Blood Analysis by VOC Sensor Array for Environmental Exposure Assessment

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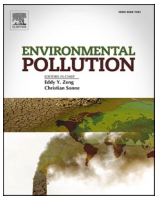

A novel human biomonitoring study by semiconductor gas sensors in Exposomics: investigation of health risk in contaminated sites☆

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ABSTRACT

Two areas in central-southern Italy *Land of Fires* in Campania and *Valley of Sacco river* in Lazio are known to be contaminated sites, the first due to illegal fly-tipping and toxic fires, and the second due to an intensive industrial exploitation done by no-scruple companies and crooked public administration offices with dramatic consequences for environment and resident people. The work is intended to contribute to Human BioMonitoring (HBM) studies conducted in these areas on healthy young male population by a semiconductor gas sensor array trained by SPME-GC/MS. Human semen, blood and urine were investigated. The fingerprinting of the Volatile Organic Compounds (VOCs) by a gas sensors system allowed to discriminate the different contamination of the two areas and was able to predict the chemical concentration of several VOCs identified by GC/MS.

1. Introduction

Pollution is one of the greatest critical issues in the world that humanity has to face and the leading environmental cause of morbidity and mortality (Briggs, 2003; Manisalidis et al., 2020). It has always a source or rather several different sources and a primary receptacle: the environment around us. Many human activities damage our environment through urbanization, industry, agriculture, transport, tourism, etc. Civil settlements are major contributors to pollution problems due to traffic and combustion emissions, water waste treatment, hazardous waste dumping, the agro-livestock sector produces pollution from nutrients, fertilizers, and plant protection products, while industry generates that from halogenated organic substances and heavy metals. The absurdity is that humans seem to have forgotten to be part of a single fragile global ecosystem and the secondary receptacle of pollution; indeed, pollutants usually reach humans through the consumption of contaminated and polluted water and food and breathing polluted air (GBD 2017 Risk Factor Collaborators, 2018; Nathanson, 2021).

The adverse effects of environmental pollution on health are more commonly documented in occupationally exposed groups, less in general population; the awareness of how living in a terrestrial contaminated ecosystem compromises health and affects the quality of life is often poor and rarely it is a subject of political debate for the safeguard of public health (Bauleo, 2019; Manisalidis et al., 2020).

Assessing the impact of environmental pollution on human health is as urgent as complex given the complexity of the interaction between multi-agent contamination and populations of residents, through multiple pathways. Exposome has been established as a conceptual model for exposure and life-course disease risk assessment (Zhang et al., 2021; Escher et al., 2017). It is now recognized that not only genetic factors, but multiple specific stressors due to external exposome (air/water/soil pollution, chemical exposures, occupation, diet, physical activity,

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tobacco, infections, drugs) and internal exposome (endogenous factors such as metabolism, gut microbiota, inflammation, oxidative stress) are involved and intricately interconnected. The disease risk due to environment pollution is significant but hidden (Escher et al., 2017; Pleil, 2012; Vineis et al., 2017). In sites contaminated by industrial activities or illegal waste dump, where a complex heterogeneity in chemical exposure predominates and contaminant levels are out of control, the disease risk of residents living nearby is higher; there, a rise in cancer-related mortality is often reported (Mudu et al., 2014). Malpractice or lack of implementation of legislation are often the real causes of contamination (Report on Eurojust's Casework on Environmental Crime, Jan. 2021, Farmer et al., 2015; Colantoni et al., 2020; Rucevska et al., 2015).

Human BioMonitoring (HBM) is more and more increasing its relevance as tool to describe exposure characterization to environmental pollution, measure known biomarkers of exposure or related-to-exposure by a targeted approach, but also discover potential novel biomarkers by an untargeted exposomic approach using omics sciences (Human Biomonitoring: fact and figures, 2015). The objective is to better understand the relationship between the internal dose due to chemical exposome and the response of the body (Ladeira et al., 2016; Angerer et al., 2007; Wilhelm et al., 2007; Biomarkers and Human Biomonitoring, vol.2, 2012; Creager, 2018; Yusa et al., 2012). The joint effort of consortium in HBM4EU project, an initiative coordinating and advancing human biomonitoring in Europe, is an example of the renewed interest in the contribute of HBM in the protection of health and environment (HBM4EU project, 2021).

