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Development of a Sensing Array for Human Breath Analysis Based on SWCNT Layers Functionalized with Semiconductor Organic Molecules

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Abstract:	<p>A sensor array based on heterojunctions between semiconducting organic layers and single walled carbon nanotube (SWCNT) films was produced to explore applications in breathomics, the molecular analysis of exhaled breath.</p> <p>The array was exposed to gas/volatiles relevant to specific diseases (ammonia, ethanol, acetone, 2-propanol, sodium hypochlorite, benzene, hydrogen sulfide, and nitrogen dioxide). Then, to evaluate its capability to operate with real relevant biological samples the array was exposed to human breath exhaled from healthy subjects. Finally, to provide a proof of concept of its diagnostic potential, the array was exposed to exhaled breath samples collected from subjects with chronic obstructive pulmonary disease (COPD), an airway chronic inflammatory disease not yet investigated with CNT-based sensor arrays, and the results were compared to those from of healthy subjects breathprints.</p> <p>Principal component analysis showed that the sensor array was able to detect various target gas/volatiles with a clear fingerprint on a 2D subspace, was suitable for breath profiling in exhaled human breath, and was able to distinguish subjects with COPD from healthy subjects based on their breathprints. This classification ability was further improved by selecting the most responsive sensors to nitrogen dioxide, which has been proposed as a biomarker of COPD.</p>
Author Comments:	
Additional Information:	
Question	Response

<p>Please submit a plain text version of your cover letter here.</p>	<p>Brescia, April 9th 2020</p> <p>Dear Dr. Goebel,</p> <p>Please find enclosed a copy of the manuscript: "Development of a sensing array for human breath analysis based on SWCNT layers functionalized with semiconductor organic molecules", which has been revised following the Reviewers comments, as detailed in the attached "Author Author Response/Rebuttal" file.</p> <p>Following the Reviewers recommendations we have:</p> <ul style="list-style-type: none"> (i) added a comment to Fig. 3,d-e and a new Reference (Reviewer1) (ii) corrected Figure 7 (Reviewer 1) (iii) Added the two References suggested by Reviewer 2 (iv) Completed Fig.S10 and S11 with the details required by Reviewer 3 (v) Fixed "volatolomics" spelling and PTFE acronym (Reviewer 3) (vi) Added details on the sampling bag (Reviewer 3) (vii) Added comments and details on the analysis of patient subsets (new Figure S15 and S16 in the S.I. file). (Reviewer 3) <p>All changes have been highlighted in the revised manuscript by RED typing. Finally, we completed the Subsection 2.4 "Statistical Analysis" at the end of the Experimental Section, as required by the Editor.</p> <p>Looking forward to hearing from you.</p> <p>Yours sincerely,</p> <p>Luigi Sangaletti and Paolo Montuschi on behalf of all authors</p>
<p>Do you or any of your co-authors have a conflict of interest to declare?</p>	<p>No. The authors declare no conflict of interest.</p>
<p>Response to Reviewers:</p>	

Development of a Sensing Array for Human Breath Analysis Based on SWCNT Layers Functionalized with Semiconductor Organic Molecules

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ABSTRACT

A sensor array based on heterojunctions between semiconducting organic layers and single walled carbon nanotube (SWCNT) films was produced to explore applications in breathomics, the molecular analysis of exhaled breath.

The array was exposed to gas/volatiles relevant to specific diseases (ammonia, ethanol, acetone, 2-propanol, sodium hypochlorite, benzene, hydrogen sulfide, and nitrogen dioxide). Then, to evaluate its capability to operate with real relevant biological samples the array was exposed to human breath exhaled from healthy subjects. Finally, to provide a proof of concept of its diagnostic potential, the array was exposed to exhaled breath samples collected from subjects with chronic obstructive pulmonary disease (COPD), an airway chronic inflammatory disease not yet investigated with CNT-based sensor arrays, and the results were compared to those from of healthy subjects breathprints.

Principal component analysis showed that the sensor array was able to detect various target gas/volatiles with a clear fingerprint on a 2D subspace, was suitable for breath profiling in exhaled human breath, and was able to distinguish subjects with COPD from healthy subjects based on their breathprints. This classification ability was further improved by selecting the most responsive sensors to nitrogen dioxide, which has been proposed as a biomarker of COPD.

Keywords: carbon nanotubes, electronic nose, breathomics, hybrid heterojunctions, COPD

1. Introduction

Breath analysis, being non-invasive and suitable for repeated measurements, might provide a powerful tool for personalized medicine, including molecular phenotyping of respiratory diseases.^[1]

Several volatile organic compounds (VOCs) have been identified in exhaled breath,^[2,3] and breathomics or **volatolomics**,^[4-7] the molecular analysis of exhaled breath, has the potential for becoming a real-time technique for assessing and monitoring physiological and pathophysiological variations of endogenous metabolites. At present, the effort to develop methods and devices for breath analysis is quite intense. Among devices for breath analysis, electronic noses (e-noses), artificial sensory systems consisting of an array of cross-reactive sensors where sensor responses are collectively analyzed as VOC fingerprints might provide a valuable diagnostic and screening tool in medicine.^[8]

Therefore, building an efficient e-nose represents an important research issue in the diagnosis and phenotyping of respiratory diseases, including lung cancer, chronic obstructive pulmonary disease (COPD), asthma, and other diseases.^[9-11] Pathophysiological metabolic changes can generate profiles of exhaled breath molecules (“breathprints”) which might be used as biomarkers of disease or for disease sub-phenotyping. As gold standard techniques for breath analysis, including gas chromatography/mass spectroscopy, are expensive and time-consuming,^[8,9] the development of user friendly techniques for breath analysis, especially in large screening programs, require novel approaches featuring low cost and quick testing characteristics. E-noses have been shown to accurately classify patients with a known diagnosis of respiratory diseases, including COPD,^[12] and to be potentially more sensitive than standard techniques, e.g. lung function testing, for assessing the effect of inhaled pharmacotherapies in patients with COPD.^[13]

