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Identification of melanoma with a gas sensor array

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Background/purpose: The relationship between diseases and alterations of the airborne chemicals emitted from the body has been found in many different pathologies and in particular for various forms of cancer. Metabolism of cancer cells is greatly altered during their lifetime; then, modification of chemicals is supposed to be large around cancer tissues. Positive hints in this direction were provided, as an example, on studying the breath composition of lung cancer-affected subjects. Besides the conventional analytical approaches, in recent years sensor arrays were also applied to these researches considering the chemical composition changes as those occurring in other applications such as for instance, those dealing with food quality measurements.
Methods: In this paper, the first application of sensor arrays to study the differentiation between melanomas and nevi, namely malignant and benign affection of melanocytary cells, respectively, is presented and discussed. The localization of lesions on the skin surface made possible the utilization of differential measurements aimed at capturing the differences between two adjacent skin regions. This approach strongly reduces the influence of skin headspace variability due to the peculiar subjective odour background and the skin odour variability. The measurement campaign involved 40 cases; 10 of these were diagnosed melanomas referred to surgical intervention. Nine of these diagnoses were further confirmed by histological examinations of the removed tissue and one was a false positive.
Results: The differences in the chemical composition of headspace were verified with a gas-chromatographic investigation, and the classification of electronic nose data provided an estimated cross-validated accuracy of the same order of magnitude as the currently used diagnostic instruments.
Conclusion: Electronic nose sensors have been shown to have good sensitivity towards volatile organic compounds emitted by skin lesions, and the method seems to be effective for malign lesions identification. The results presented in this paper encourage a second experimental campaign with a larger number of participants and a systematic use of gas chromatography mass spectrometer technology in order to identify some possible melanoma biomarkers.

Key words: non-invasive diagnosis – volatile organic compounds (VOCs) – headspace – electronic nose

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CHEMICAL COMPOUNDS exhaled from the skin surface are the result of a combination of several contributions. The most important sources are the skin glands' emission, subjected to hormonal control, and bacterial populations at the skin surfaces, which live by metabolizing and transforming organic compounds. Any alteration in this equilibrium, for instance due to some pathologies, induces changes in both the nature and the amount of volatile compounds. Because the equilibrium is subjective in character, the chemicals can considerably vary from individual to individual, giving rise to a kind of personal chemical fingerprint.

Besides metabolism changes, any alteration in the skin surface is supposed to modify the quality and the quantity of the exhaled compounds. Such changes may include non usual micro-organisms, such as fungi, or more frequently skin dots, or, more properly stated, nevi. Nevi can undergo further modifications such as melanoma, which is a particular tumor generated from the melanocytary cells. This kind of tumor is not very frequent, nonetheless, a clear increase in the number of cases has been recently observed in the last 30 years, in the white USA population, melanoma occurrence has considerably increased in about all the age ranges but with a positive five times higher rate in males older than 65 (from 18.8 to 91.9/year in 100,000) and with lower rates also for women in the same age range (1).

So far, surgery has been demonstrated to be a unique efficient therapy for melanoma and it can be considered to be resolutive when a melanoma is removed in its early stage; this is because, unfortunately, both medical-oncological therapies and radiotherapy are unsuccessful on advanced and metastatic melanoma. In order to achieve an early diagnosis of melanoma, in the last few years, the clinical ability of the dermatologists based on visual inspection of supposed melanoma has been complemented by other 'non invasive' methodologies based on imaging. The more simple of them is dermatoscopy that, combining semeiologic criteria with image analysis algorithms, improves the clinic diagnosis accuracy from about 5% to 30% (2). A more sophisticated technique is the digital system based on epiluminescence images. This procedure allows time monitoring of pigmented lesions, suggesting surgical removals only for those lesions that show substantial modifications with time (3). A recently introduced method, based on the use of confocal laser microscopy, has been applied to investigate the pigmented lesions, with encouraging results (4).

The accuracy of these methods is variable. The percentage of a correct clinic diagnosis based on visual inspection is about 70%; this value is interesting because it indicates that about 30% of the cases do not have clearly visible features.

With a dermoscopic test, undiagnosed cases are reduced to 8%. The largest number of these cases comprises both 'nevus-like' melanomas (those that have not yet shown the clinical and dermoscopic characteristics of the melanoma and thus resembling nevus) and 'hypomelanotic' melanomas (in which only a few characteristics of the vascular reticulum, if present, suggest the presence of the melanoma) (5).

Finally, it is worth noting a number of new technologies still oriented towards non-invasive techniques such as the nuclear magnetic resonance technique (6) and that are based on sentinel lymph-nodes' monitoring (7). To conclude this short overview on the newest non-invasive technologies, it is important to highlight some important works oriented towards skin characterization (8, 9) and malignant melanoma detection (10). Eventually, none of the image-based diagnosis techniques offers a diagnosis guarantee of 100%.

From the chemical activity point of view, in general, tumour cells are characterized by an altered metabolism expected to give rise to an anomalous composition of the emitted volatile compounds. In many cases, the measure of volatile compounds has been proven to provide sufficient information for the identification of tumours. For instance, lung cancer was studied for several years and a number of investigations evidenced the correlation between the anomalous concentration of a group of compounds and the presence of the disease (11).