As a novelty in HBM context, the analysis of Volatile Organic Compounds (VOCs) in body matrices (Human Biomonitoring: fact and figures, 2015; Jing et al. 2021, Drabińska et al., 2021; Cumeras, 2017) is attracting considerable interest for its potential in discovering new biomarkers useful in pollution health impact assessment. In all biological samples (blood, breath, urine, saliva, faeces, seminal fluid, etc.) there is a volatile part consisting of exogenous VOCs deriving from external factors (environment, lifestyle, diet, microbiome, drugs) and endogenous VOCs deriving from internal factors (human metabolic activities) in the organism (Aman et al., 2014; Naccarato et al., 2019; Longo et al., 2018, 2019, 2020, 2021a).

The gold analytical technique for identifying VOC patterns that can serve as diagnostic biomarkers of specific diseases or environmental exposure is of course GC/MS, but gas/VOC sensors have the undisputed advantage to be a simpler and flexible technology (Saruhan et al., 2021, Radogna et al., 2021; Nikolic et al., 2020) that could be exploited also in HBM challenge. Up to now, such electronic noses based on gas sensors have been applied mainly in disease screening, diagnosing, and monitoring for clinical uses (Capelli et al., 2016; Hu et al., 2019; Baldini et al., 2020; Broza et al., 2018), whereas few works have exploited the technologies as tool for HBM in exposomic studies for assessing population-wide exposure to hazardous chemicals with a particular interest in population living in contaminated areas (Longo et al., 2021b).

In this work a 4-gas sensor array was used for the analysis of VOCs in blood, urine and human semen samples. The different body fluid samples were collected from healthy guys living in two Italian contaminated areas with a high degree of submerged pollution as characterized by illegal dumping of waste: *Land of Fires* (LF) and *Valley of Sacco River* (VSR). *Land of Fires* is an area in the Province of Naples (Campania, Italy), where systematically since the end of the '80s, toxic wastes have been dumped by organized crime and where health risk for population living in this area have been studied (Triassi et al., 2015; Berruti et al., 2020; Pierri et al., 2020; Bergamo et al., 2016; Lettieri et al., 2020a; Mazza et al., 2015). *Valley of Sacco River* (VSR), a vast area between the south of Rome and the province of Frosinone (Lazio, Italy), represents an unprecedented environmental and social disaster in Italy. With the responsibility of unscrupulous industries that did not follow the correct treatment of waste and the complicity of careless local administration, for decades, toxic waste of industrial origin (water contamination and use for irrigation purposes) has been poured into *Sacco* river, with unavoidable consequence for exposed animals of zootechnical interest and the human population (Fantini et al., 2012; Narduzzi et al., 2020) (Fig. 1).

The study joins Ecofoodfertility initiative, an "open", participatory project, initially born in Campania region (Italy), which promotes multicenter integrated human biomonitoring studies, aiming to find a link on a scientific basis between the worrying rates of environmental pollution in contaminated areas and the worrying increase in chronic degenerative diseases recorded in recent decades (Ecofoodfertility, 2021) as well as man infertility (Bergamo et al., 2016; Lettieri et al., 2020a; Zani et al., 2019; Montano et al., 2021a).

We analyzed the sensor array response-patterns of VOCs that are present in biofluid headspaces by multivariate techniques exploring the discrimination and classification of the two groups of vulnerable population, to evaluate the peculiar characteristics of environmental pollution in these specific contaminated sites. Moreover, in an advanced data analysis step we support the data sensor results with the analysis carried out by Headspace-Solid Phase Microextraction-Gas Chromatography/Mass Spectrometry (HS-SPME-GC/MS) on the same samples of blood, urine and human semen samples, and whose results are published elsewhere (Longo et al., 2021a). Multiple Regression models were applied to the data to evaluate the ability of the gas sensor system to predict the concentration of specific pattern identified by HS-SPME-GC/MS.