Among the materials used to build the sensor arrays, metal oxides,^[14-18] conductive polymer nanocomposites,^[19,20] intrinsically conducting polymers,^[21,22] organic-based devices,^[23-25] quartz crystal microbalances,^[26] or graphene nanoribbons^[27] have been tested. A newly emerging type of

porous conductive material based on electronic conductive metal-organic frameworks has been recently considered as a highly efficient sensor material for gas/volatile analysis at room temperature (RT).^[28]

Despite the high performance of organic-based sensors, they have drawbacks, including thickness uniformity control on the nanometer-scale and degradation of properties over time due to the swelling effect and the influence of ambient conditions.^[25] In turn, carbon nanotubes (CNT) possess a rather long stability even in harsh environments, and their use as transducer part in sensors can provide cheap and flexible sensors with long-term stability, making them potentially suitable for medical applications. Indeed, due to their high physical and chemical stability, efficient transport of charge to the electrodes and the wide range of possible architectures and operational features, CNTs are widely used to develop chemiresistor gas/volatile sensors, which could be assembled in a sensor array for e-nose applications.^[5, 29-31] Their features can be matched with low power consumption and room temperature operation, which facilitate portability and integration as devices in the Internet of Things, IOT, architectures.^[32] Furthermore, CNT can be functionalized with various materials, such as metal oxides nanoparticles, polymers or organic molecules, to improve sensor response and increase selectivity to specific target molecules.^[33] Recent publications showed that noncovalent changes of nanotubes by polymer^[34] or oligomer^[35] molecules enhance the ability of an e-nose to identify various VOCs by altering the energy levels of nanotubes or the adsorption selectivity for different volatile molecules. In this work, we used different types of organic semiconductors in the form of polymers, oligomers, and monomers that are typically used in gas/volatile sensor applications because their processing is well developed, and they are suitable to create heterointerfaces with CNT layers.

Although CNT-based sensors represent a huge class of gas/volatile sensors, there is a relative lack of studies on e-noses completely based on CNT-sensors and exposed to real human breath from healthy or sick people.^[36-38] Usually, these arrays are exposed to gas/volatile mixtures, aimed to simulate human breath^[39-43] or to single gases/volatiles, proposed as possible biomarkers of

diseases. [31, 35, 39, 43-52] **Table S1** summarizes the use of CNT-based arrays in breathomics, detailing the type of sensing layers, the targeted disease, and the testing conditions. As shown in **Table S1**, at present, only three studies with CNT-based sensor arrays applied to the analysis of human exhaled breath are available. [36-38]

E-nose sensors are cross-reactive, that is each sensor can bind and respond to several different volatiles, while each volatile may interact with various sensors.^[53] Therefore, multivariate data analysis of the responses from whole sensor array is required to extract relevant information from the breathprints. As an unsupervised classification tool, Principal Component Analysis (PCA) can be a quite useful and powerful chemometrics method to analyze e-nose data.^[53,54]

The combination of a sufficient sensitivity of properly functionalized CNTs and use of PCA can be helpful to face a fundamental challenge of gas/volatile sensing, i.e. the capability to monitor environments with multiple relevant target molecules, even though individual sensors can be poorly selective.

In the present study, we address this challenge by developing a multi-purpose platform for breathomics applications based on CNT layers, deposited on inexpensive plastic substrates, operating at RT, and functionalized with selected organic molecules deposited on the CNT layers either by drop-casting or vacuum deposition. These organic layers are used to modulate the sensors selectivity to various gases/volatiles providing an additional level of discrimination.

Our study, which was carried out in two phases, had the following objectives:

(i) Investigation of the SWCNT-based sensor array behavior after exposure to individual gas/volatiles. After thorough characterization of the morphology and physical properties of each CNT-based layer, we assessed the classification ability of an 8-SWCNT-based sensor array upon exposure to individual gas/volatiles, selected for being known as potential biomarkers of diseases.

The selectivity of the sensor array towards various gas/volatiles was assessed by PCA.

(ii) Assessment of the SWCNT-based sensor array behavior after exposure to exhaled breath samples obtained from healthy and COPD subjects. Once the capability of the sensors array to

discriminate among each gas/volatile was tested, the SWCNT-based sensors array was exposed to exhaled breath samples obtained from subjects with COPD or healthy control subjects to assess its classification ability and provide a proof of concept of its potential clinical utility for exhaled breath analysis in humans, particularly in individuals with COPD.

2. Experimental

2.1 Sensors preparation and characterization

The sensors array is composed of 8 sensors based on a pristine SWCNT layer, a UV-treated SWCNT layer, and six layers where SWCNT have been functionalized with organic molecules. The main features of the 8 layers are reported in **Table 1**.

Each SWCNT film has a thickness of about 30 nm. Nanotubes were grown by the aerosol chemical vapor deposition (CVD) method and then transferred onto single polyethylene terephthalate (PET) substrates by dry method (**Figure 1**). More details on the nanotube growth are provided elsewhere.^[55] The main advantage of this method is the possibility to collect nanotube layers on any arbitrary substrate, including a membrane filter, and transfer it to any surface (**Figure 1a**) by a high throughput technique, which is described elsewhere.^[56]

PET substrates with CNTs were cut into 1.5x1.0 cm² chips by scissors. The Cr/Pd (5 nm/150 nm) electrodes were sputtered through a paper mask on the opposite sides of the substrate with a size of 1.0x0.3 cm². Pd electrode provides the ohmic contact to SWCNT layer, while the Cr few nanometers island film provides better adhesion of Pd to PET substrate. A schematic view of the SWCNT-based sensor fabrication is shown in **Figure 1b**. The SWCNT layers were, then, functionalized by organic molecules deposited through vacuum thermal evaporation (Hex-4T-Hex, PTCDA, TCTA, and TAPC) or from an aqueous (DNA, 0.5 mM) or N-methyl-2-pyrrolidone solution (PANI, 0.1 μM). The structures of organic molecules are presented in **Figure S1** of the Supporting Information (SI). For SWCNT functionalization, a low-pressure mercury lamp Svetolit-

50 (LIT, Russia) with 20 W irradiation power at 191 nm wavelength was used. The flexible substrate with SWCNT layer was irradiated by UV light in a humid atmosphere for 5 min.

