# UNIVERSITA' DEGLI STUDI DI VERONA

*DIPARTIMENTO DI*

*DIAGNOSTICA E SANITA' PUBBLICA*

*SCUOLA DI DOTTORATO DI*

*SCIENZE NATURALI ED INGEGNERISTICHE*

*DOTTORATO DI RICERCA IN*

*NANOSCIENZE E TECNOLOGIE AVANZATE*

XXX° CICLO


# E-NOSE: AN INNOVATIVE TECHNOLOGY TO EVALUATE DIFFERENT RESPIRATORY PATTERNS IN PEDIATRIC POPULATION

S.S.D. Pediatria Generale e Specialistica- MED 38


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**ABSTRACT**

Electronic nose (e-Nose) is an innovative biomimetic method that simulate the human olfactory system to identify the quality of odors, through a nano-sensor system in combination with specific processing algorithms.

The instruments used are electronic devices characterized by complex architectures.

The electronic nose can detect volatile organic compounds (VOCs) derives from hydrocarbons such as formaldehyde, methanol, ethanol, hydrogen sulphate, benzene, acetaldehyde, acetone, toluene, phenols produced during metabolic, inflammatory and oxidative stress.

In general, this tool does not allow to identify a single VOC but defines the characteristics of a group of VOCs defining a specific profile.

The sample to analyzed is exposed to the 32 nano-sensors resulting in the modification of the basal resistances and its electrical conductivity.

There are several applications of this technology in the medical field in particular pneumology for the characterization of various disease (sarcoidosis, COPD, asthma, obstructive sleep disorders) as demonstrated by several studies on adult patients. In the literature, only a few studies have been conducted on pediatric subjects, particularly in patients undergoing mechanical ventilation, therefore data on spontaneous breathing is not available and standardization of the method in this age group is not yet available.

The exhaled air contains more than 200 volatile organic substances (VOCs), many of which derive from the metabolism of endogenous substances and others from external contaminants (xenobiotic compounds). Exhaled air analysis is a new approach to evaluating metabolic disorders related to various diseases, eg. lung cancer, asthma, cystic fibrosis.

The purpose of our study is assessing the feasibility of the method on pediatric patients and to define any limits in the collection of the samples. Then evaluate if there are different respiratory pattern due to a different composition of VOCs based on the respiratory pathology of the patient.

This study has laid the ground for further refinement in the standardization of exhaled sample collection in pediatric subjects and the evaluation of pediatric disease.

## SOMMARIO

Il naso elettronico (e-Nose) rappresenta una metodica innovativa biomimetica che permette di simulare il sistema olfattivo umano nel verificare la qualità degli odori attraverso l'utilizzo di un sistema di nano-sensori in combinazione con specifici algoritmi di rielaborazione.

Le strumentazioni utilizzate sono apparecchi elettronici caratterizzati da architetture complesse.

Il naso elettronico è in grado di rilevare i composti organici volatili (VOCs) derivanti dagli idrocarburi come ad esempio formaldeide, metanolo, etanolo, solfato di idrogeno, benzene, acetaldeide, acetone, toluene, fenoli che vengono prodotti durante i processi metabolici, infiammatori e nello stress ossidativo.

In linea generale tale strumento non permette di identificare un singolo VOC ma definisce le caratteristiche di un gruppo di VOCs attraverso la definizione di un profilo specifico.

L'esposizione del campione da analizzare ai 32 sensori dello strumento provoca una modificazione delle resistenze basali modificandone la conduttività elettrica.

Diverse sono le applicazioni di questa tecnologia nell'ambito medico in campo pneumologico per la caratterizzazione di diverse patologie (sarcoidosi, COPD, asma, patologie ostruttive del sonno) così come dimostrato da diversi studi su pazienti adulti. In letteratura solo pochi studi sono stati condotti su soggetti pediatrici, in particolare in pazienti sottoposti a ventilazione meccanica, pertanto non sono disponibili dati su pazienti in respiro spontaneo e non è ancora disponibile una standardizzazione della metodica in questa fascia di età.

L'aria esalata, contiene più di 200 sostanze organiche volatili (VOCs) presenti in tracce, molte delle quali derivanti dal metabolismo di sostanze endogene ed altre provenienti da contaminazioni esterne (composti xenobiotici). L'analisi dell'aria espirata, rappresenta un nuovo approccio per la valutazione di alterazioni metaboliche legate a diverse malattie, es. tumore polmonare, asma, fibrosi cistica.

Lo scopo del nostro studio è quindi quello valutare innanzitutto la fattibilità della metodica in ambito pediatrico e definirne eventuali limiti nella raccolta dei campioni. Successivamente valutare se possono esistere differenti pattern

respiratori riconducibili a una differente composizione di VOCs sulla base della patologia respiratoria del paziente.

Questo studio ha gettato le basi per ulteriori approfondimenti nell'ambito della standardizzazione della raccolta del campione di esalato in soggetti e lo studio delle principali patologie pediatriche.

# INTRODUCTION

## NON-INVASIVE INFLAMMATORY BIOMARKERS IN LUNG PATHOLOGIES

Biomarkers are objectively and measurable indicators of the biological and pathological processes or pharmacological responses to a therapeutic intervention *(Kharitonov et al. 2001).* Measure the biomarkers in exhaled breath is a very attractive approach to monitor airways inflammation in asthma and other lung diseases.

The methods are not invasive, feasible also in pediatric population, and make sampling repeatable (*Kharitonov et al. 2001) (Barnes et al 2006).* However, there are important issues regarding reproducibility, variability and sensibilities that need to be assessed before the collection and are recommended in clinical practice. Nowadays there are relatively few knowledges about how these biomarkers interact each other in the illness evolution, how they can act in the severity of disease or clinical subtypes or response to therapy. In the future, these new techniques may be useful to predict the progression of the disease, to evaluate the response to current and future therapies, many of which are still in place way of development.

However, lung function and symptoms not always reflect the underlying airway inflammation and response to therapy *(Vijverberg et al. 2013) (Murugan et al. 2009)*; therefore, objective parameters of asthma inflammation could be important for the clinician when making a treatment choice.

Currently, diagnostic methods like bronchoalveolar lavage (BAL) and bronchoscopy are the gold standard for assessing airway remodeling and inflammation in lung disease. These methods, however, are too invasive and have a limited use, especially in pediatric care *(Vijverberg et al. 2013).* For these reasons, in the past years, research studies have focused on objective biomarkers to identify phenotype, inflammation, pathobiological pathways and to guide the clinician in the diagnosis and a personalized management of the disease *(Moore et al. 2010) (Erzurum et al. 2012).*

An ideal biomarker is easy to collect and measure, inexpensive, non-invasive, feasible in children and technically simple to applied in the clinical setting or in the treatment response evaluation.

The induced sputum technique can be considered a surrogate non-invasive method to evaluate airway inflammation. Nevertheless, this technique may be difficult to perform in pediatric patients and therefore its clinical application is still limited.

Exhaled breath condensate (EBC) and the measurement of exhaled nitric oxide (FeNO) are currently the most frequently used tests in clinical practice *(Baraldi et al. 2006)* and are feasible in the pediatric population.

FeNO is an extensively studied marker and its clinical usefulness is supported by guidelines *(ATS guidelines 2005)*. However, studies about the correlation between FeNO and asthma control or its effectiveness in the management of asthma treatment are contradictory *(Raymer et al. 1990) (Deykin et al. 2002)*.

FeNO can be helpful to evaluate asthma control in asthmatic patients and monitoring asthmatic patients in treatment. However, its suboptimal sensitivity and specificity may limit its utilization as a single monitoring tool.

On the other hand, although the efficacy and diagnostic roles of inflammatory markers in exhaled breath condensate have been studied, their clinical use is still under debate *(Robroeks et al. 2010) (Fens et al. 2009)*.

In diagnose and monitoring asthma, an approach that involves an ensemble of EBC biomarkers had better accuracy in real-life settings than a single marker. A poor to moderate association of EBC biomarkers with lung function suggests the greater importance of EBC analysis in the diagnosis of asthma in children.

Other markers that might predict asthma exacerbation are volatile organic compounds (VOCs) that reflect the degree of airway inflammation and asthma control *(Robroeks et al. 2010)*. VOCs in exhaled breath showed a potential role in predicting asthma exacerbations in children. Before adopting VOCS analysis in clinical practice, the validity of their capacity to predict asthma exacerbations should be studied in a larger cohort.

**Fractional exhaled nitric oxide (FeNO)**

In the airways, nitric oxide is mainly produced by two enzymes: constitutive nitric oxide synthase (cNOS) that generates low quantities of NO and epithelial inducible NOS (iNOS) that is induced by various inflammatory cytokines *(Turner 2015).*

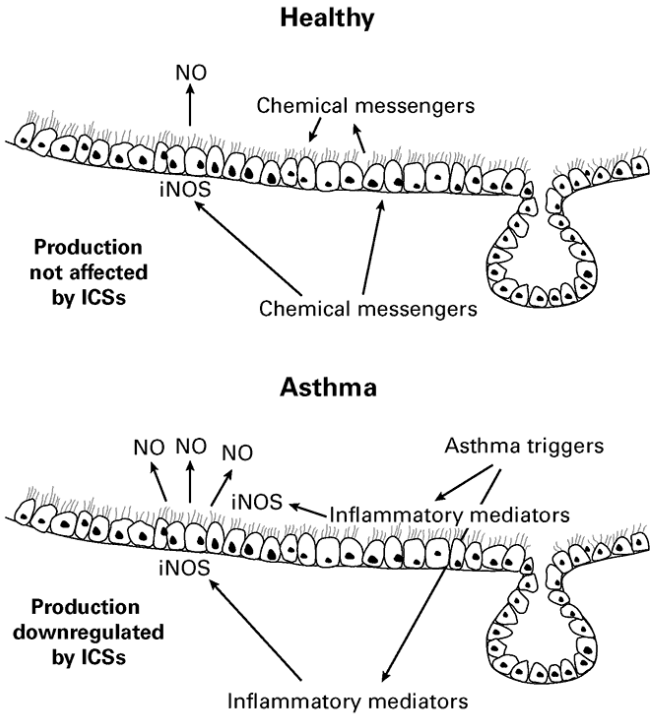



Fig. 1: FeNO pathways in health and asthmatic *(from Alving et al, 2010)*

The exhaled fraction of nitric oxide was extensively studied in asthma and has been demonstrated the correlation with eosinophilic airways inflammation pathways and its subsequent decrease after corticosteroid therapy *(ATS Workshop proceedings 2006).*

FeNO has been recognized as a marker of eosinophilic airway inflammation and its measurement has been proposed to assess the level of airway inflammation and the response to anti-inflammatory therapy *(Kharitonov et al. 1996).*

FeNO measurement is a non-invasive, repeatable and reproducible method (*Pijnenburg et al. 2008).* The gold standard for cooperative children is the single breath on-line method *(Baraldi et al. 2002)*. Other techniques have been evaluated for uncooperative children or in sedated infants *(Baraldi et al. 2002).*

On-line and off-line methods have both been used in uncooperative children without the use of sedatives. Limited experience has been described using single-breath methods in infants. However, these methods have never been validated for clinical purposes and further researches are needed to define standardized measurements in this age group.

At present, no clear evidence is available regarding the potential clinical application of NO measurements in uncooperative children, particularly regarding its potential application in association with other diagnostic tests, to predict asthma in young children *(Moschino et al. 2015).*

To standardize FeNo measurement procedures, an initial document on FeNO measurement in children was published in 2002 *(Baraldi et al. 2002),* which was jointly revised by ATS/ERS in 2005 *(ATS guidelines 2005).* The standardization of techniques may permit to collect comparable data in different centers for normal subjects and for those with diseases. FeNO levels can be influenced by various factors such as exhalation flow, nasal contamination, ambient air pollution, patient's age, height, gender and race *(ATS guidelines. 2005)*. Furthermore, spirometry or exercise performed before the measurement, diet or exposure to smoke also need to be considered.

In children, FeNO increases with age, as reported in the literature *(ATS guidelines. 2005),* and it is recommended that NO analysis should be performed before spirometry because it has been shown that can cause a reduction in transient exhaled NO levels *(ATS guidelines. 2005)*. Patients should also desist from eating and drinking before NO analysis. An increase in FeNO has been found after ingestion of nitrate or nitrate-containing foods. Several studies demonstrated that FeNO correlates positively with airway hyper responsiveness, IgE serum levels, broncho-dilator response, skin prick tests, asthma symptoms and lung function *(Covar et al. 2003) (Komakula et al. 2007).*

In allergic asthma, airway inflammation results from the activation of mast cells and Th2-mediated pro-inflammatory cytokine mechanism that results in the production of IL-4, IL-5, and IL-13 which cause epithelial inducible NO synthase expression that up-regulated via STAT-6, through corticosteroid sensitive process, a mechanism of central importance in allergic airway inflammation *(Ludviksdottir et al. 2012) (Mahr et al. 2013) (see fig.1).* Moreover, other studies showed that FeNO levels are correlated with eosinophils in induced sputum, eosinophil infiltration in the airways, blood eosinophilia, serum eosinophilic cation protein and IgE levels in atopic patients *(Mahr et al. 2013).* Asthma phenotype characterized by Th2-mediated airway inflammation, eosinophilia, and responsiveness to ICS shows high FeNO values *(Mahr et al. 2013).*

In addition to clinical history and lung function test, FeNo is also helpful in identifying patients with asthma eosinophilic phenotype and in predicting asthma exacerbation.