In case of melanoma, an indirect demonstration of the possibility to identify it through airborne chemicals has been shown by trained dogs that used their fine olfaction to locate tumours (12). Nonetheless, dog perception is rather complicated and largely based on sense integration. Hence, it is not completely clear whether only olfaction is sufficient to detect the disease. In other words, it is still not clear whether the volatile compounds emitted from a melanoma are sufficiently different from those emitted by a nevus or by a normal skin.

Besides this last example, most of the investigations about the relationship between exhaled chemicals and diseases were carried out by developing analytical methods aimed at discriminating specific compounds directly related to the presence of the disease.

On the other hand, in recent years novel methodologies based on the use of arrays of partially selective chemical sensors for the classification of complex samples appeared. Such arrays are currently identified as electronic noses because of some similarity with the human sense of olfaction.

Chemical sensor arrays were demonstrated to be able to identify a number of different diseases (13), among them, also a tumour form like lung cancer (14). Analysis of the skin was also conducted to study the evolution of ulcers and wounds (15) and the influence of metabolic alteration such as those supposed to take place in neurological disorders (16).

In this paper, the first investigation towards the identification of melanoma by measuring the volatile compounds emitted from the lesion with a chemical sensor array is presented. It is concerned with a cross-sectional study involving 40 individuals with suspect melanoma. Ten cases were diagnosed as melanoma according to a conventional diagnosis, including epiluminescence and, for some of the individuals, the histological inspection of the tissue. It has to be noted

that melanoma is a rather rare tumoural form; in Italy, in the last year, the rate of insurgence of new cases was around five to seven cases each 10,000 habitants per year, and so the goal of achieving a sufficiently large statistic may require a very long time. The data reported here show about 80% correct discrimination of melanoma from nevi, a value not far from the performance of the more diffused diagnosis methods.

Materials and Methods

The experimental campaign took place for 6 months at the 'Istituto Dermopatico dell'Immacolata' (IDI) in Rome. A total of 40 patients presenting suspected lesions were enrolled in this study. Each tested lesion was characterized by epiluminescence and for those cases requesting surgical removal the histological report was also available. A sub-group of seven individuals who underwent surgical removal of melanoma were assessed before and after the intervention. The experiment was approved by the local ethical committee, and all the individuals adhering to the experiment were fully informed about the scope of the research.

The instrument used for this experiment is a last version of the electronic nose developed at the University of Rome 'Tor Vergata'. The core of the instrument is an array of seven quartz micro balance (QMB) chemical sensors, each coated with a different metalloporphyrin (17, 18). The resonant frequency of QMB is proportional to the quantity of mass absorbed during the exposure to the sample; thus, these sensors allow measurement of the amount of molecules absorbed from the gas phase onto the sensing layer (19).

The steady-state frequency shifts of the electronic nose sensors give rise to a pattern, and a collection of measurements produces a set of patterns that can be properly analysed by some pattern-recognition algorithm for classification purposes.

One of the most important, and often underestimated, steps in electronic nose analysis is the procedure of sample uptake. The sampling methodology has to ensure sufficient reproducibilty and it should contain enough concentration of those chemicals relevant to the searched pathology.

Figure 1a shows a schematic view of the sampling system developed to sample the headspace of small skin regions. The sampler is a stainless-steel cylinder used to insulate a skin region with a diameter of 4 cm. The area fitted with the size of all the cases investigated here.

The use of the sampler was complemented by a differential measurement strategy aimed at enhancing the difference of chemicals emitted by melanoma with respect to another nearby portion of the skin used as the reference. It is worth pointing out that skin may be characterized by a large subjective variability in terms of the quantity and quality of the emitted chemicals as it was evidenced by previous investigations (20). On this basis, it is rather likely to suppose that very close cutaneous regions are characterized by the same headspace; then, for each measured lesion, a reference measurement taken in correspondence of the closer free skin region was considered.