Figure 1. Schematic illustration of the SWCNT film deposition process on PET (a) and steps on flexible sensor fabrication and modification (b)

We investigated the resistance stability of our flexible sensors during bending cycles (**Figure 2a**; see the SI and **Figure S2** for details). The resistance of the sensing film increased less than 1% during bending to $\pm 90^\circ$ angle for more than 500 cycles with a minimum radius of curvature of 3 mm. After 2000 cycles, we observed a resistance shift of about 1% that can be explained by a slight evolution of film properties or change in environmental conditions. These data show that the dependence of the resistance on the bending angle remains linear and highly reproducible upon compression and extension of the SWCNT film.

The structural and optical sample properties have been characterized by atomic force microscopy (AFM), UV-Vis, and Raman spectroscopy. UV-Vis and Raman spectroscopy were performed to reveal the structural and chemical changes in the films.

The light adsorption variation in the 4% to 7% range observed at 550 nm wavelength is ascribed to non-uniformity of CNT film coverage rather than to the film thickness (**Figure 2b**). Nevertheless,

we found several peaks in UV-vis spectra for TAPC, PTCDA, PANI, Hex-4T-Hex, TCTA molecules on CNTs (see the SI for details on UV-vis characterization).

Figure 2. (a) Resistance changes of SWCNT film covered by PANI during cycling banding for 50 (●), 500 (■) and 2000 cycles (▼). (b) UV-Vis absorbance spectra for pristine and molecular modified SWCNT films. (c) and (d) Raman spectra of pristine SWCNT film (black) and modified SWCNT layers by PTCDA (red) and Hex-4T-Hex (blue). Raman spectrum for clean PET substrate (grey) is provided as a background.

Measurements of the Raman shift show the Raman bands only for PTCDA and Hex-4T-Hex molecules (**Figure 2c-d**). Regarding PTCDA, the Raman active bands were observed at 751 cm^{-1} , 1050 cm^{-1} , 1383 cm^{-1} and 1455 cm^{-1} , in agreement with literature.^[55] Hex-4T-Hex molecules have Raman active modes at 1451 and 1487 cm^{-1} . Furthermore, we found the noticeable shift of radial breathing mode (RBM) for SWCNTs after molecule deposition, suggesting that nanotubes are covered by the organic molecules. Nevertheless, the functionalization of SWCNT does not affect the quality of the nanotubes, except for UV treated samples (**Figure S3**). The UV processing of SWCNT films results, first of all, in the oxidation of impurities, including amorphous carbon and

organic residuals, that were embedded during chemical vapor deposition (CVD) growth and transfer process. AFM topography demonstrates the decrease of the number of nanoparticles on SWCNTs surface after UV oxidation (**Figure S4**). Moreover, the number of defect sites in nanotubes has noticeably increased, consistently with the increase of the D band intensity in the Raman spectrum of UV functionalized nanotubes (**Figure S3a**) and film resistance (**Table 1**).

We carried out AFM for SWCNT films covered by organic molecules (**Figure S5**). The average thickness for organic layers is in the 5-10 nm range, which increases the measured diameter of the nanotubes. The thickness of the organic films was controlled by the evaporation time and has been calibrated through the deposition on satellite bare glass substrates. In any case, the thickness has been limited to a few molecular layers. Hex-4T-Hex, TCTA, TAPC and PTCDA molecules (**Figure S5**) are preferably deposited in the form of crystals, which is in agreement with crystal-like films observed for both films.

Table 1: Sensing layers (SWCNT+organic molecules) used to build the array, main features, and baseline resistance.

Sample type	Film characteristics	Baseline resistance (k Ω)
Pristine SWCNT (CVD grown)	30 nm film of SWCNT and SWCNT bundles, (according to AFM) I(D/G)= 0.08 (according to Raman)	5.08
UV-treated SWCNT (COOH-functionalized)	I(D/G)= 0.12 (according to Raman)	21
SWCNT covered by Hex-4T-Hex oligomers (acceptor)	Several crystalline layers of Hex-4T-Hex with 6 nm thickness (according to test on glass)	4.25
SWCNT covered by PTCDA monomers (donor)	4 nm porous PTCDA film (according to test on glass)	3.84
SWCNT covered by TAPC molecules (acceptor)	Film of 10-20 nm TAPC nanocrystals (according to test on glass)	6.77
SWCNT covered by TCTA molecules (acceptor)	Film of 6 nm TCTA nanocrystals (according to test on glass)	2.18
SWCNT covered by polymers (PANI molecules Mw=10000, acceptor)	10 nm PANI film (according to test on glass)	18.7
SWCNT covered by DNA oligomers (thrombin aptamer)	SWCNTs wrapped by 30-mer strands of aptamer (according to publications).	5.24

The parameters of the deposition and amount of the material for these experiments were almost the same, and the final thickness of the films measured via quartz resonator was about 5 ± 1 nm. It is clearly seen that Hex-4T-Hex film significantly differs from TAPC and TCTA films in lateral and height dimensions. The cluster size is larger for Hex-4T-Hex than for other ones. For TCTA film, the cluster density and the coverage area are the highest among considered molecules. When the thickness of clusters increases, they form a continuous film, as observed for PTCDA. PANI and DNA produce continuous films smoothing the irregularities of SWCNT film. TCTA and TAPC form nanocrystalline films, which increase the roughness of SWCNT layers (**Figure S5**). Comparing the results of AFM characterization and absorbance, we suggest that the nucleation of organic molecules into crystals on the surface of SWCNTs is different from nucleation on a glass substrate. Due to the porous structure of the SWCNT film, the molecules can cover the surface of nanotubes in-depth and not only on the top layer, thus tuning the electronic properties of nanotubes while providing an excellent permittivity to the gas molecules.