The ATS guidelines recommend the use of FeNO to monitor airway inflammation and to guide anti-inflammatory treatment in patients with asthma *(Mahr et al. 2013).* High FeNO values, however, are associated with allergic rhinitis, eosinophilic bronchitis and allergen or viral exposure; it is important to remind that not all high FeNO values are linked to eosinophilic asthma. The ATS/ERS document stresses the relevance of a correct interpretation of FeNO values *(Dweik et al. 2011).*

In children, FeNO values lower than 20 ppb are probably correlated to absence of response to ICS treatment. On the other hand, FeNO over 35 ppb suggests a response to ICS supporting its role in identifying Th2 airway inflammation responding to ICS treatment *(Mahr et al. 2013).* It has been widely demonstrated that there is a rapid decrease in FeNO when ICS treatment is started, with a dose-dependent mechanism, and a sudden rise when ICS therapy is interrupted *(van Rensen et al. 1999).* This trend may be helpful to monitoring patient compliance to therapy *(Pijnenburg et al. 2008).*

FeNO can also be used in patients in treatment with omalizumab. In fact, some studies showed that FeNO values together with blood eosinophils and BMI can predict response to omalizumab *(Sorkness et al. 2013).* Experimental data in

adults also showed that high FeNO values may indicate a response to treatment with human anti-interleukin-4 receptor monoclonal antibodies that inhibits interleukin-4 and interleukin-13 signaling way *(Wenzel et al. 2016).*

Despite the initial enthusiasm about FeNO in the management of asthma in children, the literature is very careful to support the use of FeNO in addition to standard symptom-based management *(Petsky et al. 2012) (Jartti et al. 2012),* and its utilization is now being reviewed and is under debate *(Bjermer et al. 2014).* Therefore, in clinical practice, FeNO may be considered a clinically useful method to identify patients with eosinophilic and Th2-mediated asthma, who are expected to respond to ICS therapy. Furthermore, it may have a practical role in predicting exacerbations and patient compliance to therapy.

**Exhaled breath volatile organic compounds**

Exhaled breath volatile organic compounds (VOCs) derive from metabolic fractioning of larger molecules. Airway VOCs originate not only from the upper and lower respiratory airways but also from the capillary bed near the alveoli *(van Mastrigt et al. 2015).* The measurement of VOCs is recently proposed in research and clinical setting to evaluated respiratory and non-respiratory diseases. The methodical approach to collect VOCs from exhaled breath requires attention to exclude from ambient air the organic compounds *(van Mastrigt et al. 2015).*

The collection of airway VOCs may be performed by on-line methods, which allow the technician to directly collect samples via inert tubes inserted into an analyzer, or off-line methods which involve the collection of exhaled air into bags, tubes or syringes. Collection devices need to be made from inert materials such as Tedlar bags *(Barker et al. 2006).*

After collecting the sample, different techniques can be used to analyze the specific content.

Gas chromatography and gas spectrometry (GC-MS) or flame ionization detection (GC-FID) are the most extensively used techniques. These methods can distinguish and quantify VOCs at low concentrations, but they require highly qualified technicians and expensive device *(Moschino et al. 2015).*

A new non-selective approach to analyze VOCs in exhaled breath is metabolomics technique, that identify and quantify all metabolites in a biological sample, without *a priori* hypothesis. Metabolomic profiles represent the interaction between genetic expression, environmental exposure, microorganisms, medication, nutrition and toxic substances *(Moschino et al. 2015) (van Mastrigt et al. 2015).* This method allows to define disease phenotype and permits the characterization and personalized therapy for the patient. This approach simultaneously evaluates many metabolites in a sample and generates metabolite profiles that discriminate between different groups of individuals, providing a defining all the biochemical processes underway in a given biological system.

More recently, simpler devices with sensor-based techniques such as electronic nose, colorimetric sensor array and gold nanoparticle sensors have been proposed. They use specific sensors with optical, chemical or electronic properties that can detect and cluster VOCs in the exhaled breath (EB) *(van Mastrigt et al. 2015).* In recent years, several studies have demonstrated the clinical application of these instruments in respiratory disease and allergy *(Dallinga et al. 2010) (Caldeira et l 2011).*

VOCs in the EB can discriminate patients with asthma from healthy children and atopic from non-atopic children *(Dallinga et al. 2010).* In children, VOCs have also been reported as can predict asthma exacerbations *(Robroeks et al. 2013).* VOCs collection is also possible in preschool children and their profiles have been shown to be different in children with recurrent wheezing as compared to controls.

Nevertheless, further studies are necessary principally to evaluate the clinical usefulness of VOCs assessment in assessing asthma severity and monitoring asthma symptoms and response to ICS therapy.

**Exhaled breath condensate**

Exhaled breath condensate (EBC) is a non-invasive method to evaluate airway inflammation analyzing markers of inflammation that can help to understand asthma pathophysiology. EBC is composed of elements from airway lining fluid

collected by the condensation of warm humid breath into a cold surface in a condensing device.


*Fig.2: Refrigerate device use in clinical practice*

EBC is composed of water vapor, unstable volatiles like $CO_2$ and $H_2O_2$, inorganic ($O_2$, $N_2$) and organic ($CO_2$) particles, exogenous and endogenous organic compounds, protein and cytokines *(Dent et al. 2013)*. In the respiratory system, $H_2O_2$ may be released from inflamed cells, including neutrophils, macrophages, eosinophils, and epithelial cells. Nitrogen redox forms such as nitrite ($NO_2$ -) and nitrate ($NO_3$ -) are present in the epithelial lining fluid of the human respiratory tract.

Concentrations of $NO_2$ and $NO_2$+$NO_3$ were significantly higher in cases of asthma, CF and bronchiectasis compared with healthy controls *(Dent et al. 2013)*.

EBC collection is typically done using a refrigerated device in compliance with ATS/ERS guidelines *(Horváth et al. 2005)*. It involves 10-15 minutes of tidal breathing during which the airways lining fluid undergoes an aerosolizing process and is then condensed in a cooled device (0 to -20°C) *(Baraldi et al. 2006).*

The most frequently evaluated parameters in EBC are pH, exhaled markers of oxidative stress and inflammation.

EBC pH is considered a non-specific marker of airway disease and normative data have been published for children from 0 to 20 years, with a median pH value of 8.0 *(Paget-Brown et al. 2006).* Some studies reported that children with stable

asthma had a lower pH in EBC than healthy controls and those suffering from severe asthma had a lower pH value than mild asthmatics *(Carraro et al. 2005) (Brunetti at al. 2008).* In addition, asthmatic patients had a lower pH than those ICS treated, and those with acute exacerbation had a higher pH after treatment with budesonide *(Carraro et al. 2005) (Brunetti at al. 2008).*

Acidification has also been reported in children with allergic rhinitis and atopic dermatitis *(Brunetti at al. 2008).*

At present, no correlation has been reported with asthma symptoms, lung function, FeNO or airway hyperresponsiveness *(Rosias et al. 2004) (Ratnawati et al. 2006).*

An important set of potential biomarkers in EBC is related to oxidative stress like $H_2O_2$, 8-isoprostane, asymmetric dimethylarginine (ADMA), aldehydes and nitrite/nitrate.

$H_2O_2$ in EBC is released from inflamed airways as superoxide anions, an unstable and reactive particle. In the respiratory system, $H_2O_2$ can be released from both inflammatory cells (neutrophils, macrophages, eosinophils) and epithelial cells. The normal level of this molecule in young, non-asthmatic and non-smoking children is 0.09 µmol *(Horváth et al. 2005).* $H_2O_2$ was found to be higher in asthmatic children during exacerbations and decreased after ICS treatment, which emphasized the hypothesis that $H_2O_2$ is a marker of airways inflammation. However, other studies don't demonstrate a significant difference in $H_2O_2$ between asthmatics and controls or in its capacity to predict exacerbations *(Trischler et al. 2012) (Robroeks et al. 2012).*

Asymmetric dimethylarginine (ADMA) is another potential marker of oxidative stress identifiable in EBC by the UPLC-MS/MS technique. It is an analogue of L-arginine that reduces, by inhibiting NOS, the synthesis of NO and increases superoxide. Asthmatic children showed higher values of ADMA than healthy ones with no difference related to ICS treatment *(Carraro et al. 2013).*

Aldehydes and lipid hydroperoxides derive from the oxidation of the phospholipid membrane and polyunsaturated fatty acid.

A study showed high levels of glutathione in the EBC of asthmatic children with exacerbation. That study reported that after 5 days of prednisolone therapy the

malondialdehyde level fell, while glutathione rose *(Corradi et al. 2003)*. These results suggest that in exacerbations of asthmatic patients there is a disparity between oxidative and antioxidant agents. In children with asthma, malondialdehyde levels is associated with air pollution, lung function and inflammatory markers *(Romieu et al. 2008).*

8-isoprostane is a product of arachidonic acid and it is also an indicator of oxidative stress *(Moschino et al. 2015)*. Children and adults with asthma present high levels of this marker, during severe asthma or asthma exacerbations *(Baraldi et al. 2003)*. The concentrations of 8-isoprostane have no correlation with ICS or leukotriene receptor antagonist therapy, lung function or FeNO.

Eicosanoids are a large group of markers derived from arachidonic acid that play a role in asthmatic inflammation. The presence of these markers in EBC can be confirmed by specific enzyme immunoassay and radio immunoassays *(Thomas et al. 2013)*.

In children with asthma leukotriene B4 (LTB4), cysteine leukotrienes (LTC4, LTD4 and LTE4) are high in EBC compared to healthy subjects *(Thomas et al. 2013)*. The role of cysteine leukotrienes (CysLT) in response to ICS therapy is under debate *(Csoma et al. 2002) (Debley et al. 2007) (Steiss et al. 2008)*. Some authors report a significant reduction of CysLT after a course of oral steroids or 6 months of ICS therapy, whereas others report no changes. A significant reduction of CysLTs has been reported after Montelukast treatment*(Montuschi et al. 2006)*.

Several other markers of inflammation and oxidative stress such as cytokines and adenosine have been studied.

Th2 cytokines are assessed using the ELISA technique. Some studies showed that the number of Th2 cytokines is higher and Th1 cytokines is lower in the EBC of asthmatic children *(Karakoc et a. 2012) (Shahid et al. 2002)*. IL-4 was higher in asthmatic children, especially in atopic, and it has been proposed as a predictor for asthma diagnosis, and IL-5 to predict asthma exacerbations *(Robroeks et al. 2012)*. Children with asthma have also been reported to present with a higher IL-4/INFγ ratio related to Th2 inflammation *(Shahid et al. 2002)*.

## ELECTRONIC NOSE TECHNOLOGY

### The e-nose analysis: rationale & applicative issues

Breath analysis performed for medical aims represents one of the most ancient clinical objectives.

Smelling breath to diagnose diseases derives from the ancient medicine. Past physicians knew that several diseases alter the odor of a patient's breath such as diabetes, liver diseases and kidney diseases.

The history of medicine since Hippocrates, during the 4th century BC, describes the use of the smell of patients to assess their health status.

A few centuries later, Galenus (2nd century AD) was able to compare breath odors from kidney or liver diseases, and in the 18th century acetonemic smell in breath has been used to define diabetic ketoacidosis.

Exhaled breath contains thousands of VOCs with exogenous and endogenous origin. Endogenous ones are associated to the physiological and pathological processes of the human body and are not only from the airway tract *(Haick et al. 2014).*

The analysis of VOCs is a non-invasive method to study metabolic, oxidative stress-related and inflammatory processes in the organism, usually linked with several biochemical reactions.

Therefore, the capacity to define pattern of exhaled VOCs to specific clinical conditions could accelerate and simplify clinical approach for diagnosis and monitoring a disease. The breath analysis principle is based on the physiology of olfactory system and, by the application of modern artificial sensors technology, with the purposes to create devices able to identify volatile compounds of clinical interest, even those that are not detected by human smell (e.g., nitric oxide).

The term 'electronic nose' (e-nose) is known since 1988 and was introduced by Gardner and Bartlett, who defined it as 'an instrument that comprises an array of electronic chemical sensors with partial specificity and appropriate pattern recognition system, capable of recognizing simple or complex «odors»' *(Gardner et al. 1994).*

The interest in the electronic nose is demonstrated by many scientific articles that explain and use this new technology in research in medical and clinical practical

applications. E-nose and VOCs analysis were also being applied in different fields such us for volatile emissions assessments, homeland security, environmental protection, biomedical diagnoses, personnel safety and in product development research *(Scarlata et al. 2015).*

Breath analysis aims to identify compounds in different samples of exhaled breath to recognize and differentiate physiologic and pathologic conditions. A crucial question is the identification of the VOCs in the complex exhaled breath mixture. Only 1% of the about 3000 different compounds present in human breath is shared to all individuals *(Horvarth et al. 2009)*, whereas the other mixtures are formed from the interaction between human behaviors, environment factors, and lifestyles. Identify disease markers in or out of that 1% is not the ability of e-nose technology because it doesn't identify the specific molecules.

Gas chromatography (GC) approach consents the identification of each single VOC in different group of patients (e.g., healthy/diseased) *(Scarlata et al. 2015).*

Mass spectrometry lets the measurement of low-molecular-weight particle to identify metabolites that are correlated with a specific illness.

Most popular current VOCs analysis are based on the analysis of each compounds, however an approach that evaluated the mixture of compounds may offer more information on the differences between subjects with different diseases. In this perspective, breath analysis is a 'pattern recognition' problem where the acquired patterns are related to a population of samples.