2. Experimental

2.1. Subjects

The study, conducted within EcoFoodfertility project, was designed to recruit a small representative sample of *Land of Fires* (LF) and *Valley of Sacco River* (VSR) residents. The volunteers were all young healthy male students about 18 old, who gave their written informed consent for biosamples collection for the specific research purposes of this study. All participating subjects were students, so that only general environmental exposure conditions of the population were considered, excluding occupational exposure conditions. Excluding factors were habit of smoking or alcohol drinking, chronic diseases (diabetes or other systemic diseases), reproductive system malfunctions (varicocele, prostatitis and so on) and high body mass index (BMI >33 kg/m^2). For this study, 50 healthy young male volunteers in fertile age from Land of Fires (35) and Valley of Sacco River (15) were enrolled. All subjects were asked to provide all three biological samples of interest (human semen, blood, and urine), but only a part met this request by providing one or two of the requested biosamples. The partition of the different biosamples provided by the sample population living in the two

Fig. 1. Map indicating the geographical location of the two contaminated sites *Land of Fires* and *Valley of Sacco River*.

contaminated areas, is reported in Table 1. In total 101 samples were collected (33 from seminal liquid, 44 from blood and 24 from urine). The study carried out in accordance with the Code of Ethics of the World Medical Association (World Medical Association, 2013) was approved by the Ethical Committee of the Local Health Authority Campania Sud-Salerno, and at the "San Francesco d'Assisi" Hospital (Oliveto Citra, Salerno, Italy) and at the Italian Association of Blood Volunteers (AVIS, Frosinone office, Frosinone, Italy).

2.2. Sample collection

Human semen was collected via morning masturbation after 3–4 days of sexual abstinence in sterile containers. Morning fasting blood samples were collected via venipuncture into sodium citrate tubes and gently shaken. First morning urine samples, also called 8-h specimens, were collected when the subjects first wake up in the morning, having emptied the bladder before going to sleep; each subject provided a first morning urine samples in a 50 mL sterile PVC container.

In a subsequent aliquoting step, aliquots from the different biofluids (250 μL of semen ejaculated, 1 mL of whole blood and 1 mL urine) were taken from the standard collection containers and transferred to gas-tight glass containers suitable for collecting the volatile fraction of the specific biosample in the vial headspace. In particular, 5 mL headspace vials (Shimadzu™, cod.27319-U Sigma-Aldrich) capped with an assembled screw cap with hole with PTFE/silicone septum were used as collecting vial. The vials were immediately frozen and stored at – 80 °C. The frozen samples were sent to Gas Sensor Lab at CNR-IMM in Lecce (Italy) for the joint VOC analysis by gas sensors and HS-SPME-GC/MS.