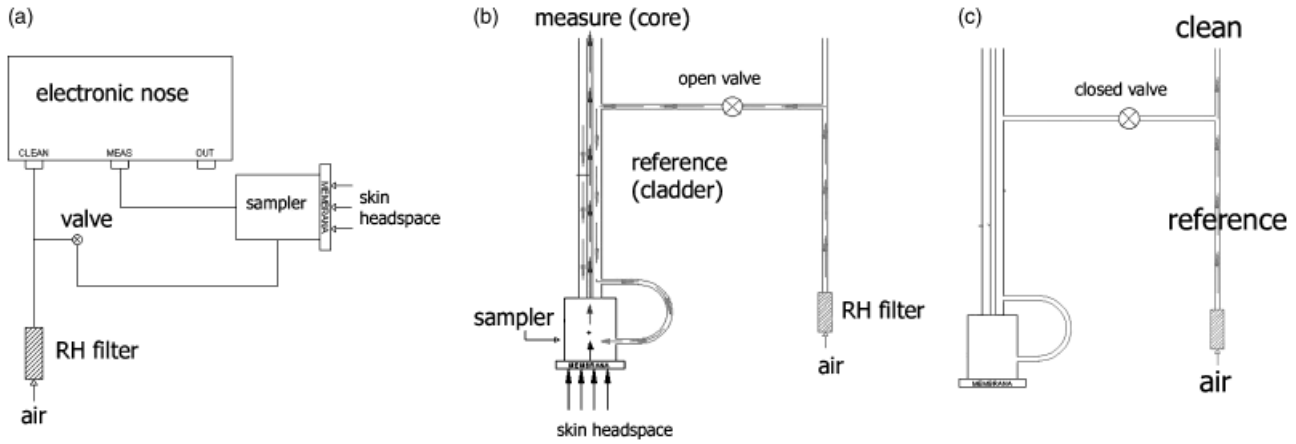

Fig. 1. (a) Schematic overview of the measurement set-up elements and their pneumatic connections. RH filter is a standard CaCl filter mainly for ambient water vapor content attenuation. (b) Skin odour is probed by washing the skin surface with the filtered ambient air circulating in the external part of the coaxial tube of the sampler. Skin odour is then uptaken into the sensor cell by the electronic nose internal pump.(c) During the flushing of sensor cell, the ambient air, filtered by the CaCl cartridge, is directly injected into the electronic nose inlet.

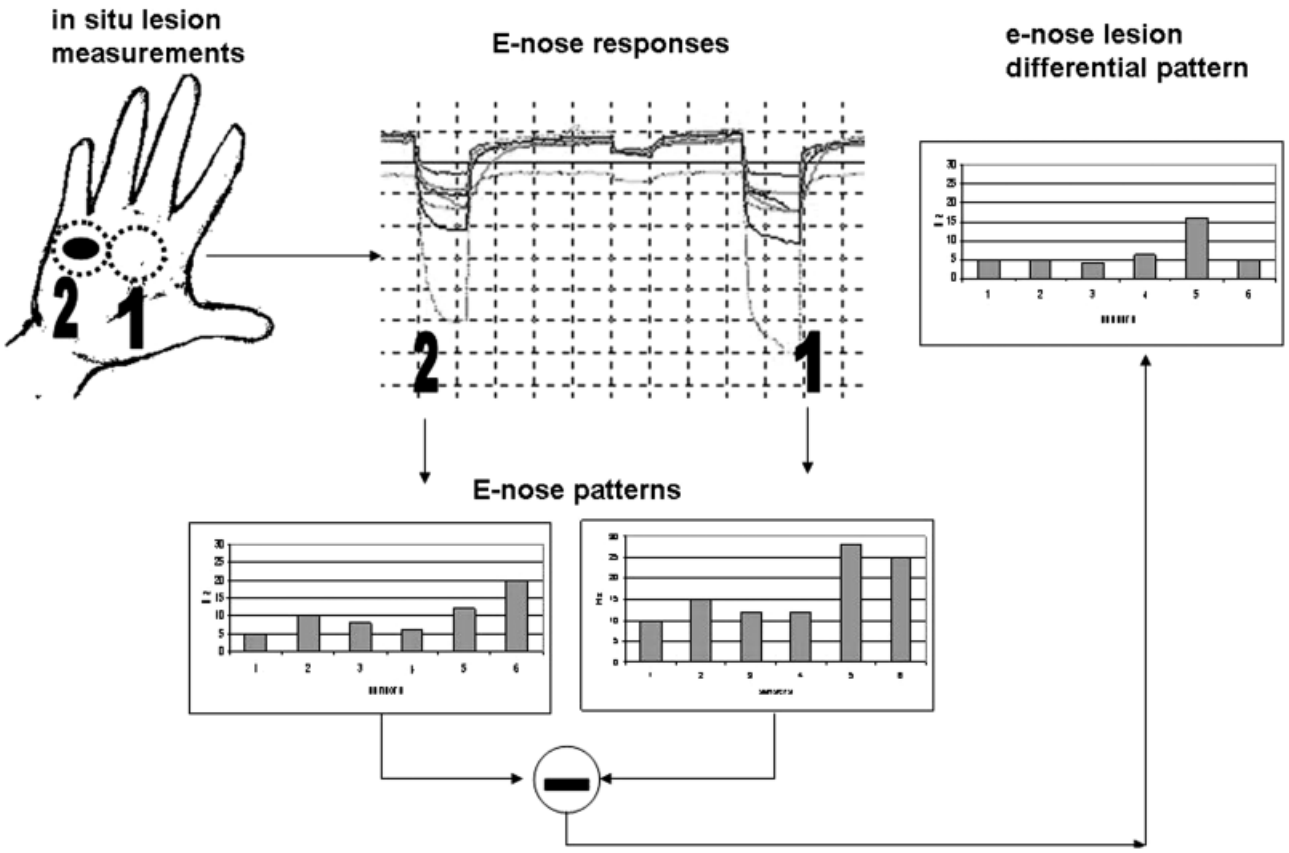

Fig. 2. Conceptual sketch of the differential measurement. A region as close as possible to the lesion to be measured is used as a reference. Sensors are then exposed to the air collected from the two surfaces and signals are subtracted to form the differential pattern used in the subsequent data analysis.