Further investigation of the morphological and structural properties of selected samples was carried out by transmission electron microscopy (TEM) on a LEO 912 AB Omega TEM (Carl Zeiss, Germany). For these measurements we used the SWCNT films transferred, just after the CVD growth, from paper filter to copper TEM grids.

In particular, we collected TEM images and diffraction patterns of the film to show the presence of organic molecules on the nanotubes and discuss the heterojunction formation between organic films and carbon nanotubes (**Figure 3a-b**). The thin films of organic materials were deposited via the same procedure as for SWCNTs on the substrates. In these images it is possible to observe that CNTs are covered by aggregates of organic materials. For PTCDA and TAPC samples on SWCNT, we observe the presence of additional peaks in electron diffraction patterns (**Figure 3d-e**). For PANI, we see only carbon ring and amorphous phase inside the ring (**Figure 3f**). The diffraction patterns confirm the formation of PTCDA and TAPC crystals on SWCNT surface, while PANI forms a polymer film. The crystals of small organic molecules like PTCDA and TAPC have a

different orientation on the curved nanotube surface. They could form superstructures with several symmetry domains. ^[57]

Figure 3. TEM images of SWCNTs covered by PTCDA (a), TAPC (b), and PANI (c) films. In the inserts: the magnified images of individual nanotubes with corresponding organic molecules marked by green squares of (a-c) respectively. The residuals of iron catalyst are observed as dark-gray nanoparticles. (d-f) The electron diffraction patterns for SWCNT-organic films heterojunctions measured in the whole areas of (a-c).

2.2 Volatile sensing measurements

The sensor array was hosted on a properly designed board (**Figure 4a**), with 8 independent channels for the simultaneous detection of each sensor's response. In addition to these channels, relative humidity (RH) (humidity sensor HHH-4000 series - Honeywell Sensing) and temperature (Thermistor NTC PCB 5K - Murata) sensors were available on the board (**Figure 4a**).

CNT-based sensors can behave differently on varying relative humidity conditions, which significantly affects the sensitivity to a specific gas/volatile.^[58] As the exhaled human breath can reach the 100% value of relative humidity, it is quite important to discriminate the effect of humidity not only while sensing single molecules but also while sensing the mixture of molecules

present in the exhaled breath; thus, we exposed the array also to water molecules and, furthermore, in all measurements we tracked the relative humidity through the commercial Honeywell sensor.

The sensor signals have been acquired using a dedicated software, written in the LabVIEW environment. The gas/volatile sensing characteristics were analyzed by exploiting the chemoresistive responses upon gas/volatile exposure. Indeed, the sensitive element, consisting of bundles of SWCNTs (bare or functionalized), can change its resistance in the presence of certain gases/volatiles. The basic electronic circuit for chemiresistor gas/volatile sensing measurements consisted of a load resistor (R_L) in series with the sensor, to which a constant voltage ($V_c = 5\text{ V}$) was applied. By monitoring the output voltage (V_{OUT}) across the sample, the sensor resistance R is measured. Then, the chemo-resistive responses $\Delta R/R_0 = (R-R_0)/R_0$ are measured, where R_0 is the baseline sensor resistance before exposure, while $\Delta R = R-R_0$ is the resistance variation occurring during gas/volatile exposure. **Figure 4b** shows a typical change in the sensor resistance during a gas/volatile exposure and how ΔR , R , and R_0 are determined. A scheme of the readout circuit developed for each sensor is shown in the inset of **Figure 4b**.

A typical signal recorded from the CNT-COOH sensor during a sequence of 1-minute exposures to 30, 20, and 10 ppm ammonia concentrations is shown in **Figure 4c** (adapted from Ref. [58]). The recovery after the volatile exposure is clearly visible.

In order to probe the sensor array capability to discriminate different target molecules, which were selected on the basis of their abundance in human exhaled breath and their possible use as biomarkers of respiratory diseases, the sensors array was exposed to ammonia (NH_3), water (H_2O), ethanol ($\text{CH}_3\text{CH}_2\text{OH}$), acetone (CH_3COCH_3), 2-propanol ($\text{CH}_3\text{CHOHCH}_3$), sodium hypochlorite (NaClO), benzene (C_6H_6), hydrogen sulfide (H_2S), and nitrogen dioxide (NO_2). The dataset was analyzed through multivariate statistical approaches, as detailed below.

Exposure to ammonia, water, ethanol, acetone, 2-propanol, and sodium hypochlorite was carried out in laboratory air, while the exposure to benzene, hydrogen sulfide, and nitrogen dioxide was

carried out in a sealed test chamber for safety reasons; all measurements were performed at room temperature. Gas/volatile concentrations are reported in the SI (**Table S2**).

2.3 Exhaled breath measurements

Twelve subjects with COPD and 9 healthy subjects were included in a cross-sectional observational clinical study. Subjects attended the Pharmacology Unit, Catholic University of the Sacred Heart, University Hospital Agostino Gemelli Foundation IRCCS, Rome, Italy, on one occasion for exhaled breath sampling and lung function testing. Diagnosis of COPD was based on GOLD guideline criteria (Global initiative for chronic obstructive lung disease).^[59] Demographic data of study participants are reported in **Table S3**.