Electronic noses represent an innovative, relatively cheap and easy technique for VOCs pattern analysis *(Montuschi et al. 2013).* The device is characterized by a sensor array and an in-built processor *(Rock et al. 208).* Functionally they are like the biological olfactory receptors and cannot recognize singular VOC but are able to relate molecular patterns and discriminate exhaled gaseous samples based on their molecular fingerprints. The exhaled volatile compound pattern is frequently referred to as 'breathprint' *(Bikov et al. 2015).*

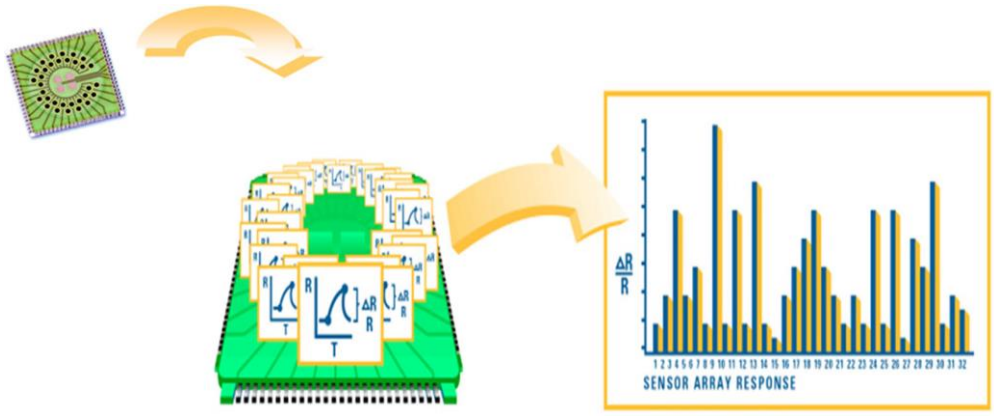



*Fig. 3: The mixture of the signal from all sensors generates the so-called "breathprint" (from Dragonieri et al, 2016)*

## Existing devices

The devices for VOC collection/analysis now existing are summarized in table 1.

| Device (producer) | Type of sensor | Availability | Notes |
|---|---|---|---|
| Cyranose320® (Intelligent Optical Systems, Inc. Baldwin Park, CA, USA) | 32 chemical sensors composed by an inorganic conductor (carbon black) and insulating organic polymers | Commercial | Measurement is based on a resistance variations in each chemical sensor when exposed to a VOC mixture. The differential responses across the array (resistance shifts) are composed in patterns and analyzed by pattern recognition algorithms |
| LibraNose (University Tor Vergata, Rome, Italy) | 8 quartz microbalance gas sensors coated by molecular films of metalloporphyrins | Prototype | Sensors detect the concentrations of chemicals absorbed in the sensitive films through the changes of resonant frequency that is proportional to the absorbed mass |
| MOSES II e Nose® (GSG Messund Analysengeräte GmbH, Bruchsal, Germany) | 8 metal oxide sensors and 8 quartz microbalance Sensors | Commercial | Provides complementary information on the adsorbed volatile compounds |
| TFDS-870 (VST Ltd., Petah Tiqwa, Israel) | 10 pairs of circular interdigitated gold electrodes | Commercial | 14 GNP sensors with different organic functionalities were mounted onto a custom PTFE circuit board to form the nanosensor array |
| The Nanoscale Artificial Nose (NA-NOSE)® | Five combined inorganic nanomaterials such as metal nanoparticles, silicon nanowires and carbon nantubes, with nano- or micrometric organic functionalities | Commercial | Potentially providing an extremely high chemical selectivity |
| **Hybrid systems** | | Combining the high sensitivity of classical e-noses based on chemical sensor arrays with the high specificity of different e-nose techniques including Gas Chromatography/Mass Spectrometry | |
| Prometheus® (Alpha MOS, Toulouse, France) | The sensor array consists of 18 metaloxide sensors arranged in three chambers each containing six sensors. The fingerprint mass spectrometer consists of a quadruple mass filter and an electron impactionizer | Commercial | Combines a sensor array with a fingerprint mass spectrometer |
| The Z-Nose® (Electronic Sensor Technology, Newbury Park, CA, USA) | Available in a portable version | Commercial | Combines a surface acoustic wave detector with Gas Chromatography |

*Table 1: Current available devices (from Scarlata et al, 2015)*

Some devices are commercially available but others, those with nonchemical sensors, are prototypes developed by different centers working in this field *(Scarlata et al. 2015).*

The kind of sensor technology used play an important role in the exhaled breath analysis.

The common sensors used in exhaled breath analysis are made with sensitive materials obtained from organic molecules (organometallic compounds and polymers) that are very conductive and permit to transducer the signal based on mass variations or on optical properties *(Scarlata et al. 2015).*

The combination between organic molecules and gold nanoparticles or carbon-black allow the sensors to transform the chemical signal in an electric resistance change. These sensors in e-nose are usually minor than hundred and are not 108 as in the human olfactory system.

Each sensor of the e-nose is composed by sensitive material that 'translates' the variation of chemical signal into an optical or electrical signal. Electronic device allows the recognition and amplification of very low concentrations of VOCs. The final output must be analyzed using multivariate data analysis techniques.

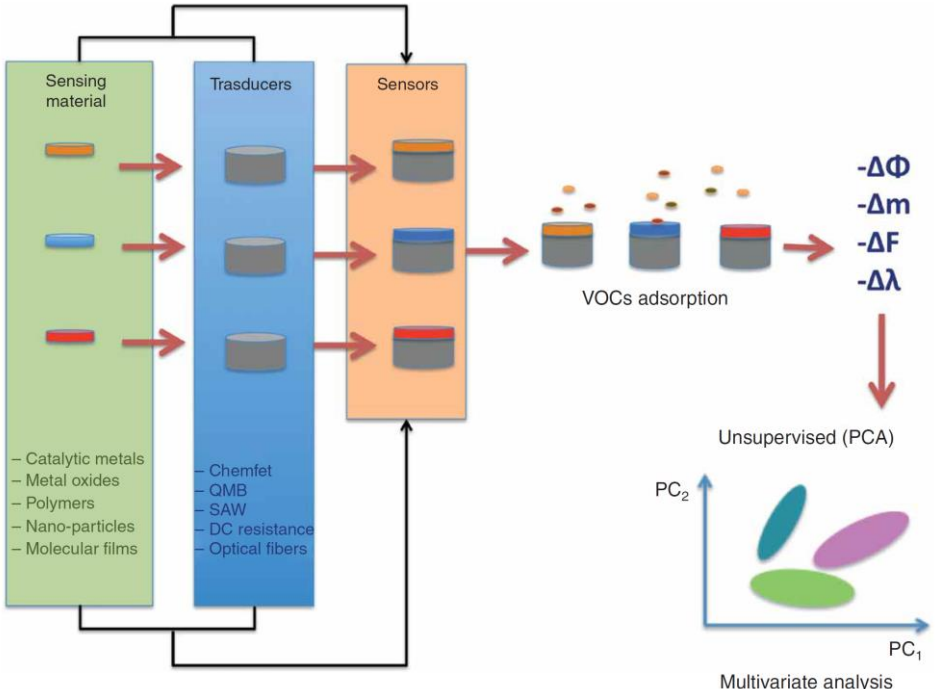



*Fig.4: steps in electronic nose measurement (Scarlata et al. 2015)*

**Sensor systems in Cyranose 320®**

The most usually used electronic nose in respiratory disease is Cyranose 320® that is applied in different research laboratory and studies. The instrument has an optimal number and arrangement of sensors, sensor layer material and vacuum pressure *(Lewis et al. 2004).*


*Fig.5: Cyranose 320*

Cyranose 320® is characterized by a carbon black conducting polymer array of 32 sensors which work on electrical resistance changes from steady-state induced by the volatile particle exposure to the sensor *(Lewis et al. 2004).*

The sample is aspirated, during the measurement, by an in-built pump, and VOCs induced swelling of the polymer film.

This increases the electrical resistance of the composite which produces an electrical signal.

Sensor responses (dR) are computed based on the change in resistance caused by the attachment of molecule and background according to the following formula:

$$dR = (Rs - R)/R$$

where Rs is the response to the sampled gas and R is the response to the baseline reading.

Molecules produce different responses based on their chemical characteristic such as molecular shape, size, volume, dipole moment and hydrogen bonding capacity *(Lewis et al. 2004)*. The sensors response to VOCs is a linear combination of responses to analytes together by the mole fraction absorbed into the polymer and the concentration of the volatile substance and are independent from the background *(Hopkins et al. 2001)*.

Cyranose 320 can differentiate volatile substances in the concentration range between 100 ppb and 100 ppm.

The selectivity of carbon black polymer sensors for volatile substances is not influenced by humidity in exhaled breath but the rise of water vapor pressure decreases its sensitivity *(Hopkins et al. 2001)*.

This possible confounding factor may be resolved excluding from analysis sensors 5, 6, 23, 31 that are the most sensitive to the water sensors in Cyranose 320 *(Maciejak et al. 2002)*.

The polymer sensors are also influenced by the temperature of the sample gas, so monitoring temperature is mandatory during analysis *(Knobloch et al. 2009)*.

## Technical issues: Breath sampling systems, available devices for collection and storage

The procedure of exhaled breath analysis by an electronic nose needs to consider some important and critical aspects:

- ✓ contamination of the sample by exogenous VOCs;
- ✓ collection and storage methods;
- ✓ standardization and reproducibility of the procedure.

*Contamination of the sample by exogenous VOCs:*

Exogenous volatile compounds in the exhaled breath can be reduced by using specific filter that purified the air before entering in the system (wash-in procedure) or pre-breathing the air purified by the subject and/or using a

restrictive protocol for the subject pretreatment in relation to food or drinks intake before the examination *(Gordon et al. 1985) (Dragonieri et al. 2009).*

The wash-in approach needs specific equipment and often is not possible in clinical practice. The patients absorb volatile exogenous compounds also by through the skin and then they excreted in the breath and that are not eliminable.

Another method, easier, to remove exogenous VOCs includes the use of a cartridge (Cartridge N7500-2; North Safety Products, Cranston, RI, see figure 6), connected in series to the inspiratory system of the sampling mechanism. This cartridge can remove 99.99% of the VOCs present in the inhaled air *(Kataoka et al. 2013) (Spanel et al. 2013).*


*Fig. 6: Cartridge*

Nowadays it is undefined the use of nose clips in the influence sampling in electronic nose analysis, but it is used in exhaled breath condensate (EBC) analysis *(Philips et al.1997).*

Ambient air, particularly in laboratories and hospitals, may contain variably high levels of acetone, acids, aldehydes and benzene derivates so a filter may be used to exclude them from exhaled breath *(Bajtarevic et al. 2009).*

In literature two procedures are described to collect the alveolar air sampling. The first one needs that the subject exhale the entire vital capacity through a mouthpiece connected to a 2-way valve (Vitalgraph, Maids Moreton, UK; Hans Rudolph 2700, Hans Rudolph, Kansas City, MO) *(Kataoka et al. 2013),* connected in series with a filter for inhaled VOCs (A2, North Safety, Middelburg, NL) *(Buszewski et al. 2007)* and to a system (silica gel) for eliminating water from the sample. A specify bag for the collection of the sample completes the system (fig. 7).

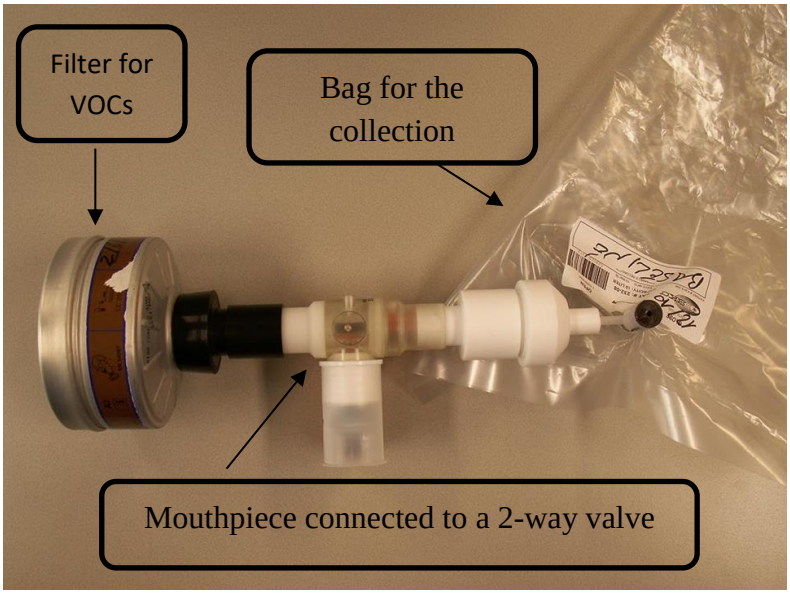



*Fig.7: System to collect the sample*

In the second procedure, the mouthpiece is connected to a filter for VOCs and to a three-way valve *(D'Amico et al. 2010)*. The two free outputs of the valve are linked to a collection bag. The air can easily enter the first bag, while a valve prevents access to the second one. When the volume in the first bag reaches the estimated volume of the dead space, alveolar air can enter the second bag *(de Lacy Costello et al. 2014) (van de Kant et al. 2012).*

Exhaled VOCs can be measured also in real-time, during exhalation, without the problems related to storage and breath-to breath variations. Therefore, in research studies sampling from bags is more common.

The collection of breath air depends on the sampled volume of exhaled breath: alveolar air, or air from both alveolar air and respiratory dead space (mixed air). Mixed air is easier to be collected but at more risk of contamination by external agents.