Lesion and reference measurements were performed with respect to dry air, which was chosen as the reference gas. The final sensor response for each lesion was calculated as the difference of two frequency shifts calculated as the steady-state frequency shift between the lesion and dry air, and reference skin area and dry air, respectively. The whole measurement protocol is graphically shown in Fig. 2.

The differential strategy used for the estimation of the final Δf response permits to separate to a large extent the contribution to the skin headspace due to the measured lesion disregarding the skin headspace composition peculiar to the individual and the skin region.

The dry air flow during sensor measurements and flushing is schematically shown in Figs. 1b and c. During the measurement, the valve switches the dry air flow inside the cladder of the coaxial tube. In this way, the dry air works as a carrier gas for the sample. The skin headspace is finally up-taken inside the core of the coaxial tube to reach the sensors' cell.

Although strongly reduced by the differential measurement, the use of perfumes and detergent may interfere with the measurement. For this reason, the skin headspace sampling protocol was complemented by conditioning of the patients. Individuals adhering to the tests were requested to observe simple hygienic rules. The skin portion under analysis was lightly washed no $<$ 2 h before the measurement with a neutral soap. A single brand of a commercial soap was provided in advance to all the participants.

Gas chromatography investigations were carried out by a gas chromatography mass spectrometer (GCMS) instrument (Shimadzu QP 2010, Shimadzu, Kyoto, Japan), in order to support electronic nose data interpretation and to engage the research for mark identification. The methods used by the few research groups that have published some studies on this matter are very different; in the present work, all the reported methods have been tested and synthesized in the optimal protocol for the tasks and the constraints of this experiment.

Zhang et al. (21) developed a sampling device to analyse the chemicals emitted from the skin of limbs using the solid phase micro extraction (SPME) with GCMS; this sampling device consisted of a canister enclosing the limb. Similar

investigations were carried out for the identification of methane, ethylene, ethane (22) and ammonia (23) released by the skin. Curran et al. (24) also tried to identify the volatile organic compounds present in human skin odour using SPME-GC/MS. They aimed at characterizing the various types of compounds present in the headspace above axillary sweat samples, and also at studying the qualitative differences and similarities between male and female subjects. This last protocol is the one that best fitted the experimental conditions of the present work. The main modification is relative to the storage time of the sample at ambient temperature before SPME extraction.

Samples were collected by wiping the skin surface with a sterile $10 \times 10\,cm^2$ gauze pad from Amukinmed, following the same procedure adopted by the Scent Transfer Unit-100 (STU-100) developed to aid US law enforcement with forensic tasks (25).

Gauze pads were stored and immediately sealed in 20 mL glass vials, crimp seal vials with PTFE/silicone septa (Supelco, Bellefonte, PS, USA). All samples were stored in ice to transport them, and were then stored in a freezer until analysis. Each sample was stored at room temperature for 9 h before extraction.

Divinylbenzene/carboxen on a polydimethylsiloxane (DVB/CAR on PDMS) 50/30 μm fibre (Supelco, Sigma-Aldrich Group, St. Louis, MO, USA) was used to extract the volatile organic compounds from the headspace of the vials. Exposure was performed at room temperature for 15 h by inserting the fibre into the silicon septum of the vial.

A Shimadzu GCMS-QP2010 gas chromatograph mass spectrometer was used with an EQUITY-5 column, 30 m length, 0.25 μm thickness, and 0.25 mm diameter, with helium as a carrier gas. The analytes, adsorbed in the fibre, were desorbed in the injection port of the GC, for 3 min at an inlet temperature of 250 °C, in the splitless mode. The GC method was initiated with an initial oven temperature of 40 °C for 5 min. The temperature was then ramped at 10 °C/min until it reached 300 °C, and then was held at 300 °C for 2 min (total run time: 33 min). The mass spectrometer was used with a quadrupole analyser in the full scan mode (range: 50–550). The interface and ion source temperatures were maintained at 250 °C. The solvent cut time was 3 min. Mass spectra were obtained in the TIC mode by electron impact. The compounds were identified by comparison with mass spectra from the NIST library database (26).

Results and Conclusion

Forty subjects participated in the experiment, and 47 skin lesions were measured. Seven lesions were measured before and after the surgical intervention.

Lesions were classified according to the epiluminescence report, and, in some cases, by the histological analysis of the removed tissues. The largest number of lesions were nevi, and 10 lesions were diagnosed as melanoma. It is worth pointing out that one of the 10 epiluminescence-diagnosed melanomas was negative on successive histological inspection; then, it may be considered as a false positive.

The differential measurement protocol was optimized by measuring several nevi in different body regions of a single subject. The amount of sensor signals was rather variable with the body surface; nonetheless, some features of the differential patterns were found to be constant in any parts of the body, such as the ratio of the intensities of different sensors. As a consequence, it is possible to suppose that a sort of 'sensors pattern shape' is a characteristic of the subject, while the intensity of the response changes with the skin surface region.

These tests indicate that the differential method and the sampler ensure repeatable measurements maintaining the sensor pattern characteristics for the subject and the body surface region.