Breath sampling was carried out in disposable polytetrafluoroethylene (PTFE) bags, a very inert material, containing the sensor array. The volume of the bags was 0.6 liters. After inserting the sensing unit, the bag was zipped on the side where the cable was connecting the unit to the data logger. Subjects inhaled to maximal inspiration and inflated the PTFE bag through a plastic straw. After the bag was inflated, the straw was extracted and the bag was properly sealed to preserve the integrity of the sample, and avoid cross contamination between room and breath sample for the whole sampling time. The sensor array inside the bag was exposed to exhaled breath for about 180 seconds to let all sensors fully interact with the target molecules. A scheme of the experimental set-up is shown in **Figure S6**.

Then, the bags were opened, and the sensor array was cleaned with dry air before the next measurement to allow the sensors to return to baseline resistance values (R_0). Two consecutive breath samples were collected from each study participant 1 hour apart for a total of 42 samples from 21 (12+9) subjects.

Informed and written consent was obtained from study participants. The study was approved by the Ethics Committee of the Catholic University of the Sacred Heart, University Hospital Agostino Gemelli Foundation IRCCS, Rome, Italy (Prot. 18300/16, ID: 1180).

(a)

(b)

(c)

Figure 4. (a) Picture of the 8-sensor array board. From left to right: S01: DNA, S02: PANI, S03: TCTA, S04: TAPC, S05: PTCDA, S06: Hex-4T-Hex, S07: COOH, S08: CNT. Humidity and temperature sensors are located in the middle of the array. (b) Typical response of a p-doped CNT layer upon exposure to a reducing gas, e.g., ammonia (NH_3). Inset: read-out scheme applied to each of the 8 array channels. (c) $\Delta R/R_0$ response measured by the CNT-COOH sensor following a sequence of 4 exposures to ammonia at concentrations of 30, 20, and 10 ppm (adapted from Ref. ⁵⁸).

2.4 Statistical Analysis

Data were analyzed with a multivariate statistical approach implemented into the R software.^[60] PCA, an unsupervised multivariate statistical method which represents the main linear technique for dimensionality reduction, partitions the total response in a set of principal orthogonal components (PCs); PC1 represents the dimension with the greatest variance, PC2 the second greatest variance, and so on. In general, only the first relevant PCs need to be considered; when only two relevant PCs, obtained by plotting PC1 as a function of PC2, are present in the resulting space, the response data to each exposure could cluster in separate regions and enable to discriminate different target molecules or groups of subjects, who share similar breathprints, belonging to different classes, e.g., healthy subjects vs. subjects with COPD. If necessary, additional coordinates can be taken into account. For instance, if PC3 is also considered, the clustering is studied in a 3-D space rather than in a 2-D space.

Data to feed the PCA has been pre-treated only with the column mean-centering.

For PCA on data obtained from exposure to single target molecules, a set of 37 exposures was considered. For PCA on exhaled breath, 42 samples from 21 subjects have been considered. In this case, after PCA was carried out, the boundary line between the two study groups was drawn on the basis of the Support Vector Machine (SVM) algorithm detailed in Ref. ^[61]

3. Results and Discussion

As an example of the sensor array response, the resistance change measured upon ammonia and nitrogen dioxide exposure is shown in **Figure 5 a-b**. These two target molecules are often included in the testing of CNT-based sensors due to their reducing or oxidizing behavior, respectively.^[62] In our study, all the sensors increased their resistance during ammonia exposure, while they decreased their resistance when exposed to nitrogen dioxide, and response height and recovery time varied

among the various sensors. Sensor recovery after nitrogen dioxide exposure was always slower than the recovery observed after ammonia exposure.

A recovery of at least 80% compared to baseline R_0 values was observed after 1 hour from ammonia exposure, while recovery from NO_2 exposure was still limited after 15 hours. In this case, the slow sensor recovery can be ascribed to different factors. First of all, the fact that sensors worked at room temperature, as we avoided to heat the sensors to speed-up recovery because of the use of plastic foils as substrates. Then, the difference between nitrogen dioxide and ammonia responses might reflect nitrogen dioxide reactivity with CNTs, which makes nitrogen dioxide desorption more difficult and, therefore, sensor recovery slower. This effect is well known, and previous studies addressed this issue by using UV light post-nitrogen dioxide exposure irradiation to speed up recovery.^[58] Finally, under our experimental conditions, nitrogen dioxide exposure was carried out in a sealed chamber for safety reasons. The use of a large volume (about 2000 cm^3), which required a relatively long time to purge the chamber, might have increased sensor array recovery time after nitrogen dioxide exposure.

Datasets similar to those shown in **Figure 5** were collected for exposures to the various target molecules. Results are summarized in **Figure 6 a-c**, which shows the responses of the 8 sensors array to the 9 selected target molecules expressed as the $\Delta R/R_0$ variations. Data are grouped into three panels as the responses to ammonia and nitrogen dioxide (**Figure 6a**) are by far larger than the responses to sodium hypochlorite, water, benzene, and hydrogen sulfide (**Figure 6b**). The lowest responses were recorded upon exposure to acetone, ethanol, and 2-propanol (**Figure 6c**). Of note, the response to alcohols was low as compared to other target molecules, as usually observed with functionalized SWCNT-based sensors.^[63] Actually, alcohols do not provide an effective charge transfer to CNTs and proper functionalization of SWCNTs is thus needed. Error bars are estimated on the basis of the signal to noise ratio. They are more evident in **Figure 6c**, where a lower signal upon exposure to target molecules was detected.

All the sensors changed their resistance following exposure to all volatiles which were tested, except pristine CNT, DNA, TAPC, TCTA, and PTCDA exposed to sodium hypochlorite. In this case, a resistance change was not observed. In general, the whole sensor array shows a relevant response, especially to ammonia and nitrogen dioxide.