Mixed air provides more information in asthma, alveolar air to diagnose lung cancer *(Montuschi et al. 2010) (Santonico et al. 2012).*

The kinetics of VOC from the airways may change at various flow rates, but proper modelling for VOC flow-dependence has not been done *(Boshier et al 2011).*

*Representative collection and storage methods*

Plastic bags internally covered with chemically inert materials such as polyvinyl fluoride (PVF, Tedlar), polytetrafluoroethylene (Teflon), polyester-aluminum, polyethylene terephthalate (Mylar, Nalophan) are usually used for collecting and stored the exhaled breath. Tedlar bag are the most usually used.

Some attentions to the environment status must be taken in consideration: direct exposure to sunlight or uncontrolled heating may cause the emission of hydrocarbons, nitrogen, N-dimethylacetamide and phenols from these bags.

VOCs when collected into the Tedlar bags reduce over time their concentration, the volume decreases by 10% of the starting sample 52 h after collection.

The sampling bags are very cheap but not consent the preservation of breath samples for more than a few hours (six hours in Tedlar bags) *(Amman et al. 2010).*

*Standardization and reproducibility of the procedure*

In clinical practice standardization and reproducibility are crucial during a measuring process.

The lack of an acceptable level of standardization does not permit to replicate the results and so the procedure is impossible to be considered feasible for routine clinical practice.

The reproducibility of measurements, also, should be considered of pivotal importance *(Bikov et al. 2015).*

Some studies examined the reproducibility of Cyranose 320 by repetitive breath monitoring and both a good short- and long-term reproducibility was reported but more studies have been needed, in particular in pediatric field.

*Interpretation of exhaled VOCs patterns: the breath fingerprint*

There are two types of methods to analyze the multidimensional pattern of exhaled VOCs. A direct method that compared the breathprint (BPs) of different groups and an indirect that observed BPs over the assessment of correlations between BP and known marker of a specified condition or disease.

*Direct interpretation*

The graphical or numerical analysis of breath fingerprints offers a diagnostic indicator of a specified condition.

There are alternative approaches:

- The radar plot approach represents the individual BPs through the graphical displays of responses by the different sensors (fig. 8).

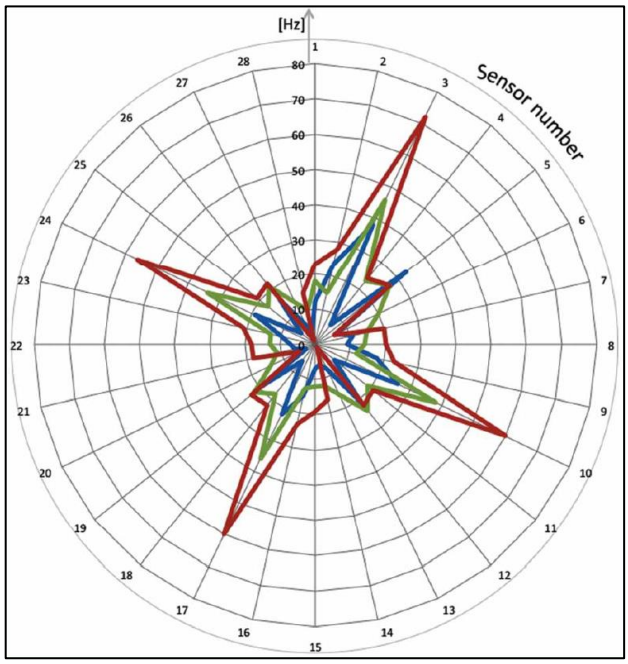



*Fig.8: Radar plot (from Scarlata et al., 2015)*

Each radar plot is formed by equiangular radii, each characterize the response of an individual sensor. The radius length is straight proportional to the size of the response. The radar plot 'profile' consists of a line drawn connecting the end of each radius. This approach has the advantage of providing comprehensive view of individual breath prints that permits a comparative analysis of different BPs and distinguishing behaviors of selected sensors (fig. 8) (*Scarlata et al. 2015).*

Another method is evaluated simple histogram showing both directionality and extent of changes in BPs recorded by individual sensors (fig. 9) using box plots.

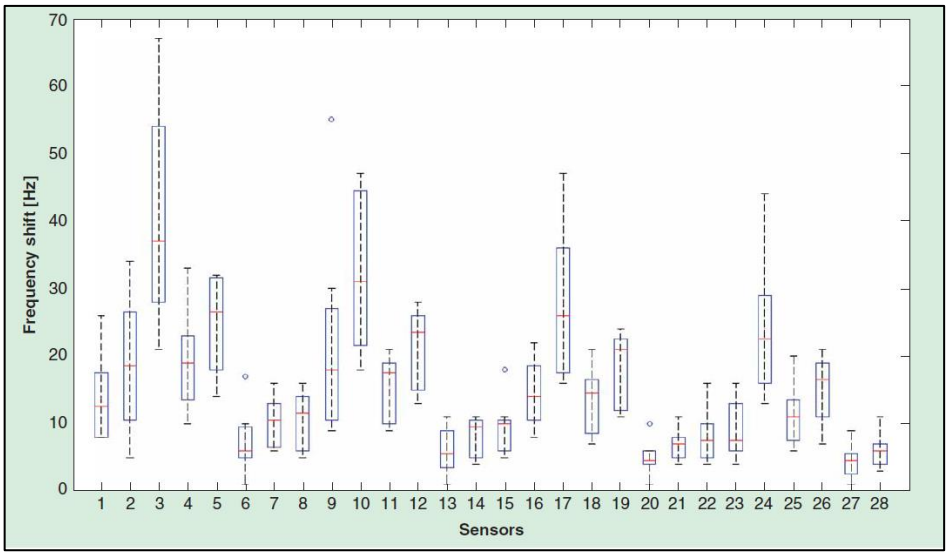



*Fig. 9: Simple histogram*

This graphical presentation is well appropriate for analysis of changes in BPs. Analyzing box plots, many different characteristics of the data can be showed: statistical support for data pretreatment and cleaning, raw comparison of different populations, anomalous and/or prevalent contribution of some sensors (*Scarlata et al. 2015).*

The more appropriate graphical representation to compare BPs of different populations is Principal Component Analysis (fig. 10) that evidence the clusters studied.

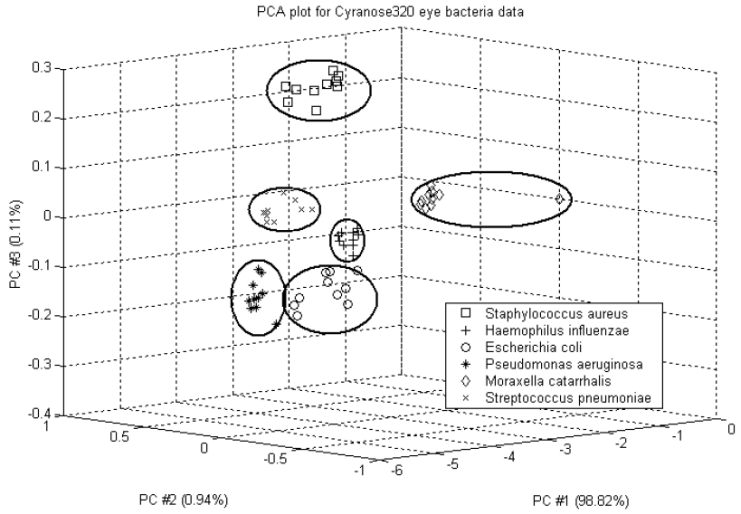



*Fig.10: Example of PCA blot*

## ELECTRONIC NOSE TECHNOLOGY IN RESPIRATORY DISEASES

Electronic nose technology has been studied in different medical field, especially in respiratory disease in adult population.

Nowadays only few studies evaluated this technology in pediatric population, so the use of this new technique in children must been evaluated *(Benedek et al 2013)* as non-invasive and innovative method.

*Asthma*

The evidence of the use of e-nose in asthma diagnosis was described in different studies. Dragonieri et al evaluated mild and severe asthmatic adults from healthy controls (accuracy 100 and 90%, respectively) using e-nose technology *(Dragonieri et al. 2007).* Nevertheless, the e-nose could not efficiently distinguish mild from severe asthma *(Dragonieri et al. 2007)*. Fens et al evaluated molecular profiling from adults with asthma and with COPD (accuracy 96%) and control subjects *(Fens et al. 2009)*. Authors demonstrate a potential role of electronic noses in the differential diagnosis of obstructive airway diseases and in the risk assessment of asymptomatic smokers.

Wagener et al. instead differentiated between eosinophilic and non-eosinophilic asthma with an accuracy of 85% *(Wagener et al. 2013).*

In the last years Plaza et al. discriminated different asthma phenotypes (eosinophilic, neutrophilic, and paucigranulocytic) in induced sputum of asthmatics adult patients *(Plaza et al. 2015)* with e-nose.

De Vries et al. have developed a standardized method to integrate e-nose technology with spirometry (Spiranose). This new technology discriminated controls and asthmatic, COPD and lung cancer with promising accuracy *(de Vries et al. 2015).*

| Study (year) | Study design and aims | Main finding | R |
|---|---|---|---|
| Bofan *et al.* (2013)[†] | Within-day and between-day repeatability of an e-nose made from 32 sensors in patients with stable COPD was assessed | In patients with stable COPD, the e-nose has acceptable within-day and between-day repeatability which varies between different sensors | |
| Antonelli Incalzi *et al.* (2012)[‡] | 25 senior subjects (5 patients with COPD for each GOLD stage and 5 healthy controls) e-nose study through a seven sensor system and respiratory function tests at times 0, 7 and 15 days | VOC patterns were highly reproducible within healthy and GOLD 4 COPD subjects, less among GOLD 1–3 patients. VOC patterns significantly correlated with expiratory flows but not with residual volume and total lung capacity. E-nose might conveniently be used to assess COPD severity and, likely, to study phenotypic variability | |
| Timms *et al.* (2012)[†] | To distinguish patients with GORD from those without GORD in the common obstructive lung diseases and healthy controls | The E-nose distinguished exhaled breath profiles of obstructive lung disease patients without GORD from obstructive lung disease patients with GORD ($p = 0.023$, accuracy 67.6%), asthmatic patients with reflux from asthmatics without GORD (85%, $p = <0.015$, interclass M distance >2.8), but did not reach acceptable performance for patients with COPD and COPD with GORD ($p = 0.047$, accuracy 64%) | |
| Hattesohl *et al.* (2011)[†] | Exhaled breath condensate and pure exhaled breath of patients with COPD with ($n = 10$) and without ($n = 23$) AAT deficiency and healthy controls ($n = 10$) were analyzed | Smell prints of patients with AAT-deficiency were different from those with COPD in exhaled breath condensate (Linear Discriminant Analysis (LDA): $p < 0.0001$, sensitivity of 100%, specificity of 100%) and in pure exhaled breath (LDA: $p < 0.0001$, sensitivity of 100%, specificity of 100%). The e-nose can reveal differences in smell prints of COPD patients with and without AAT deficiency | |
| Bikov *et al.* (2014)[†] | To assess changes in Exhled Breath Condensate pH during Exercise Induced Bronchoconstriction. 22 asthmatics who reported breathlessness following exercise and 16 healthy individuals. pH, dilution factor and volatile compound pattern measurement pre-exercise and at 0, 10, 20 and 30 min after physical exercise challenge | The development of EIB was related to acute changes of EBC pH, which suggest the role of airway pH decrease in the pathophysiology of EIB. Exercise-induced changes in exhaled biomarkers suggest methodological precautions to avoid physical exercise before performing exhaled breath tests | |
| Fens *et al.* (2011)[‡] | Relationships between exhaled compounds, eNose breathprints and sputum inflammatory markers were analyzed and receiver operating characteristic (ROC) curves were constructed in 28 COPD patients with mild/moderate disease | Exhaled compounds were highly associated with sputum cell counts. eNose breathprints were associated with markers of inflammatory activity in GOLD stage I. ROC analysis for eNose showed high sensitivity and specificity for inflammatory activity in mild COPD but not for moderate COPD. This suggests that breath analysis may be used for assessment and monitoring of airway inflammation in COPD | |

*Table 2: Studies on the breath analysis in asthma and COPD (from Scarlata et al., 2015)*

*Respiratory infection*

During the last years there was an increasing interest on exhaled VOCs profiling in respiratory infections disease.