The differences among the chemicals emitted by melanomas and nevi have also been studied with the GCMS. Two cases are illustrated in this paper related to two patients affected by melanomas at a different stage and located on the right shoulder. Three points were sampled for the measurement: they were skin cancer on the right shoulder, a nearby portion of clean skin and a nevus close to the same area. The chromatograms relative to these samples are reported in Fig. 3. As expected, the chromatograms were very similar with very subtle differences; nonetheless, a limited number of compounds appear only in the chromatogram related to skin cancer. The relative abundance of these peaks is low, and as a consequence the identification of the compounds is not reliable. The complete identification of compounds is beyond the scope of this paper and it

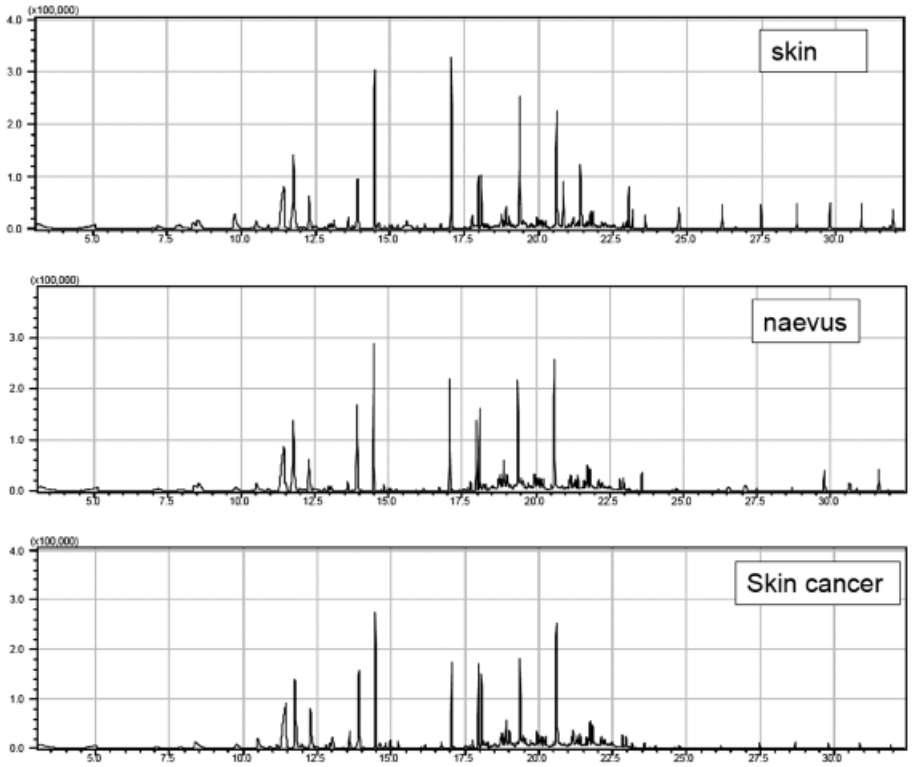

Fig. 3. Three chromatograms relative to three measured points (melanoma, 'free' skin and nevus) of the same individual. The similarity shows the reproducibility on the same subject. Certain very small peaks indicate the differences detectable in the volatile organic compounds composition.

will be a subject of an investigation in the near future. It is worth noting that one of the compounds present occurring only in the skin cancer chromatogram is probably propanal; this compound has been previously indicated to be a marker of UVR-induced lipid peroxidation, a process involved in many forms of cancer (27).

A qualitative analysis of GC/MS data has been performed partitioning the chromatogram in a pre-definitive set of retention time intervals and analysing the data with multivariate statistical methods. We started from an initial set of about 180 variables statistically reduced to 12. On the final dataset, a principal component analysis (PCA) model was built. The scoreplot of this model is illustrated in Fig. 4. Two main kinds of information can be obtained from Fig. 4: nevi and clear skin data are grouped into a single cluster and this cluster is rather well distinct from the skin cancer data. Despite the small number of investigated cases and the lack of compound identification, GC/MS analysis suggests that in case of melanoma, there is an anomalous composition of the volatile compounds released by the skin; a measure of these compounds could then help in the identification of the disease. This result partially supports the previous experiments with animals (12) and provides a basis for the application of chemical sensors for melanoma diagnosis.

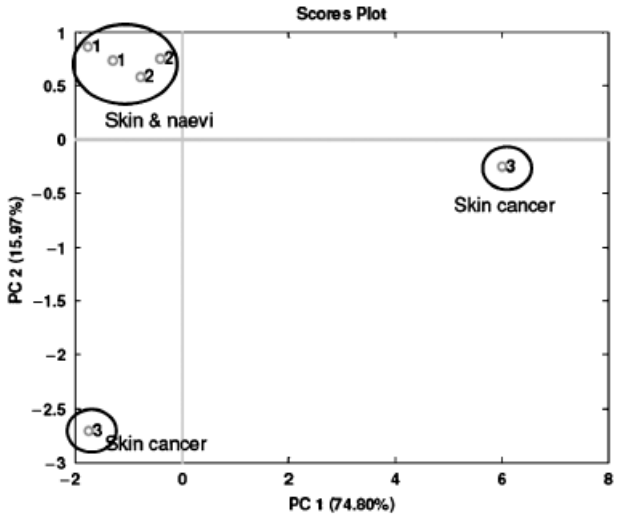

Fig. 4. Scoreplot of the first two PCs of the principal component analysis model built on the gas chromatography mass spectrometer data. A good discrimination can be observed between the 'healthy' cluster ('free' skin and nevi) and the two skin cancers.