The volatiles selected for the present study (ammonia, nitrogen dioxide, sodium hypochlorite, water, benzene, hydrogen sulfide, acetone, ethanol, and 2-propanol) are known to be related to specific diseases and could be potentially considered as biomarkers. Indeed, 2-propanol, benzene, ethanol, and acetone in the exhaled breath have been reported elevated in lung cancer,^[47] whereas acetone has been shown to be elevated in patients with diabetes.^[64,65] A high concentration of ammonia in human breath is related to liver or kidney disease,^[4, 23, 66, 67] and nitrogen dioxide in exhaled breath has been detected in patients with COPD.^[31, 68, 69] Hydrogen sulfide has been proposed as a biomarker of asthma.^[70,71] By reacting with water as it occurs in ambient air and exhaled breath, sodium hypochlorite can dissociate in hypochlorous acid, that increases chlorine concentration, and in sodium hydroxide, that increases sodium concentration; both chlorine and sodium concentrations have been shown to be elevated in the exhaled breath in children with asthma and cystic fibrosis.^[72]

As shown in **Figure 6**, functionalization can dramatically affect the response of the CNT layer to target molecules, suggesting possible ways to increase the layer selectivity. As compared to the other layers, the COOH sensor was much more responsive to 2-propanol, TCTA displayed a high response to hydrogen sulfide and water; the TAPC showed the highest response to ethanol and, unlike most of the sensors, PANI showed a good response to sodium hypochlorite. Furthermore, the opposite sign of sensor response upon exposure to the same molecule is also helpful for the discrimination of a single gas/volatile. TCTA and COOH showed the best response to hydrogen sulfide, but positive and negative deflection of $\Delta R/R_0$ from the baseline, respectively; PANI and TCTA displayed a positive response to benzene exposure, while COOH displayed a resistance decrease.

The organic molecules can dope carbon nanotubes either by direct charge transfer^[73] or by ion-selective gating.^[74] PTCDA molecules can transfer an electron from a nanotube via π - π stacking, while TCTA and TAPC provide charge injection (**Figure 7a**) and ion gating. To support this diagram, we covered the conductive channel of SWCNT-based field effect transistor with organic molecules using the same deposition procedure. The two opposite behavior types of current-voltage characteristics of backgated SWCNT transistors were observed for PTCDA and TCTA molecules (see Supporting Information, **Figure S7**). This can be explained by the effect of electrostatic doping from molecules. PTCDA and TCTA molecules could penetrate in interlayers between SWCNTs and decrease the number of metallic contacts changing the I_{ON}/I_{OFF} ratio of the source-drain current. Therefore, PTCDA which forms π - π stacking on SWCNT surfaces can extract charges from the nanotube leading to additional p-type doping and shift of the threshold voltage (V_T) to more positive values. On the other hand, TCTA deposition shifts the V_T to more negative values, providing n-type doping of the nanotube.

This coverage provides a controllable change in the CNT sensor properties that is important for better discrimination analysis in an e-nose. For example, while both TAPC and PANI transfer holes to SWCNT, the difference in the energy gap and the majority charge carrier density for these molecules provides better discrimination to reducing and oxidizing gases. Thus, PANI, being a p-type semiconductor, weakly reacts with oxidizing molecules, while in the presence of reducing molecules like ammonia it increases the response of SWCNT (**Figure 7b**). On the contrary, the TAPC doping effect is more pronounced in the case of interaction with nitrogen dioxide, but due to the lower density of majority charge carrier, it responds to both oxidizing and reducing gases.

While a polymer film like PANI is commonly used in sensors, the use of oligomers is limited due to their low conductivity. PANI is a p-type semiconductor that can be easily deprotonated when exposed to ammonia. The response to hydrogen sulfide is clearly lower, because of weaker interaction and minor charge transfer.^[24] On the contrary, oxidative gases/volatiles like sodium hypochlorite, nitrogen dioxide, and water vapor dope PANI and increase its conductivity.^[21]

Figure 5. Response of the 8 SWCNT-based sensor array to 30 ppm ammonia (NH_3) (a) and 10 ppm nitrogen dioxide (NO_2) (b) exposures. The shaded areas denote the exposure time.

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Figure 6. Response of the 8 SWCNT-based sensor array to selected target molecules. Nitrogen dioxide and ammonia (a); sodium hypochlorite, water, benzene, hydrogen sulfide (b); acetone, ethanol, 2-propanol (c).

Furthermore, the small size of molecules can provide more precise control on deposited layers down to a single molecular layer that can increase the sensor response.

Opposite responses of SWCNTs sensors to ammonia and nitrogen dioxide have been observed (**Figure 6a**) according to their well-known reducing and oxidizing effect, respectively. [62] The doping by organic molecules can modulate the level of response. Thus, PANI, as shown in **Figure 7b**, can increase the response of pure SWCNTs to ammonia more than twofold and decrease the sensitivity to nitrogen dioxide (**Figure 6a**). This modulation effect was observed for most of the organic molecules used.

Figure 7. (a) Energy band diagram for SWCNT and organic molecules. **(b)** Schematic energy levels of SWCNT/molecule junctions after Fermi level alignment in the presence of nitrogen dioxide (NO₂) and ammonia (NH₃) molecules.

SI reports additional information on the sensing characteristics of the array. In particular, when exposed to equivalent concentrations of analytes (0.25 ppm), significant resistance variations over the baseline were observed with ammonia, nitrogen dioxide, benzene and hydrogen sulfide with the highest sensor response observed after nitrogen dioxide exposure (**Figure S8**). The detection limit, estimated from calibration curves to ammonia, nitrogen dioxide, benzene and hydrogen sulfide, as

described in the SI for the best performing sensors was usually good, i.e. in the sub-ppb range (**Figure S9**). In most cases, the sensors response was fitted with a Freundlich curve. When exposed to increasing ammonia concentrations, all sensors showed a linear response, except PANI and COOH, which displayed a sub-linear response (**Figure S10**).