E-nose in correlation with a neural network system was tested to successfully identify mycobacterium tuberculosis but also anaerobic bacterial cultures in the blood *(Pavlou et al. 2004).*

Lai et al. used e-nose to recognize upper respiratory infections and to differentiate the common upper respiratory bacterial pathogens (Staphylococcus aureus, Streptococcus pneumoniae, Haemophilus influenzae, and Pseudomonas aeruginosa) in vitro *(Lai et al. 2002).*

Other studies in vivo, evaluated exhaled breath of patients with pulmonary tuberculosis and controls with Cyranose 320. *(Nakhleh et al. 2014).*

Thaler et al. identify the bacterial species in patients with sinusitis, and other authors evaluated VOCs profiling in the diagnosis of Ventilator Associated Pneumonia (VAP), aspergillosis in patients with cystic fibrosis and in subjects with prolonged chemotherapy-induced neutropenia *(Thaler et al. 2005) (Hanson et al. 2005) (de Heer K et al. 2013).*

| Study (year) | | |
|---|---|---|
| Hockstein *et al.* (2004)† | Testing the breath of 25 patients receiving mechanical ventilation and its potential diagnostic aid in the detection of patients with VAP | In the first analysis, in which a training set was identical to a prediction set, the accuracy of prediction results was greater than 91.6%. In the second analysis, in which the training set and the prediction set were different, the accuracy of prediction results was at least 80%, with higher accuracy depending on the specific parameters and models being used |
| Hanson *et al.* (2005)†† | To determine whether breath test analysis Using an electronic nose correlates with a clinical pneumonia score in a sample of 19 mechanically ventilated surgical intensive care patients | The score predicted by the electronic nose showed good correlation with The actual pneumonia score (r = 0.81) |
| Hockstein *et al.* (2005)† | To determine the potential use of an electronic nose as a diagnostic adjunct in the detection of ventilator associated pneumonia. 44 mechanically ventilated patients were studied. Fifteen patients had pneumonia scores of 7 or greater | With Fisher discriminant analysis and K-nearest neighbor analysis, the Electronic nose was able to discriminate between the 2 groups |
| Bos *et al.* (2014)† | To assess whether eNose would be able to discriminate patients with VAP from those without VAP based on analysis of headspace air from TAs. TAs were collected every third day from 45 intensive care unit patients who were ventilated for more than 7 days | Fourteen patients developed VAP, 14 patients had airway colonization but did not develop VAP and 17 patients developed neither VAP nor airway colonization. E-nose discriminated VAP with a sensitivity of 94%; specificity of 79%, PPV 91%; NPV 85% |
| Humphreys *et al.* (2011)†† | To investigate the potential use of an E-nose for the diagnosis of VAP by detecting microorganisms in BAL fluid in a prospective comparative study of E-nose analysis and microbiology on 44 patients following a minimum of 72 h mechanical ventilation | E-nose fingerprints correctly classified 77% of the BAL samples, with and without microbiological growth from patients not on antibiotics. Inclusion of patients on antibiotics resulted in 68% correct classification. Seventy per cent of isolates, cultured in the laboratory from the clinical samples, were accurately discriminated into four clinically significant groups. E-nose technology can accurately discriminate between different microbial species in BAL samples from ventilated patients |
| Fend *et al.* (2006)†† | To investigate the potential of an EN (gas sensor array) to detect different *Mycobacterium* spp. and *Pseudomonas aeruginosa* in the headspaces of cultures, spiked sputa, and sputum samples from 330 culture-proven and HIV-tested TB and non-TB patients. The data were analyzed using principal-component analysis, discriminant function analysis, and artificial neural networks | EN differentiated between different Mycobacterium spp. and between mycobacteria and other lung pathogens both in culture and in spiked sputum samples. The detection limit in culture and spiked sputa was found to be 1 × 10(4) mycobacteria ml(-1). After training of the neural network with 196 sputum samples, 134 samples (55 *Mycobacterium tuberculosis* culture-positive samples and 79 culture-negative samples) were used to challenge the model. The EN correctly predicted 89% of culture-positive patients; the six false negatives were the four ZN-negative and two ZN-positive patients. The specificity and sensitivity of the described method were 91% and 89%, respectively, compared to culture |

*Table 3: Studies on breath analysis in respiratory infection (from Scarlata et al., 2015)*

*Lung and pleural cancer*

Electronic nose has been used also to analyze patients with lung cancer and healthy controls as described by Di Natale et al *(Di Natale et al. 2013).*

D'Amico et al., compared exhaled VOC spectrum of subjects with lung cancer and with other lung diseases (COPD, idiopathic pulmonary fibrosis, pulmonary arterial hypertension, and pulmonary sarcoidosis) obtaining an accuracy of 85.7% *(D'Amico et al. 2010).* Similar values were obtained in patients with and without metabolic comorbidities, such as diabetes, obesity, and dyslipidemia and showed a large sensitivity to lung cancer at stage I with compared to other stages (II/III/IV) *(Gasparri et al. 2016).*

These studies underline the importance of the application of this new technology for screening patients at early stages of lung cancer.

Two studies evaluated e-nose technology in the field of Malignant Pleural Mesothelioma (MPM). Dragonieri et al. compare exhaled breath samples of patients with MPM in individuals professionally exposed to asbestos compared to healthy controls *(Dragonieri et al. 2012).*

*Obstructive Sleep Apnea Syndrome*

Several studies investigated exhaled VOC patterns using Cyranose 320 in detecting obstructive sleep apnea syndrome (OSAS) *(Greulich et al.2013) (Benedek et al. 2013) (Kunos et al. 2015).*

Greulich et al. showed a significant difference between OSAS and healthy controls and also that exhaled VOC pattern changed after 3 months of CPAP therapy from untreated OSAS *(Greulich et al.2013).*

Benedek et al. use e-nose in children and controls, showing an accuracy of 64% and an AUC of 0.83 *(Benedek et al. 2013).*

Dragonieri et al. has distinguished between the exhaled profile of obese patients with OSAS from non-obese healthy controls but the e-nose could not extract VOC spectrum of obese patients with and without OSAS *(Dragonieri et al. 2016).*

*Other Respiratory Diseases*

Other studies evaluated exhaled VOC profiles in other respiratory disease.

Paff et al. evaluated the exhaled molecular profiles in patients with cystic fibrosis, primary ciliary dyskinesia, and controls *(Paff et al. 2013).*

E-nose could also be applied in the field of lung transplant in the follow-up of lung transplant receivers *(Kovacs et al. 2013).* Moreover, e-nose help in the diagnostic tool of pulmonary embolism. Dragonieri et al. evaluated also patients with pulmonary sarcoidosis *(Dragonieri et al. 2013).*

## METHODS

### Aim of the study

*Primary objective:*

The primary objective of the study is to evaluate the feasibility of this new non-invasive method in pediatric population.

The electronic nose (e-nose) is an emerging technology that detects volatile organic compounds (VOCs) in exhaled breath. It uses sensors that react with different VOCs and generate a specific "breathprint" for each individual.

In literature a limited study evaluated this method in pediatric care.

This new technique could improve and promote early diagnosis and follow up in children with different diseases.

We evaluated the capacity of children to properly perform the expiratory maneuver for collecting an exhaled air sample and if the ability to perform the technique will correlate with the patient's characteristics (age, gender, BMI).

In this phase we evaluated also the limits and the possible changes that could be made in the collection method to make it applicable in the pediatric field.

*Secondary objective:*

The secondary objective is to investigate the existence of different respiratory patterns between a pediatric population with clinical and instrumental diagnosis of respiratory disease and a population of healthy children.

**Study design**

The study was conducted at the Pediatric Respiratory Department of A.O.U.I Verona.

This is a pilot study to evaluate the feasibility of new non-invasive technique, the electronic nose, in pediatric population.

For the *first objective*, we enrolled 48 children aged between 6 and 16 years old (27 males and 21 females) who were conducted to the Pediatric Allergology and Pulmonary Clinic.

Afterwards, this population, based on the objective examination and respiratory function tests, was divided into two groups: asthmatics and controls.

Inclusion criteria for enrollment were:

• Children of both sexes, aged between 6 and 16, who needed a pediatric broncho-pneumologic outpatient visit;

• Signature of informed consent by parents or legal guardian for participation in the study.

Asthma was diagnosed according to the GINA guidelines. The classification of asthma was based on clinical history, physical examination, functional respiratory tests and the β2 agonist reversibility test.

Exclusion criteria for both groups were:

• Patients with oxygen therapy

• Patient collaboration failure during biological collection

• Presence of other chronic respiratory diseases other than asthma.

The group of asthmatic patients was composed of 27 children, 16 males and 11 females, while the control group consisted of 17 patients, 8 males and 9 females (table 4).

At recruitment, after verifying the inclusion and exclusion criteria, physical examination for anthropometric measurements (height, weight, BMI), familiarity about asthma or allergies, skin prick test for diagnose allergic disease, evaluation of ongoing therapy and exposure to passive smoking have been collected.

All children performed, lung function test and exhaled breath sampling collection.

The study was approved by the Ethical Committee of Verona and both children and their parents gave informed consent (CESC564).

| | Asthmatic | Controls |
|---|---|---|
| Total Number | 27 | 17 |
| Male | 16 | 8 |
| Female | 11 | 9 |
| Medium age (yrs) | 11.4 | 10.9 |

*Table 4: Patients characteristics*

For the *second objective* we enrolled we enrolled 47 children aged between 6 and 16 years old (34 males and 13 females) who were conducted to the Pediatric Allergology and Pulmonary Clinic for respiratory disease (obstructive or restrictive disease). This population, based on the objective examination and respiratory function tests, was divided into four groups: patients with obstructive asthma (controlled, partially controlled, uncontrolled asthma) and restrictive pattern (congenital heart disease exposed to surgery) (*fig.11).*

| | Restrictive pattern | Obstructive pattern | Controls |
|---|---|---|---|
| Total Number | 9 | 28 | 10 |
| Male | 8 | 22 | 3 |
| Female | 1 | 6 | 6 |
| Medium age (yrs) | 12 | 11 | 10 |

*Table 5: Patients characteristics*

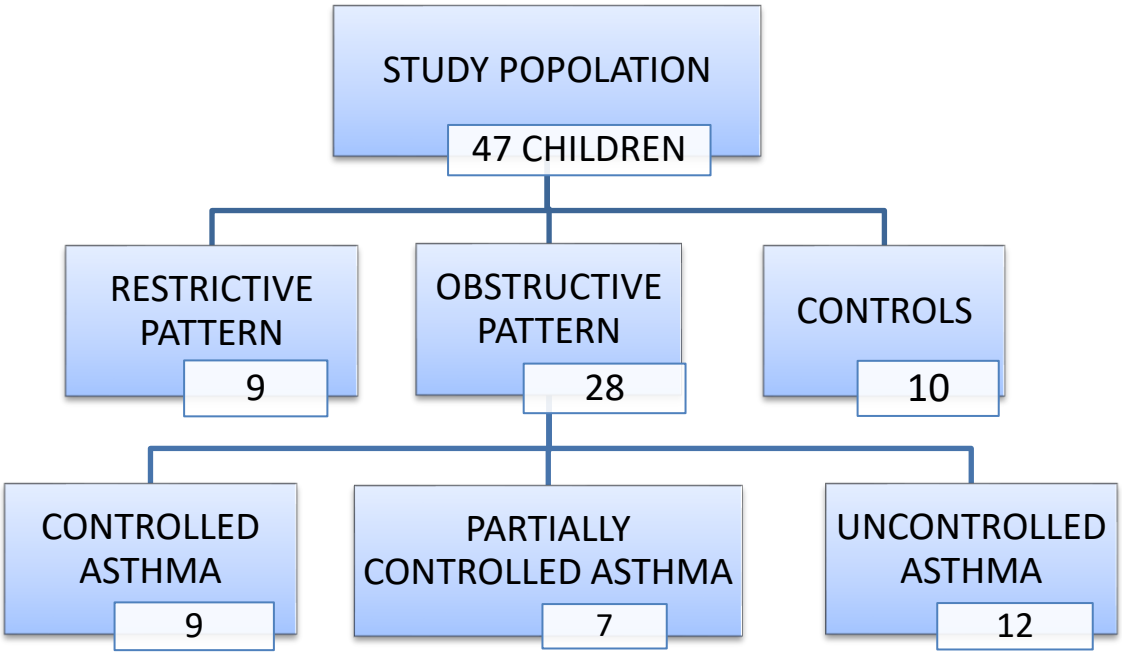



*Fig.11: Study design*

Inclusion criteria for enrollment were:

• Children of both sexes, aged between 6 and 16, who needed a pediatric broncho-pneumologic outpatient visit;

• Signature of informed consent by parents or legal guardian for participation in the study.

Asthma was diagnosed according to the GINA guidelines. The classification of asthma was based on clinical history, physical examination, functional respiratory tests and the β2 agonist reversibility test.

Restrictive pattern was diagnosing on clinical history, physical examination, functional respiratory tests in patients with congenital heart disease that have one or more surgery in early age.

Exclusion criteria for both groups were:

• Patients with oxygen therapy

• Patient collaboration failure during biological collection

• Presence of other chronic respiratory diseases other than asthma or restrictive disease.

At recruitment, after verifying the inclusion and exclusion criteria, physical examination for anthropometric measurements (height, weight, BMI), familiarity about asthma or allergies, skin prick test for diagnose allergic disease, evaluation of ongoing therapy and exposure to passive smoking have been collected.
All children performed lung function test and exhaled breath sampling collection.

**Respiratory function**

Spirometry was performed following the American Thoracic Society (ATS) and the European Respiratory Society (ERS) guidelines, by a Masterscreen IOS (Jaeger; Wuerzburg, Germany) spirometer. It was calibrated daily with a 3L syringe (Cardinal Health; Germany 234 GmbH), to test the accuracy of volume measurement and to check ambient temperature and humidity.
To evaluate bronchial reversibility, four separate doses (total dose 400 mcg) of salbutamol were delivered at 30 s intervals and the respiratory tests were repeated 10–15 min after. Forced expiratory volume in one second (FEV1), forced vital capacity (FVC), maximum mid-expiratory flow (FEF25%–75%) and Tiffenau index (FEV1/FVC) were considered for the evaluations.

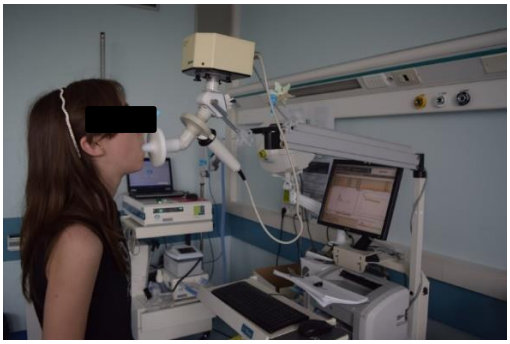


*Fig. 12: Spirometry*

**Exhaled breath collection**

The patients breathed through a mouthpiece with the nose clipped into a 2-way non-rebreathing valve (Hans Rudolph 2700, Hans Rudolph, Kansas City, Mo) with an inspiratory VOC filter (HONEYWELL SPERIAN A2 ALUMINIUM FILTER CANISTER A2, North Safety, Middelburg, NL) and an expiratory silica reservoir to dry the expired air.
The expiratory port was connected to a Tedlar bag.