Chemical sensors provide, as a result of a single measurement, a pattern of seven values, each corresponding to the frequency shift due to the exposure to the lesion and to the reference

clean skin area. The whole experiment then produced a data matrix with 47 rows (the samples) and seven columns (the sensors). Before applying multivariate analysis, it is interesting to study the behaviour of each sensor, in particular considering the distribution of each sensor output value with respect to the two main classes of samples: melanomas and nevi.

Figure 5 shows the distributions of nevi and melanomas data in a representation called a boxplot. In a boxplot, some statistical descriptors are graphically reported: the extension of each box indicates the variance, the central line indicates the mean value, the dotted lines are the limits of the confidence interval and the crosses indicate the measurements exceeding the confidence interval that can be considered as potential outliers. Boxplots offer an immediate comparison between distributions indicating whether two distributions are different or not. In Fig. 5, the boxplot of the data of each sensor divided into nevi (5a) and melanomas (5b) is shown.

Figure 5 suggests that the variance of the melanoma data is larger than that of nevi. The largest melanoma variability could be linked to several factors such as the typology of melanoma, its stadiation and the surface conditions, all contributing to displace the headspace profile from that of the clean area. Less evident is the behaviour of the mean values because only sensors 1 and 5 show a significantly larger mean value for melanomas with respect to nevi.

On this basis, it is also interesting to compare the patterns related to the same patient and in the same skin location, but taken before and after the surgical treatment. These measurements were performed on seven patients and the second measurement was performed 15 days after surgery; in these cases also, the histological report was available. Large differences could be observed in the patterns relative to measurements performed before and after surgery. An example is reported in Fig. 6. Figure 7 illustrates the case of the false positive, mentioned previously, corresponding to an epiluminescence melanoma diagnosis not confirmed by histological analysis. In this case, the sensor responses do not show a relevant change.

The results obtained in the pattern analysis have been confirmed by the multivariate discriminant analysis-solved partial least squares discriminant analysis (PLS-DA). PLS-DA represents

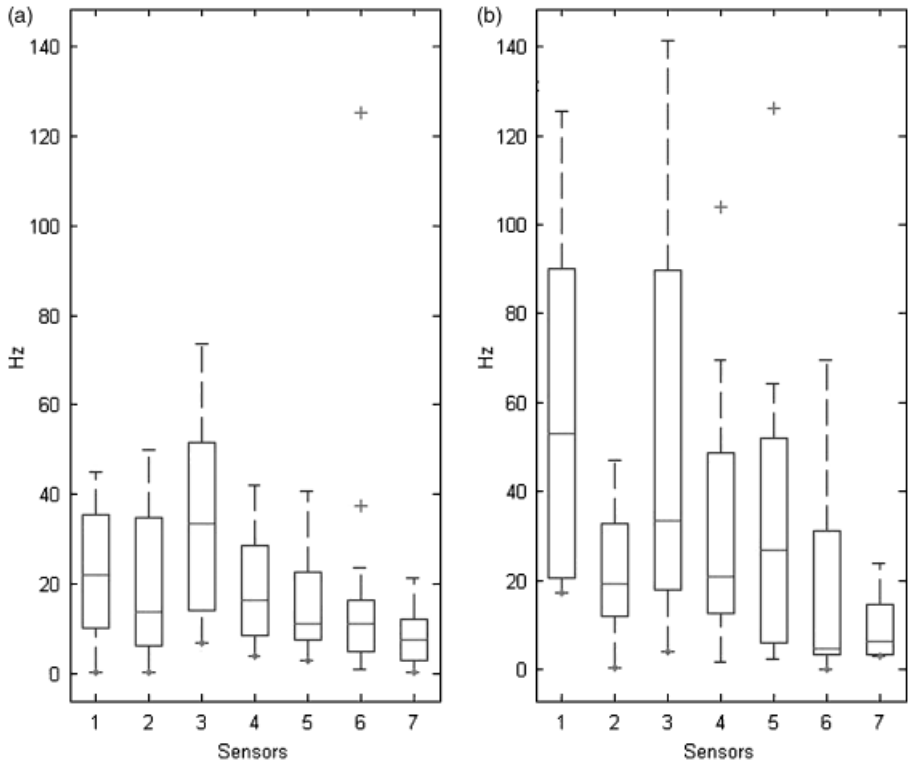

Fig. 5. The responses of sensors to all the cases are reassumed in the boxplots in the figure. Data are separately considered: nevi (a) and melanomas (b). Although limited by the different population of the two distributions, the boxplots display the largest variability of distribution observed for melanoma. Details of the meaning of the boxplot are given in the text.

