly, ejection fraction and systolic pulmonary arterial pressure, which impairment produce a tissue hypoperfusion with metabolic effects. Further, NYHA is characterized by a poor reliability [12], especially in decompensated CHF, where the classification is not representative of the usual health status of the patient, thus misclassification may have hidden the association between breath-fingerprint and NYHA classes.

On the other side, results in COPD are encouraging. Results of the study described in *Chapter 4* confirm the association between the breath-fingerprint and the expiratory flows already proposed [13]. The importance of these findings may be due to the effect of airways inflammation on expiratory flows and VOC production. However, it is not the only determinant of the breath-fingerprint. Clustering COPD patients basing on their breath-fingerprint we identified three unsupervised phenotypes of patients with different characteristics, not only that about lung function. Indeed, comorbidities are another determinant of the breath-fingerprint, as happens for obstructive sleep apnea syndrome [14], supporting the hypothesis that human diseases are associated with a change in the VOC release and hence that breath analysis is an informative test about subject health status. Moreover, having a longitudinal design, the study in *Chapter 4* allowed us to investigate the short-term effect of the inhalation therapy on breath-fingerprint and to conclude that these medications affect the VOCs produced, in line with the evidence of Montuschi and colleagues [15], in a different manner depending on the presence of inhaled bronchodilators alone or in combination with inhaled corticosteroids. Indeed, while inhaled bronchodilators modify the area but not the shape of the breath-fingerprint, inhaled corticosteroids impact on the shape, probably because of their effect on airway inflammation. This evidence is important because underscores the importance to take into account the potential confounding effect of the inhalation therapy in future studies evaluating the breath-fingerprint of COPD patients, but also for clinical purposes. There are several inhalation devices on the market and pulmonologists should prescribe the correct device to the correct patient, teach the correct inhalation technique and check periodically if the administration is performed correctly, however nowadays there is not a tool able to give information about the effectiveness of the inhalation therapy. If our result will be confirmed in future and larger studies, breath analysis would represent a useful and rapid tool to check the effectiveness of therapy and therefore facilitate the decision about continuing or withdrawing the device. Furthermore, mean BODE index was different in the three clusters, suggesting a potential association between breath-

fingerprint and patients' prognosis. In the study described in *Chapter 5* we investigated the ability of breath-fingerprint to predict quartiles of 6' minute walking distance, normalized per squared height (n6MWD), and classes of BODE index. Both these variables are proxy of patients' functional status and prognosis and have been utilized because of the lack of data about mortality due to the shortness of the follow-up. 6MWD has been normalized per height because it allows a correction for height, and partially sex, difference which impact on the walked distance but are not associated with the prognosis of the patients; similar approach has already been proposed for the FEV1 by Miller and colleagues [16]. Interestingly, breath-fingerprint predicts correctly the quartile of n6MWD with an accuracy of 0.79 (95%CI 0.67-0.88, p-value:<0.001) and classes of BODE with an accuracy of 0.86 (95%CI 0.75-0.93, p-value:<0.001), and identifies also whose patients are poised to worsen, more accurately than GOLD classification. The study was a "proof of concept" and thereby carried out in a small size and without external validation. Further studies are warranted to confirm these preliminary results, but the ability of VOCs analysis to predict COPD patients' prognosis is intriguing and deserves further attention.

METHODOLOGICAL CONSIDERATIONS

The bulk of results on exhaled breath analysis comes from mono-centric studies, with small sample sizes and without external validation, hence evidence is still too weak to consider the approach ready for clinical use. Nevertheless, the application of breath analysis for medical purposes elicits a great interest worldwide and the rapidly increasing number of registered clinical trials, the cumulative number of which almost reached 450 in 2017, would probably help to have firm conclusions at least for some of the fields of application. However, more efforts are needed to address some technical and non-technical issues that are slowing down the applicability of breath analysis in clinical practice. Firstly, it is pivotal to gain a deeper understanding of the mechanisms