The subject, connect with mouth to the system, performed an inspiratory capacity maneuver and exhaled the full expiratory vital capacity into the bag with an expiratory resistance of 20 cmH2O to close the soft palatum and to obtain an expiratory flow of 0.1 to 0.2 L/s. Within 20 minutes, the bag was connected to the electronic nose for the analysis.

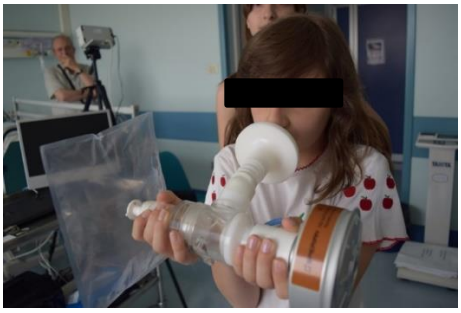


*Fig. 13: Exhaled breath collection*

**Electronic nose**

Exhaled breath samples were analyzed by a commercially electronic nose (Cyranose 320; Smith Detections, Pasadena, Calif) with a nanocomposite array of 32 organic polymer sensors.

When these sensors are exposed to a mixture of VOCs the polymers are swelling, inducing a change in their electrical resistance.

The changes in resistance of each of the 32 sensors produce data that are collected in an onboard database, producing a distribution (smellprint) that describes the VOC mixture and that can be used for pattern-recognition algorithms.

*Fig. 14: E-nose analysis*

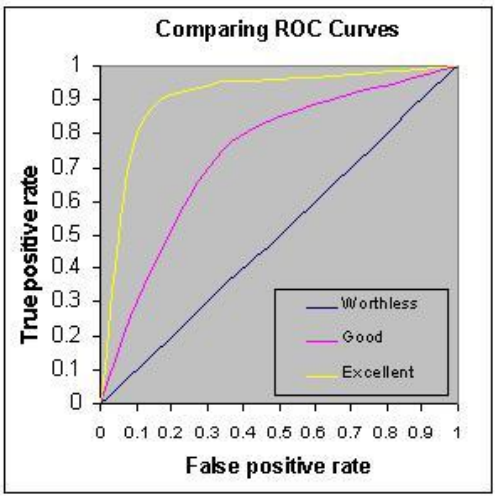
## RESULTS

### Statistical analysis

The signals of the 32 sensors for the 47 study patients were analyzed by PCA to reduce the data from 32 individual sensors to a set of five principal component scores aimed to finding the factors capturing the largest variance in the dataset. PCA was used as an exploratory analysis and was plotted in 2-dimensional graphs to visualize between-group separations.

Once the PCA factors had been calculated, these factors were used to estimate penalized logistic regression for the construction of a pattern recognition algorithm *(Friedman et al. 2010)*.

Discrimination between patient groups were evaluated using the Area Under the Receiver Operating Characteristic curve (AUC-ROC). The graph below shows an example of three ROC curves representing excellent, good, and worthless diagnostic tests plotted on the same graph.




The accuracy of the diagnostic test depends on how well the test separates the group being tested into those with and without the disease in question. Accuracy is measured by the area under the ROC curve. An area of 1 represents an excellent test; an area of 0.5 represents a bad test. A rough guide for classifying the accuracy of a diagnostic test is the traditional academic point system:

- 0.90 - 1 = excellent
- 0 .80 - 0.90 = good
- 0.70 - 0.80 = fair, moderately good
- 0.60 - 0.70 = poor
- 0.50 - 0.60 = bad

The optimal cut-off on the ROC curve was estimated using the approach suggested in *(Youden et al. 1950)*, which maximizes the Youden's J statistic:

$$J = \text{sensitivity} + \text{specificity} - 1.$$

Data were analyzed by Stata software version 15.0 *(StataCorp. 2017. Stata Statistical Software: Release 15. College Station, TX: StataCorp LLC)* and R version 3.4.2 *(R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.https://www.R-project.org/).*

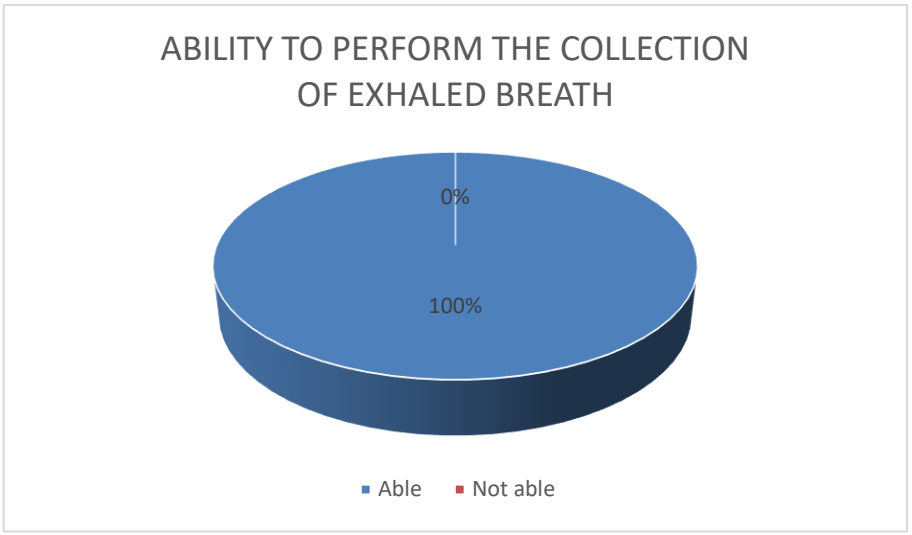
**Evaluation of feasibility of the new method in pediatric population**

Electronic nose is a relatively recent method and, in the literature, only very few studies were conducted on pediatric subjects. However, one of these were conducted in subjected in mechanical ventilation, therefore data on spontaneous breathing patients are not available.

The primary objective of our study was to evaluate the feasibility of this new non-invasive method in the pediatric population.

100% of the children examined were able to correctly perform the necessary maneuver for collecting the sample.

This result, associated with low cost and ease of use, makes this method suitable for use in a pediatric clinical setting.




*Analysis of the breathprint obtained*

For each of the 45 patients we observed the measurements provided by the 32 sensors. The data matrix has 45 rows and 32 columns, and we first used a Heatmap chart.

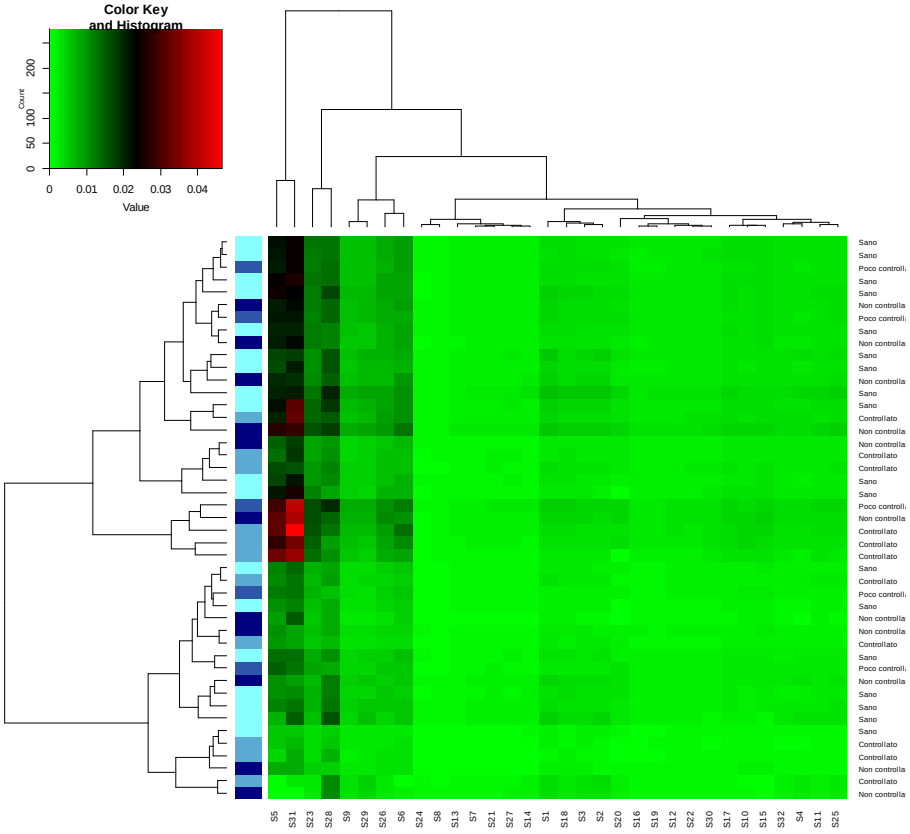



*Fig.15: Heatmap chart*

This type of representation associates different color to each numeral value of the matrix using a rule that is defined by the Color Key, represented at the left top of the chart.

In this heatmap, two clusters have been applied: on the lines (patients) and on the columns (breathprint). The heatmap seems to identify two clusters of individuals based on their spectrum, one of which is characterized by higher values in S5, S31, S23 and S28 sensors (fig. 15).

However, this subdivision does not coincide, even partially, with the two groups of controls and asthmatic patients in the study.

We tried then to represent, in a two-dimensional graph, a first clustering with two groups, based on the breath using the Partitioning Around Medoids (PAM) method (fig. 16).

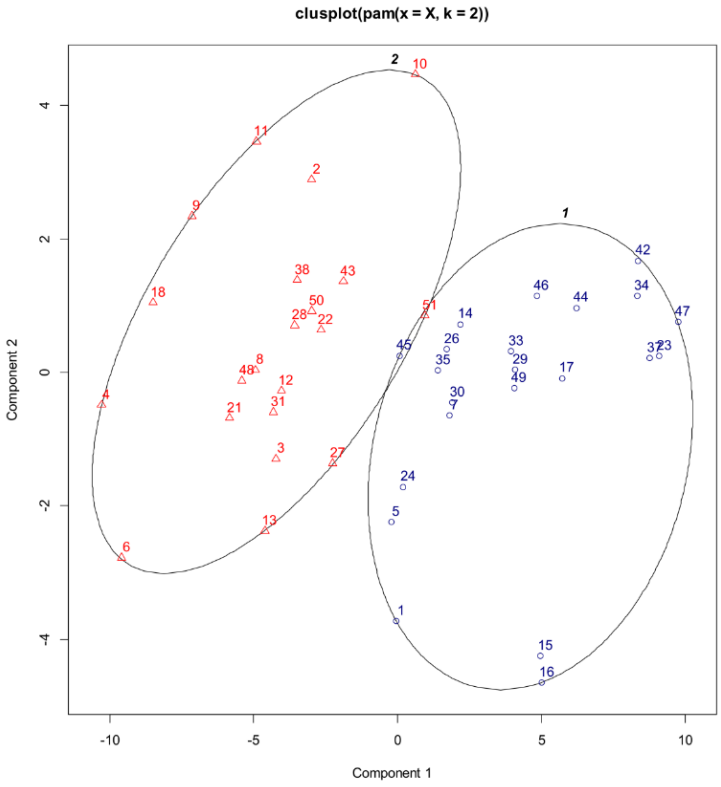



*Fig.16: Partitioning Around Medoids (PAM) method*

On the aisles, the first two main components of a PCA are represented (the variability of subjects explained by these components is 95%) and the points are highlighted with different colors and symbols based on their clusters.

Then we characterize the individual subjects within the two groups, focusing on specific characteristics of patients such as age, exposure to smoke and other characteristics. In this way, we noticed that the two clusters behaved differently depending on the ages considered.

In children under the age of 9 years it is possible to differentiate them in the two clusters, asthmatics and controls. In older children this classification was not possible. The explanation of this behavior is probably linked with a bias on the volume of exhaled collect in the bags.

In fact, the total expiratory volume of a young children (<9 years old) is about 1 liter and consequently the 1L Tedlar bags are sufficient to contain the entire lung volume, this does not occur in older children with larger total exhalations volumes. In this case, the bags only contain the exhalation present in the upper airways and not the lower pathways most affected by the inflammatory processes.

At this point we collected exhaled breath from children in three different Tedlar bags: 1 liter, 2 liters and 5 liters.

We analyzed the more representative volume contains in the three different bags and define that in children aged 6-16 years the most suitable is 2 liters; and this was an important bias in the prior analysis. For the second objective, therefore, we used 2 liters Tedlar bags.

**Evaluation of the existence of different respiratory patterns in a pediatric population**

The subject characteristics of the 3 groups analyzed (obstructive, restrictive patterns and controls) are described in Table 5 and figure 11. Patients with obstructive pattern (asthma) were divided into 3 groups (controlled, partially controlled and uncontrolled asthma) about the symptoms controls.

A sample of exhaled breath could be obtained in all subjects.

The measurements were made in the same room with fixed temperature and humidity. A silica filter was used to limit the influence of humidity on the sensor

signals. Patients don’t eat any food, coffee, or other drinks during the 2 hours before the test.

First, we examined whether exhaled breath from patients with restrictive pattern could be discriminated from others.

The first graphical representation (fig. 17) obtained by PCA shows that patients with restrictive pattern have smellprints that significantly differ from those of the other patients for almost all the 32 nanosensors. In particular, the median value of the nanosensor signal is constantly higher for patients with restrictive pattern (fig. 17-18-19).