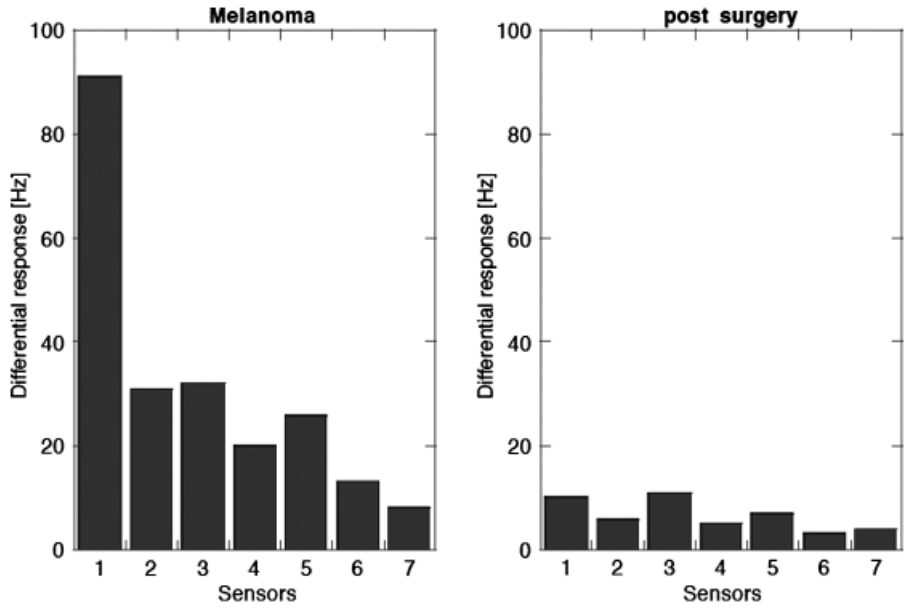

Fig. 6. Differential pattern of the same patient examinated before and after the surgical, complete removal of melanoma. The sensor response in the two cases is considerably different and indicates the absence of tumor cells after surgery.

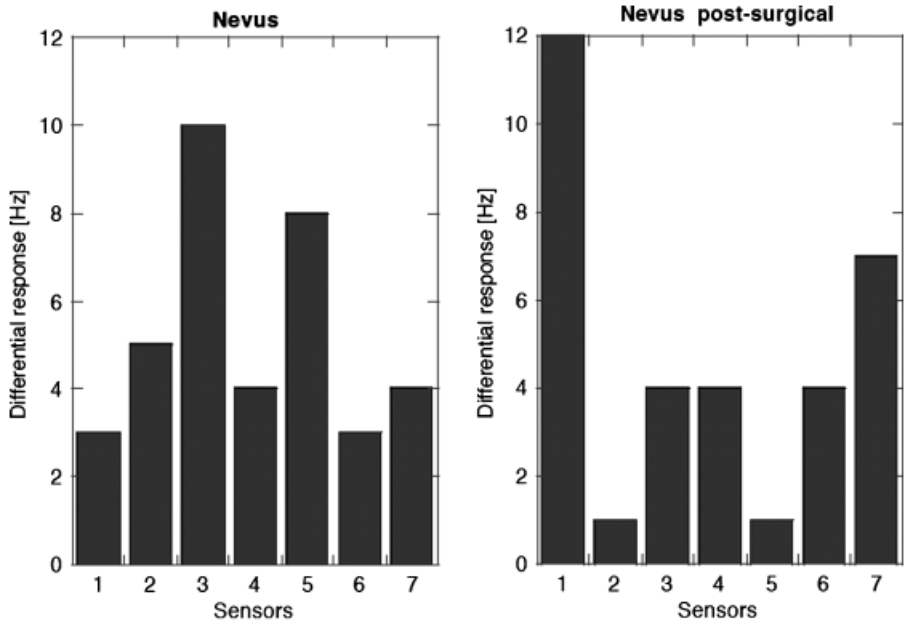

Fig. 7. Differential pattern of a false-positive patient measured before and after the removal of nevus. Differential sensor signals are smaller with respect to melanoma, and the magnitude does not change after surgery. The electronic nose correctly identified this case as a nevus.

a particular use of PLS, an algorithm originally developed for quantitative regression, as a pattern-recognition tool (28). One of the drawbacks of the application of multivariate techniques is the relationship between the number of variables and the number of samples. In general, an increase in the number of variables reduces the reliability of multivariate techniques based on covariance matrix estimation (such as PCA). Nonetheless, in this regard, it has been demonstrated that when the number of variables exceeds the number of samples and when the variables are highly correlated with each other, another typical occurrence for sensors in electronic noses, PLS-DA is more reliable than linear discriminant analysis (29).