Further selectivity of the sensor array can be achieved by PCA, which can be applied to both analysis of the sensor array responses to single volatile and exposures and analysis of human breathprints generated by several molecular species.^[41]

The distribution of sensor response ($\Delta R/R_0$, as defined in **Figure 4a**) to all gases/volatiles at various exposure concentrations in the PCA 2D-space is shown in **Figure 8**. Here PCA performs an unsupervised analysis of the dataset collected by an array of n -sensors (8+humidity) exposed to m -gases (9 selected molecules) at various concentrations (**Table S2**). PCA enables dimensionality reduction and maximizes data variances. The goal of this method is to achieve a clustering of the distribution of single gas/volatile exposures to show that the e-nose has the potential for detecting their presence in complex biological mixtures such as human exhaled breath.

In **Figure 8**, PCA of the sensor array responses is shown as PC1 vs PC2, i.e. the weight of each exposure in the 2-dimensional space of the PCA coordinates with the largest variance. In most cases, exposures to various concentrations of the same volatile were considered in order to enrich the dataset; moreover, since CNT-based sensors can behave differently in varying conditions of relative humidity,^[58] the response of a relative humidity commercial sensor was included in data analysis in addition to the CNT-based gas sensor responses. The highest nitrogen dioxide (2.5 ppm), ammonia (60 ppm), water (30 ppm), benzene (0.25 ppm), and hydrogen sulfide (2.5 ppm) exposure concentrations are indicated. Exposure concentrations decreased as the points approached the axis origin, as shown by the arrows (**Figure 8**), suggesting a relationship between the position of the data points in the PCA space and the target gas concentration. This relationship is shown in **Figure S11**, in which the monotonic dependence of PC1 vs ammonia, benzene, nitrogen dioxide and

hydrogen sulfide concentrations is reported. When the RH sensor was excluded from data analysis, discrimination capability of the SWCNT-based sensor array was reduced, as shown in **Figure S12**.

Figure 8 shows that the contribution of ammonia and nitrogen dioxide are well separated in the PCA plot, consistently with the opposite behavior of these volatiles while interacting with CNTs, and, as the volatile exposure concentrations increase, data distribution of both volatiles roughly followed an arch line. Regarding other volatiles, exposure distribution of benzene, hydrogen sulfide and water clustered away from that of ethanol, acetone, sodium hypochlorite, and 2-propanol. These results show good discrimination ability of the 9 (8+RH) sensors array for ammonia, nitrogen dioxide, hydrogen sulfide, benzene and water. By contrast, exposures to 2-propanol, acetone, ethanol, and sodium hypochlorite concentrations were not separated, as the data for these analytes clustered in a narrow region of the PCA plot. However, a preliminary analysis of the subset of exposures to 2-propanol, acetone, ethanol, and sodium hypochlorite (**Figure S13** of the SI) showed that they are not overlapped. Although larger datasets are required to draw conclusions, these data suggest that there is room for improving the SWCNT-based sensor array ability to discriminate these volatiles.

The overall variance of data in the PCA was 96,99%.

Once the capability of the sensor array to discriminate each gas/volatile based on its exposure distribution had been shown, the SWCNT-based sensor array was exposed to exhaled breath samples obtained from 12 subjects with COPD and 9 healthy subjects.

Figure 8. PCA of the sensor array responses to exposure to nitrogen dioxide (0.29-2.5 ppm), hydrogen sulfide (0.12-2.5 ppm), benzene (0.035-0.25 ppm), ammonia (0.3-60 ppm), water H₂O (28-30 ppm) concentrations. The arrows indicate decrease in exposure concentrations (from the highest to the lowest). Gas/volatile exposure concentrations decrease moving towards the axis origin. The highest exposure concentration (ppm) for nitrogen dioxide, hydrogen sulfide, benzene, ammonia, and water is provided by proper labels.

Typical breathprints registered from the COOH sensor during exposures of breath samples from a healthy subject or a subject with COPD is shown in **Figure 9a**.

Under our experimental conditions, recovery after breath exposure was always observed. Of note, the response to exhaled breath from healthy subjects was larger than the response to exhaled breath from subjects with COPD. This difference might be related to higher breath nitrogen oxide concentrations in patients with COPD compared with healthy subjects as reported in previous studies.^[31, 68, 69] This evidence, combined with the fact that all the 8-SWCNT-based sensors showed a pronounced negative response to nitrogen dioxide exposures (see **Figure 6a**), might explain the lower overall response observed in patients with COPD.

The potential effect of lung function variations on exhaled breath volatile exposure levels and, consequently, on sensor response size is a general issue in e-nose applications to respiratory medicine and needs to be addressed. In the present case, between-group significant differences in forced vital capacity (FVC) were registered (**Table S3**). However, the reduced volume of the sampling bags hosting the sensor array (0.6 liters) allowed all subjects to fully inflate the bags in few seconds, thus excluding effects of exhalate volume on between-groups signal differences. Lung function between-group differences might also result in airflow variations in the rate of volatiles to which the sensors were exposed. Differences in the exhalation rate might affect the signal rise time at the early stage of exposure (0-5 seconds). However, in the present study, we assessed the stable sensor array response observed after 180 seconds, which is unlikely to be modified by airflow changes.

The influence of water vapor on sensor behavior, which was previously discussed in relation to single target volatile exposure (**Figure 8** and **Figure S12**), is even more relevant for the analysis of exhaled breath, which mostly consists of water vapor. Usually, the impact of water vapor on breath analysis is reduced by collecting samples after a 5 min wash-in phase in which subjects are asked to breath tidally through a mouthpiece connected to a 2-way non-rebreathing valve with an inspiratory VOC filter to reduce inhalation of ambient VOCs and an expiratory silica reservoir to reduce sample water vapor.^[13] In the present study, principally aimed at testing the technical feasibility of a new e-nose based on SWCNT, we followed a simplified breath sampling procedure not involving any humidity or ambient air filtering. However, to exclude any bias in the measurements caused by ambient humidity, we investigated possible relationships between study group classification and the exhaled breath sample water vapor content as detected by the RH sensor included in the SWCNT-based sensor array. As shown in **Figure S14**, RH sensor responses in healthy and COPD subjects were similar. Indeed, the mean $\Delta RH/RH_0$ values and the variability in the two study groups are virtually identical, suggesting that changes in water vapor concentrations, as reflected by the RH sensor responses, contribute little, if any, to the observed between-group classification.