underpinning VOC production and release through exhaled breath, as well as factors affecting them, focusing particularly on smoke, food and drugs, but it is also important to fix technical issues in breath sampling and storing, which can potentially affect the result of the measurement due to the release of compounds in the collected mixture [17]. Moreover, there is the need to compare all different available platforms (devices and types of statistical analysis) used to analyze VOCs. This would indeed represent a paramount step towards the application of breath analysis in clinical practice, because it will provide information on whether and what type of electronic nose array (or analytic technique) outperforms others in terms of reliability, feasibility and cost-effective ratio; on strengths and limitations of different statistical approaches mining information from the compound analysis and last but not least, it will offer the opportunity to compare different trial results in order to achieve more powerful results and a higher level of evidence on its use. Nevertheless, it is important to modify the design of future trials in order to target pathological traits of individuals rather than use 'umbrella' terms (e.g. COPD) which in fact have demonstrated conflicting evidence in the last decades. In this perspective, a closer cooperation among research groups is expected in order to reach the goal, because it would be necessary to enroll a higher number of patients and also to share expertise, so to allow a comprehensive characterization of patients. Only a multidimensional assessment in fact, including information derived through breath analysis, could improve our ability to manage multimorbid patients in the future. However, this approach will produce a huge amount of data to store and to analyze, thus larger memories (e.g. cloud storage) and higher computation power will become mandatory, rather than optional as they are now.

CONCLUSIONS AND FUTURE PERSPECTIVES

In conclusion, breath analysis is definitely complex and challenging, but the clinical applications of VOC analysis in COPD and CHF, as well as in other diseases, are so breakthrough to ensure further

efforts towards its development. In the era of 'precision medicine', where developing disease risk assessment, prognosis and therapy are tailored to patients' genotype and phenotype characteristics, VOC analysis, providing information on subjects' metabolism, and potentially on his microbiome, without invasive procedures, at bedside and with limited costs, will surely play a key clinical role. Furthermore, breath analysis is easy to be performed and patients can perform the test without the need of clinical supervision, therefore with the improvement of the electronic nose technology in the next future is not utopian to imagine people breathing into their device from the comfort of their own home and continuously tele-monitored by their doctors, with positive impact on individuals' health and on the costs for the health care system.

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CURRICULUM VITAE

Dr. Panaiotis Finamore is resident in Geriatrics since November 2018 at Campus Bio-Medico University of Rome, Italy. He graduated in Medicine cum summa laude plus special mention for the academic records in July 2015 at the Campus Bio-Medico University of Rome. From November 2015 he attended the Ph.D. program in Science of Aging and Tissue Regeneration at the same university. During his Ph.D. he spent 6 months in CIRO, Centre of expertise for chronic organ failure, Department of Research and Education, Horn, the Netherlands, under the supervision of Prof. Emiel F.M. Wouters, Prof. Martijn A. Spruit and Dr. Daisy J.A. Janssen. There, he participated to the courses on pulmonary rehabilitation organized by CIRO and endorsed by the European Respiratory Society. During his Ph.D. he participated as sub-investigator in a multi-center randomized control trial, he acquired a good command of *R* software for statistical analysis and expertise in lung function tests, breath and body composition analysis. He speaks fluently Italian and Greek, quite well English (B2-C1 level) and a bit of French (A2 level). His research interests mainly concern the volatile organic compound (VOC) analysis in respiratory and cardiac diseases of elderly people, nutrition and pulmonary rehabilitation in chronic obstructive pulmonary disease (COPD) and palliative care medicine. He is a member of the Italian geriatric society (SIGG). On February 2019, he is author of nine publications in international peer-reviewed journals. He presented posters and gave oral presentations in national and international congresses.

LIST OF PUBLICATIONS

1) Scarlata S, Finamore P, Giannunzio G, Santangelo S and Antonelli Incalzi R 2017 Chest ultrasonography in health surveillance of asbestos related pleural disease Lung Cancer Amst. Neth. 111 139–42

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