A PLS-DA model was built in matlab. The model was aimed at classifying nevi from melanomas. The model was properly optimized by the leave-one-out cross-validation method. The percentage of correct classification in prediction was 87% largely comparable with other diagnosis

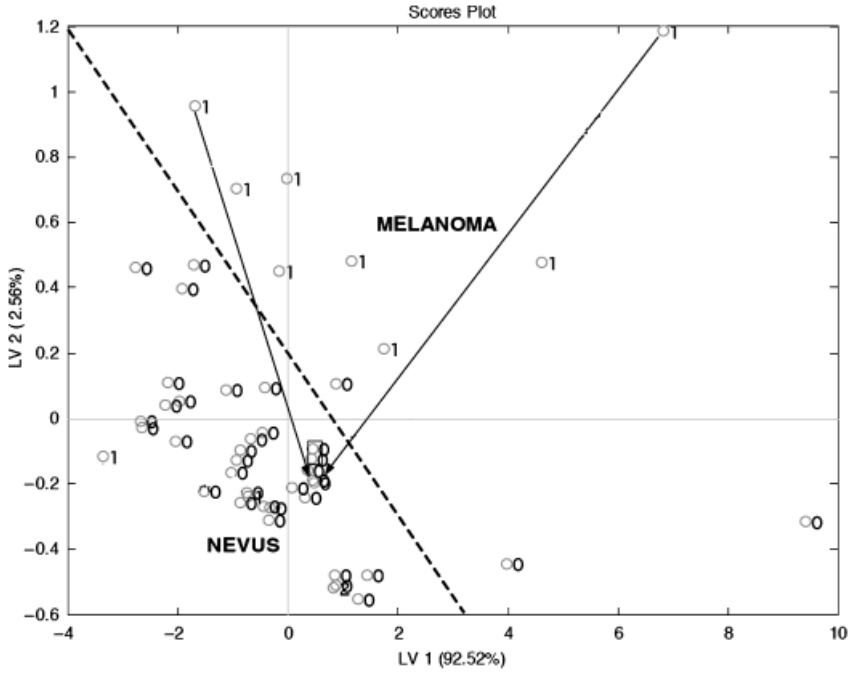

Fig. 8. Score plot of the first two latent variables of the partial least squares discriminant analysis (PLS-DA) model. The full PLS-DA model minimizes the error with four latent variables. Nonetheless, the plot of the first two variables provides a simple visualization of the classification properties. On the same plot, the arrows show those cases measured before and after the surgical removal (one of them shown in detail in Fig. 6) and the false positive whose differential patterns are shown in Fig. 7.

methods currently in use for melanoma. Nonetheless, this figure is biased by the different population in the two classes; indeed, the benign lesions are correctly identified in 90% of the cases, while the correct prediction of the melanoma is only 70%. The score plot of the first two latent variables of the PLS-DA model is reported in Fig. 8, which shows the separation between the distribution of nevi and melanoma. The discrimination is rather promising; in fact, a small number of melanoma cases fall in the nevi region and vice versa. The large disperions of melanoma data are in agreement with the boxplot behaviours, and as discussed previously, this spread can be explained by the differences among the tumoral form, different stadiations and different morphological states of the examined lesions. The temporal evolution of tumours introduces a further variable that needs to be taken into consideration because tumours emerge from the normality only at a certain development stage. The definition of this turning point is of paramount importance to evaluate the real potentiality of any early diagnostic method. As an example of the importance of stadiation in recognition, it is worth mentioning that one of the misclassified melanomas is a very early stadiation malign lesion.

In Fig. 8, those measurements performed before and after surgical intervention are also shown in evidence. This result is rather similar to a previously reported investigation about lung cancer where individuals assessed after the surgical removal of tumours were classified into the control group class (14).

Conclusions

Experiences with trained dogs suggest that melanoma cells produce a different 'bouquet' of volatile compounds. Nonetheless, the complexity of dogs' perception does not allow for a straightforward connection of the identification with the natural olfaction mechanisms. As a consequence, these experiments did not univocally demonstrate the correlation between volatile compounds and melanoma. In this paper, evidences that the gas chromatogram of the headspace of the skin surface is different in the presence of a melanoma or a nevus have been shown. Such differences are large enough to be captured by a chemical sensor array; the application of a linear pattern recognition was able to identify melanoma with an accuracy in prediction of about 80% comparable to that achieved by the diagnostic methods currently in use.

Sensor array result are rather underestimated because the array was composed by as yet unoptimized sensors and only a linear pattern-recognition method has been used. Concerning the sensor, it is worth noting that the sensor coating was not chosen to maximize the sensitivity towards some particular compound basically because these compounds are still unknown.

From the electronic nose measurement point of view, melanoma identification is a favourable experimental condition because it is a very localized alteration that can be insulated and measured with respect to a close unaltered skin region. The application of a differential strategy avoids, in principle, any contamination of the manifold causes contributing to determining the skin headspace composition.

The results presented here have to be considered as a first step and some further aspects need to be clarified. First of all, GC findings have to be followed by an identification of the compounds occurring either in anomalous concentrations or appearing in melanoma headspace. This identification is of primary importance to correlate the findings to the biochemical processes occurring in the altered cells. Another important issue resulting from these studies is the scarce number of available cases. In the study presented here, melanoma represented only 25% of the cases. The relative rarity of the disease does not allow a fully satisfactory characterization of the method according to the usual accumulation of experience typical in other electronic nose applications; the identification of the chemicals, altered by the presence of melanoma, will then be necessary for a better evaluation of the actual sensitivity of the sensors and also for the optimization of the chemical sensors implemented in the array more suitable for this particular aspect.

Despite the mentioned problems that deserve particular attention, the melanoma identification by a gas sensor array seems to offer a promising opportunity in the field of medical applications.

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