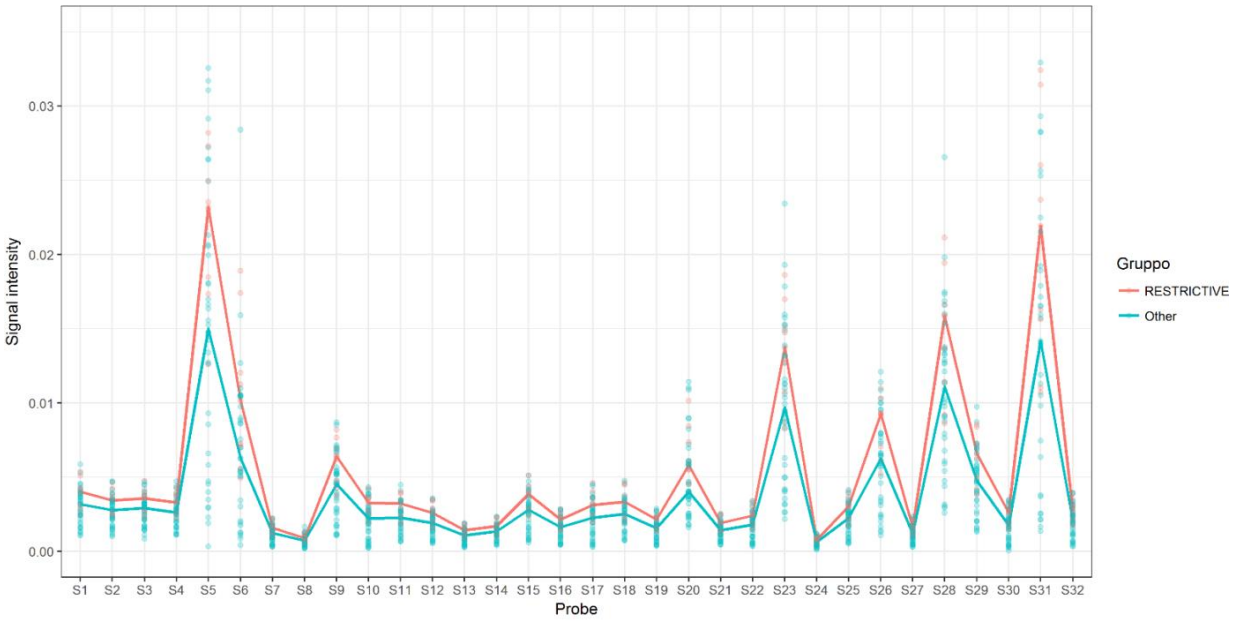



*Fig. 17: Graphical representation of smellprint median values in the 32 nanosensors for patients with restrictive pattern (red line) and other patients (blue line)*

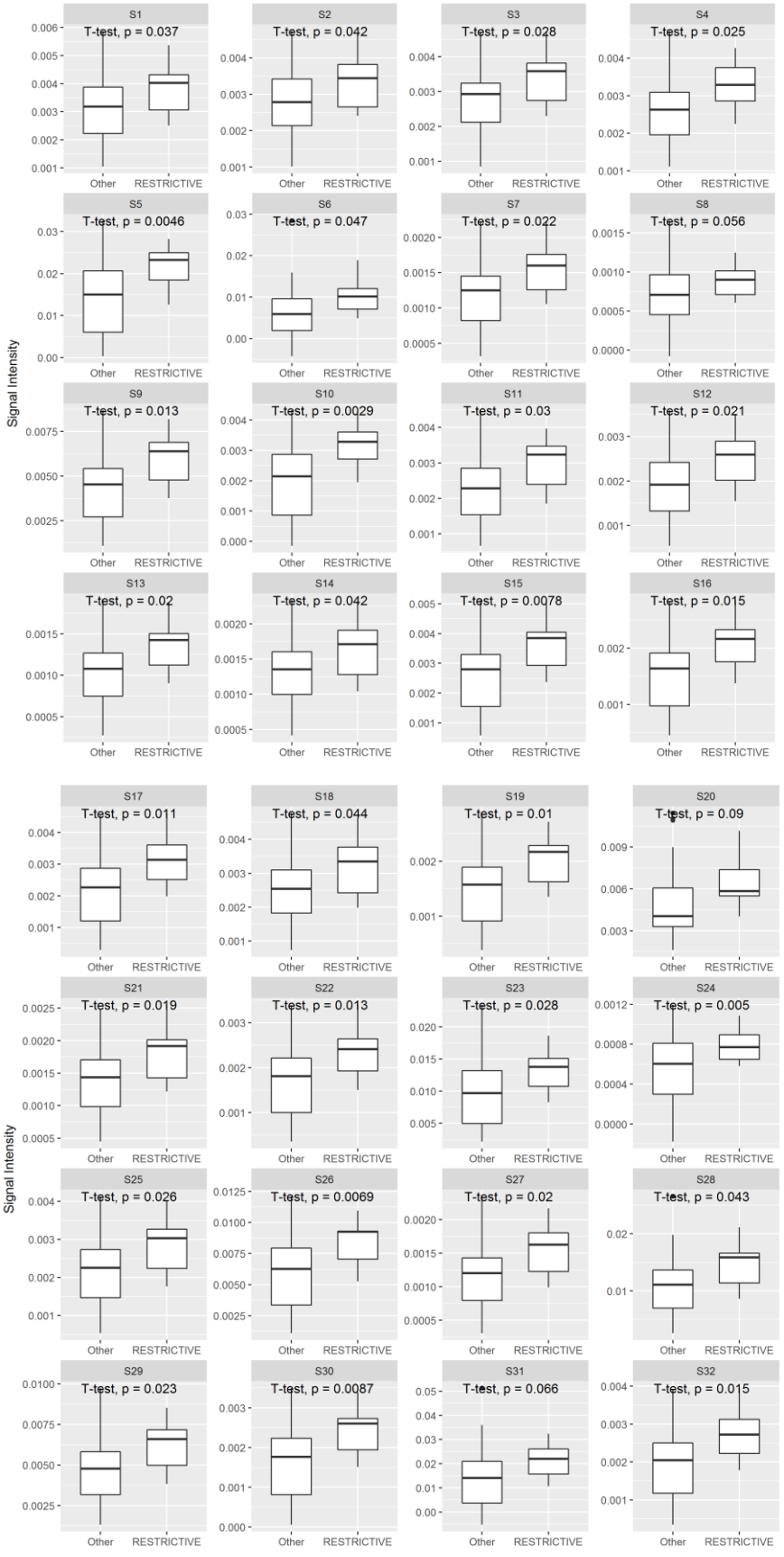
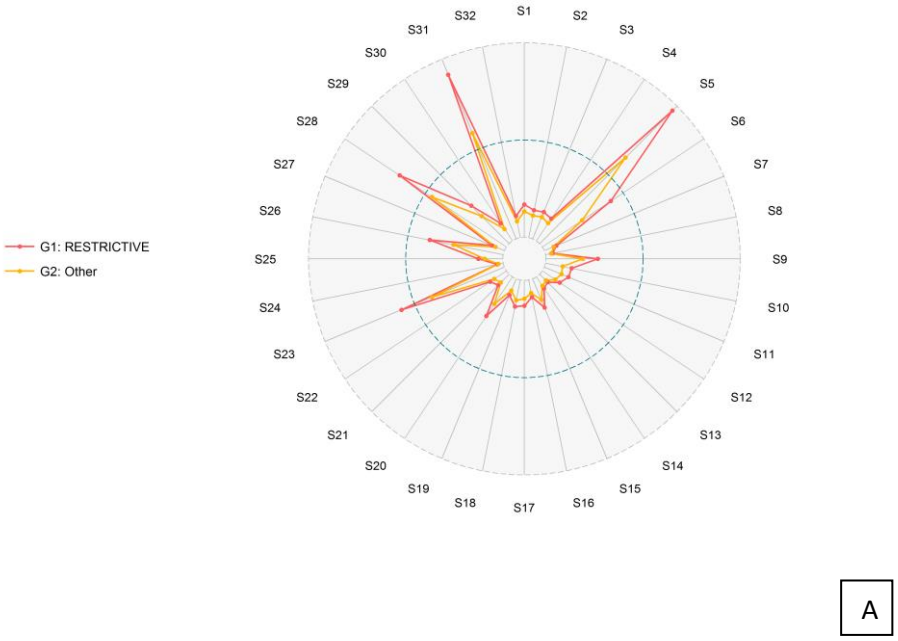



*Fig. 18: Boxplot of smellprints of 32 nanosensor*



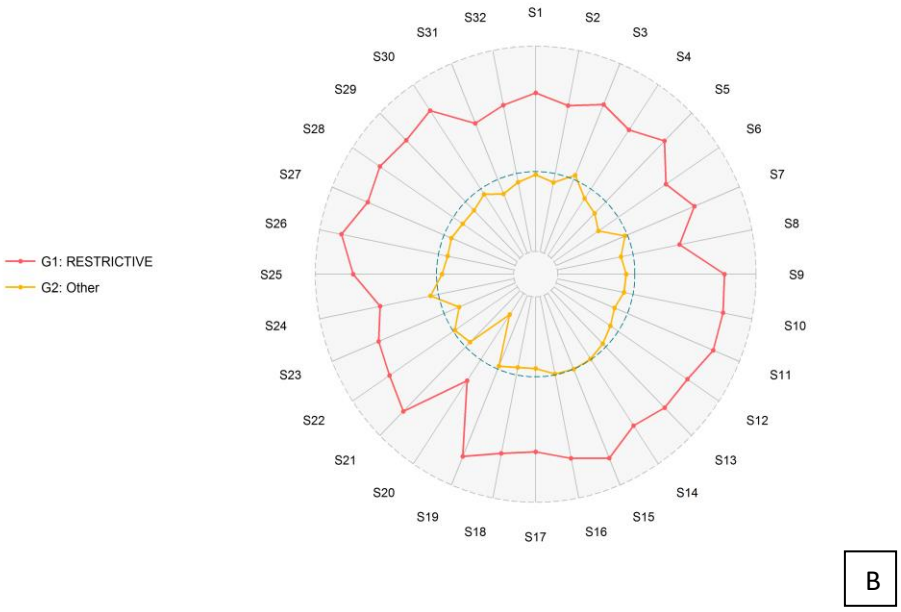




*Fig. 19: Radar plot of smellprint median values for the untransformed (A) and standardized (B) signals of the 32 sensors.*

Principal component analysis (PCA) was performed on unstrasformed data and the first 5 principal component scores were calculated.

A penalized logistic regression model for discriminating patients with restrictive pattern was estimated using the 5 PC scores. The selected scores were the first (Score1) and the fifth (Score5) (fig. 21). The decision rule given by the penalized model was:

if (Score5-0.01972441*Score1- 0.0008513515>0) then (patient=Restrictive)

otherwise (patient=Other)

The area under the ROC curve for this model was 0.84 (95%CI= 0.67 – 1.00, P<0.001). The sensitivity and specificity of the model at the Youden cutpoint were 0.78 and 0.95, respectively.


*Fig. 20: Graphical visualization of the discriminative ability of the proposed predictive logistic model. The dashed line represents the discrimination curve of the model. Score1 and Score3 are the predictive PC scores used in the model*

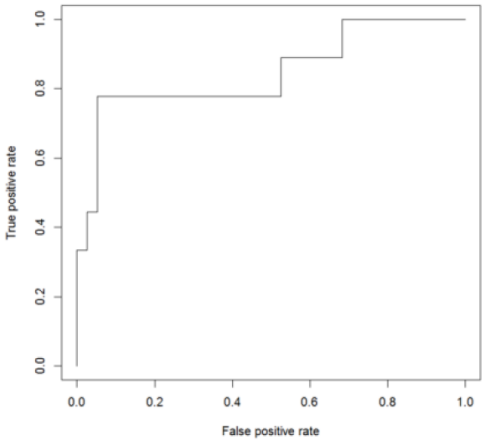



*Fig. 21. Receiver operating characteristic (ROC) curve for the penalized logistic regression model aimed to discriminate between exhaled biomarker profiles of restrictive patients and controls.*

Afterwards, we examined whether exhaled breath from patients with obstructive pattern could discriminate patients with controlled or not controlled asthma.

The first graphical representation (fig. 22) obtained by PCA shows that asymptomatic (control patients and patients with controlled asthma pattern C+AC) have smellprints of the sensor 20 (S20) that significantly differ from those symptomatic (asthma partially controlled and not controlled, APC+ANC). In particular, the median value of signal of S20 is higher for C+AC patients.