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3 As shown in **Figure 9b – left panel**, a good separation between breathprints from healthy subjects
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5 and those from subjects with COPD is obtained from the PCA analysis of our dataset. These results
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7 provide a proof of concepts of correct classification between healthy and COPD subjects with a
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9 SWCNT-based array, suggesting that our e-nose is potentially suitable for identifying patients with
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11 COPD based on their exhaled breath volatile profiles. However, the sample size of this study,
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13 including 12 subjects with COPD and 9 healthy subjects, is limited and results require validation in
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15 larger clinical studies. On the other hand, the use of standardized procedures for breath sampling ^[13]
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17 and analysis, including filtering of ambient air and exhaled breath, and measurement of sensor
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19 responses to exposure to filtered ambient air used as a baseline to be subtracted from the breath
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21 sample responses, is expected to improve SWNCT-based e-nose performance.

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27 In spite of the limited sample size, PCA analysis carried out on selected subsets of patients (gender,
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29 age, and smoking habits) showed that unmatching between the number of COPD and healthy
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31 control subjects in these groups did not affect the overall classification, as discussed in the
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33 Supporting Information (Fig.S15 and Fig.S16).
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37 Finally, to provide information on the most relevant breath volatiles for driving the observed
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39 discrimination between COPD and healthy subjects, we carried out a PCA on breathprints
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41 selectively recorded by sensors which showed the highest response to nitrogen dioxide selective
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43 exposure, that is TAPC, Hex-4T-Hex, CNT and COOH sensors (**Figure 6a**) as shown in **Figure 9b**
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45 – **right panel**. Interestingly, this selective approach clearly improved the e-nose capability of
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47 discrimination between breathprints from healthy subjects and subjects with COPD, suggesting that
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49 breath nitrogen dioxide might be a relevant driver of classification.
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Figure 9. a) COOH sensor responses recorded during exposure to two consecutive exhaled breath samples obtained from a healthy subject (first 2 exposures – green curve) or from a subject with COPD (last 2 exposures - red curve). Sensor response pairs are shown in the same panel. **b)** PCA of exhaled breath samples from 9 (green dots) and 12 COPD subjects (pink rhombus). For each individual two consecutive breath samples, labeled with the same number, were collected and included in the statistical analysis to enrich the dataset. Left panel: analysis carried out on the basis of the responses of all sensors. Right panel: analysis carried out by considering only the sensors with the highest sensitivity to nitrogen dioxide, i.e., TAPC, Hex-4T-Hex, CNT and COOH. Each dot represents a breath sample labeled with the study number of the individual from whom it was obtained. Average and SD values of PC1 projection for the two study groups are shown as horizontal bars. The dotted line represents the between-group boundary as assessed by support vector machine (SVM) algorithm used for classification.

This improvement is also evident from the analysis of the mean values and variances of the PC1 as shown in **Figure 9b**. Indeed, when only sensors with the highest sensitivity to nitrogen dioxide are included, the spread of the data along the PC1 axis is reduced, i.e. the variance decreases from 4.14 to 2.99, for the healthy patient cluster, and from 1.99 to 1.11, for the COPD cluster, allowing for a better discrimination and identification of the boundary line between the two clusters. These findings suggest that the use of selective sensors, more sensitive to specific target volatiles, can improve the discrimination capability of the sensor array. On the other hand, these findings also highlight the need and the importance of the sensor testing upon exposure to molecules known as potential biomarkers of diseases, as was done in the first part of the present study.

4. Conclusions

An array of functionalized SWCNT sensors has been produced to explore its potential applications in breathomics. Functionalization with different semiconducting molecules makes the SWCNT sensor array suitable for discriminating volatile substances. The sensor array was exposed to various target volatiles that can be related to specific diseases. PCA shows that this SWCNT-based array of sensors, operating at room temperature and developed on plastic substrates, detects different target volatiles, making this platform compatible with flexible electronic technology, suitable for the production of functionalized chemiresistive sensors based on SWCNTs and scalable. The capability to test several sensors at a time can facilitate the discovery of new VOC-selective sensors, based on the selected functionalization of CNTs. In this respect, we showed a remarkable sensitivity of PANI-covered CNT to acetone, TAPC-covered CNT to ethanol and SWCNT-COOH to 2-propanol.

On the basis of its good ability to discriminate selected gases/volatiles, this e-nose has been successfully tested on human exhaled breath. The e-nose resulted to be able to distinguish healthy subjects from subjects with a known diagnosis of COPD, not yet investigated with CNT-based

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3 sensor arrays, based on their breathprints. However, larger clinical studies are required to formally
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5 assess and validate the classification performance of this e-nose, using training and testing datasets,
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7 and to assess its potential clinical utility in the diagnosis and phenotyping of individuals with
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11 12 13 14 15 **Supporting Information**

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17 Supporting Information is available from the Wiley Online Library or from the author.
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35 00401 - fabrication of functionalized CNT sensors, and No. 17-19-01787 - synthesis of carbon
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37 nanotubes).
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ToC text

A sensors array based on heterojunctions between semiconducting organic layers and CNT films was produced to explore applications in the field of breathomics, i.e. the molecular analysis of exhaled breath. The array was successfully validated on the diagnostics of chronic obstructive pulmonary disease, a major lung disease so far not yet investigated with CNT-based arrays.

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