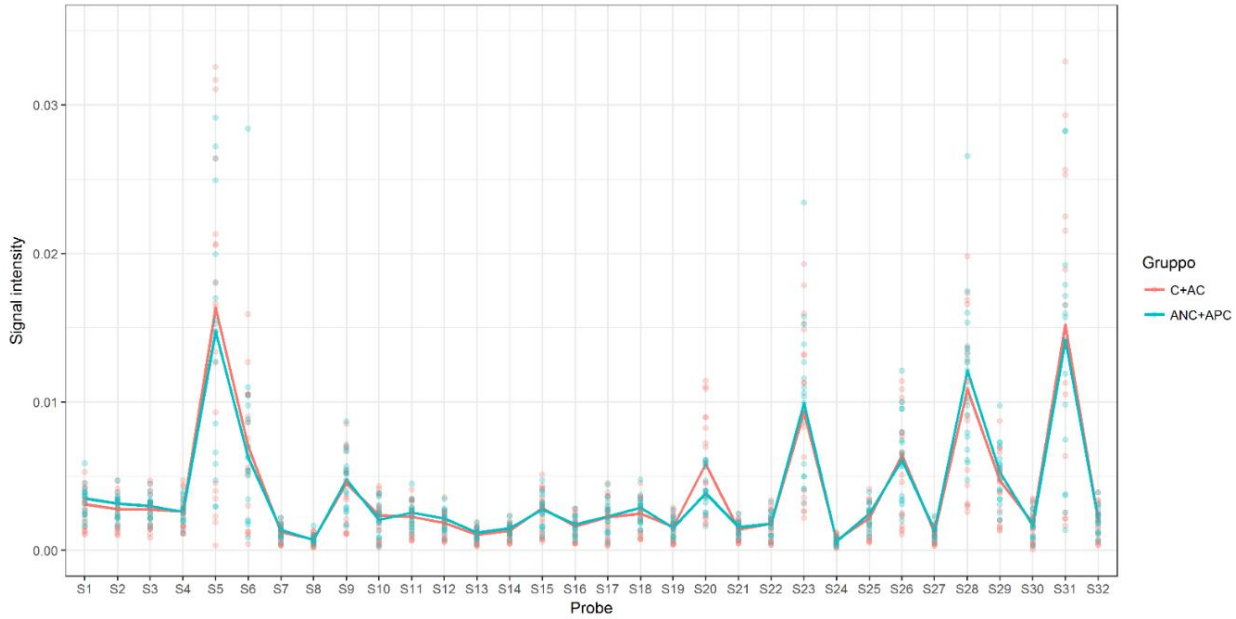
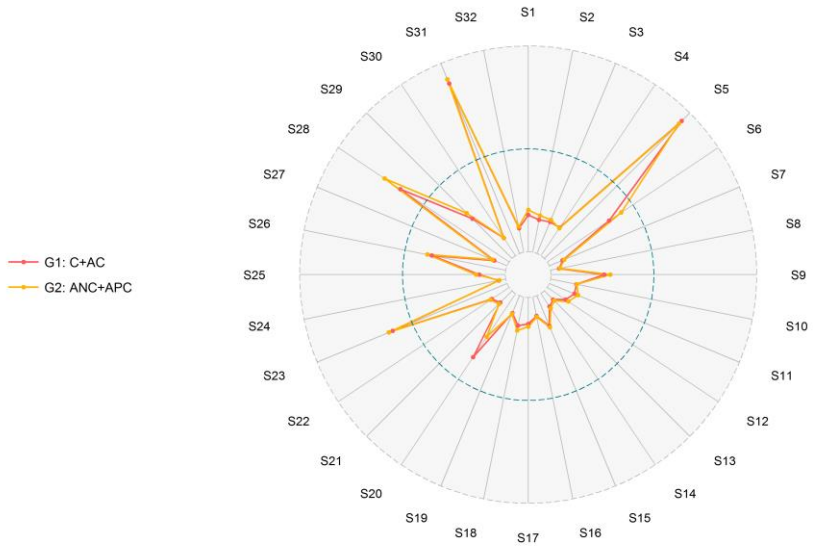
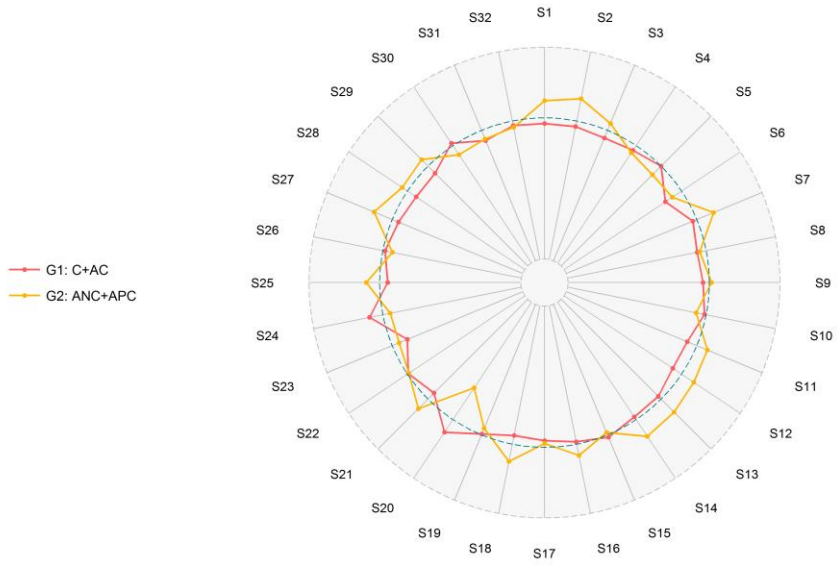



*Fig. 22: Graphical representation of smellprint median values in the 32 nanosensors for control patients and patients with controlled asthma (red line) and partially controlled or uncontrolled asthma patients (blue line)*




A




B

*Fig.23: Radar plot of smellprint median values for the untransformed (A) and standardized (B) signals of the 32 sensors.*

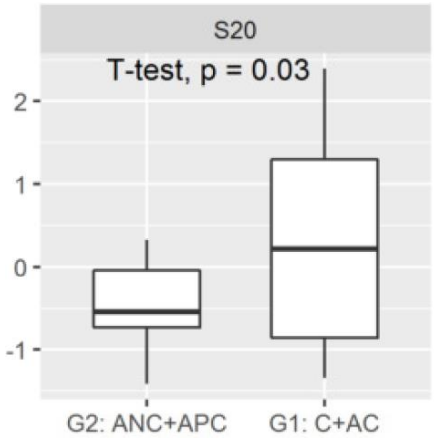



*Fig. 24.: Boxplot of smellprints of sensor 20 (S20)*


*Fig. 25: Graphical visualization of the discriminative ability of the proposed predictive logistic model. The dashed line represents the discrimination curve of the model. Score2 and Score3 are the predictive PC scores used in the model*

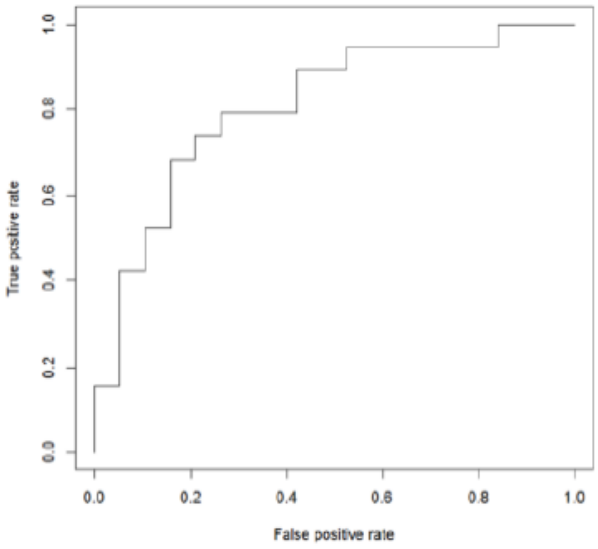



*Fig. 26: Receiver operating characteristic (ROC) curve for the penalized logistic regression model aimed to discriminate between exhaled biomarker profiles of AC+C vs APC+ANC*

Principal component analysis (PCA) was performed on untransformed data and the first 5 principal component scores were calculated.

A penalized logistic regression model for discriminating AC+C patients was estimated using the 5 PC scores. The selected scores were the second (Score2) and the third (Score3) (fig. 26). The decision rule given by the penalized model was:

if (Score3+0.000134485>0) then (patient=AC+C)

otherwise (patient=ANC+APC)

The area under the ROC curve for this rule was 0.81 (95%CI= 0.67 – 0.95, P<0.001). The sensitivity and specificity of the model at the Youden cutpoint were 0.78 and 0.95, respectively.

## DISCUSSION

The electronic nose (e-Nose) is an innovative biomimetic technology that simulates the olfactive system to identify compounds in different samples with complex data analysis system and processing algorithms.

This technique has been applied for the characterization of various pathologies (sarcoidosis, COPD, OSAS and others), as reported in some studies on adult patients. In literature, only a study was conducted on pediatric subjects in spontaneous breathing.

The primary objective of our study was to evaluate the feasibility of this new non-invasive method in the pediatric population. 100% of the children examined were able to correctly perform the necessary maneuver for collecting the sample. This result, coupled with low cost and ease of use, makes this method suitable for use in a pediatric clinical setting.

Based on the results obtained in adults by Dragonieri *(Dragonieri et al. 2007)* and his team, the next goal of our study was to identify different respiratory patterns in a pediatric population with clinical and instrumental diagnosis of obstructive or restrictive disease and a population of healthy children.

However, the smellprint obtained through e-nose analysis showed the presence of two distinct clusters of patients without any correlation, even partial, with the previous subdivision of children into asthmatics and controls.

At this point we re-analyzed the possible bias that influence the practical technique to collect the exhaled breath sample.

We characterized the individual subjects within the two cluster, focusing on age, exposure to smoke and other characteristics. In this way, we noticed that the two clusters behaved differently depending on the ages considered.

The bias found was linked to the exhalation volume collected in the bags used of 1-liter Tedlar bags. In fact, if the total expiratory volume of young children is quite small and consequently the 1L sachets are sufficient to contain the entire lung volume, this does not occur in older children. Probably the 1 L bags in older children only contain the exhalation present in the upper airways and not in the lower ones.

The size of the bags can be considered the first limit of this new pediatric method.

Therefore, in the second phase of our study we used bigger Tedlar bags (2 L) to collect exhaled breath sample.

Our study shows that electronic nose can discriminate exhaled breath of patients with restrictive pattern from others. Moreover, in the group of obstructive patterns, the electronic nose could adequately discriminate between symptomatic and asymptomatic asthmatic patients. These findings indicate that the mixture of exhaled volatile organic compounds is different in different respiratory inflammation forms.

To our knowledge, this is the first study using pattern analysis of exhaled VOC mixtures in exhaled breath in pediatric population. Interestingly, we observed a complete separation of smellprints between patients with restrictive pattern and other groups as well as between symptomatic patients in obstructive pattern.

Nowadays, electronic noses have been used in a variety of medical fields, including the detection of sinusitis, respiratory disease, urinary tract infections, cancers and diabetes mellitus.

The application of electronic noses in respiratory disease showed high specificity and moderate sensitivity in the identification of lung cancer and high accuracy in the diagnosis of tuberculosis infections and pneumoniae.

Therefore, the analysis of VOCs present in exhaled breath may be an important source of biomarkers for the diagnosis of respiratory diseases, including asthma especially in pediatric population.

In this study, attention was paid to methodologic aspects such as the collection of the sample and selection of pediatric patients.

This is the one of the first studies, to our knowledge, where exhaled breath samples collected from children were successfully analyzed by an E-nose.

All the patients were well characterized by using subjective and objective criteria such as the presence of symptoms, reversible airways obstruction or restrictive characteristics.

Patients with controlled and uncontrolled asthma were selected based on their medication use, lung function and asthma symptoms control.

All subjects inhaled VOC filtered air then exhaled in a flow- and pressure-controlled way to close soft palate and the dead space to collected breath originates from the alveolar region.

E-noses were able to discriminate breath samples of patients with bronchial asthma *(Dragonieri et al. 2007),* BPCO *(Fens et al. 2009)* and OSAS *(Greulich et al. 2012)*. For the first time, we used breath analysis in pediatric patients with obstructive and restrictive disease. The biomarker profile of breath collected from the lower airways in children with obstructive and restrictive airways pattern and controls were analyzed by e-nose.

In our study the electronic nose was not able to make a clear distinction between controlled, partially controlled and uncontrolled asthma and controls but only between controls and asthma controlled (C+AC) and partially and uncontrolled asthma (APC+ANC). This may suggest that the state of bronchial inflammation related to clinical manifestations probably are the major determinant of exhaled breath smellprints.

However, our results suggest that new studies with higher sample sizes may be required to obtain optimal results by electronic noses in pediatric patients with various asthma patterns.

Volatile compounds are cellular metabolites produced during multiple pathways in inflammation-related oxidative stress in the airways *(Miekisch et al. 20004)*. Additionally, the composition of exhaled breath is also influence by volatile substances from the blood stream to the alveolar space.

Recent studies described how e-nose can identify exhaled biomarker pattern and the associations between airway and systemic inflammatory markers *(Fens et al. 2011)*.

Other analytical methods to define VOCs in exhaled breath are gas chromatography/mass spectrometry (GC-MS) or magnetic resonance spectroscopy *(Boots et al. 2012).*

More than 3000 different VOCs are being evaluated by GC-MS analysis in the human exhaled breath. The use of an inspiratory VOC filter permits to detected VOCs derived from physiologic and pathophysiologic metabolic pathways *(Moser*

*et al. 2005).* These derives from the airways and the lungs or can represent systemic metabolites from elsewhere in the body.

The markers in exhaled breath are influenced by the change of several metabolic pathways during chronic airways inflammation *(Kharitonov et al. 2001).* For instance, asthma is associated with elevated levels of pentane, markers of oxidative stress and eicosanoids in exhaled breath condensate *(Paredi et al. 2000).*

Our results demonstrate that exhaled biomarker pattern can be collected in a pediatric population and can distinguished children with lung obstructive and restrictive disease, suggesting that airway and systemic metabolic changes are present in the diseases. Further large-scale studies are needed to validate our results and investigate exhaled biomarker pattern analysis by an E-nose as an additional tool in monitoring airway inflammation in children with airways disease.

We acknowledge the limits of the study. The number of patients recruited is relatively small therefore validation studies for exhaled breath pattern discrimination should include a higher number of children to better discriminate different obstructive pattern.

Electronic is a handheld, non-invasive, rapid and feasible in children device that make this tool suitable for pediatric clinical practice. In addition, validation of electronic noses in other diseases, not only on exhaled breath, seems to be mandatory.

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