2.3. Sensor array measurements

To correlate in stringent way, the analysis of VOCs performed with the gas sensor array with that performed by HS-SPME-GC/MS, the two methods of analysis have been applied in succession, by carrying out the measurements on the same vial containing the biosample. As for HS-SPME-GC/MS analysis we analyzed the samples in triplicate. All the human biosamples (human semen, blood, and urine) were treated with the same standardized VOC extraction protocol. In particular, at the time of analysis, each vial containing a biosample was thawed at room temperature and next immersed in water bath onto a magnetic stirrer hotplate at 60 °C overnight (Heidolph MR 3001 series); rubber ring mounts were used to keep the vials afloat during thawing, and a gentle stirring guaranteed the same agitation to all samples. After this incubation phase, the vial was first subjected to the VOC extraction phase with SPME followed by the measurement run defined for the identification of VOCs with GC/MS (Longo et al., 2021a). Next, the vial has been connected to a test bench for gas sensor calibration, where a gas mixing station with mass flow controllers, pipelines and electropneumatic valves, performs a vial headspace sampling protocol using a total flow of dry air of 25 sccm; during the measurement the vial was kept at a constant temperature (25 °C) in a water bath. To transport the volatile compounds, the air flow, used as carrier, enters by a needle in the PTFE/silicone septum of the vial and exits by another needle stripping the volatile compounds into the steel airtight sensor chamber (able to house up to 4 microsensors) of the measuring system. Four commercial gas microsensors mounted on TO39 sockets were housed in the test cell; sensor 1: cod. MICS 5135, e2V Technologies; sensor 2: cod. AS-MLK, AppliedSensor GmbH; sensor 3: cod. AS-MLV, AppliedSensor GmbH; sensor 4: cod. AS-MLC, AppliedSensor GmbH. All the commercial sensor component chips are fabricated using Si-micromaching silicon technology; the heater and interdigital electrode structures are placed on micromachined membrane to achieve the lowest possible power consumption. The electrical currents of the 4-sensor array (polarized at 1 V) were acquired by an electrometer (Keithley 6517A) equipped with a multiplexer module (Keithley 6521). A LabVIEW software controls the gas line system (MKS 674B with 4 channels) and headspace sampling process and acquires the sensor signals via IEEE board. The sensor array responses were processed by statistical multivariate techniques.

Table 1
Partition of the biosamples collected from the sample population living in *Land of Fires* (LF) and *Valley of Sacco River* (VSR).

	Total subjects (n)	Subjects *Land of Fires* (LF) (n)	Subjects *Valley of Sacco River* (VSR) (n)
Human Semen (HS)	**33**	**27**	**6**
Blood (B)	**44**	**29**	**15**
Urine (U)	**24**	**20**	**4**
HS ∩ B ∩ U	**14**	**12**	**2**
HS ∩ B	**29**	**23**	**6**
HS ∩ U	**17**	**15**	**2**
B ∩ U	**15**	**4**	**19**

3. Results and discussion

3.1. Gas sensor results

Each vial, as soon as the SPME procedure was completed, was connected to the sensor system and the measurement protocol for stripping all the VOCs, collected in the vial headspace into the sensor chamber, started. In Fig. S1 a picture of a gas sensor inserted in the gas-tight measuring chamber was reported.

Since the volume of VOCs extracted and concentrated in the fiber by ad/absorption processes after reaching equilibrium is minimum, the headspace composition can be considered unaltered by the exposure of the SPME fiber in the vial headspace. Therefore, for each biosample the gas microsensors in the test chamber were exposed to the same headspace whose composition was scanned and identified with combined GC/MS analysis. The sensors, based on a chemoresistivity principle, provided a response signal in terms of electrical resistance decrease when exposed to the overall set of VOCs in the headspace through the air flow that conveys them; the response signals recovered in air returning reversibly to the equilibrium electrical resistance values in conditions of non-exposure to VOCs.

The protocol of VOCs exposure/recovery (5 min VOCs exposure/10 min recovery) was repeated more times. All the 4 sensors of the array showed a wide variation in electrical resistance up to more than one order of magnitude under exposure to the vial headspace containing the different biofluids (human semen, blood and urine) (Fig. 2). As expected, the first resistance variation is higher than the following ones, as it corresponds to a headspace richer in VOCs as its obtained at higher temperature and for a longer time. Subsequent variations in electrical resistance are smaller than the first but comparable to each other in that they correspond to the formation of the headspace in the same time interval (10 min during the recovery phase in the air). A modulation of the sensor responses was got by using the 4-sensor array as consequence of the different properties of sensitivity towards gas/VOCs, although two sensors of the array (sensor 2 and 3) exhibited similar behaviour probably due to similar gas sensing characteristics of their sensitive layers. Thus, the modulated sensor responses patterns obtained from all the samples have been essential for next statistical multivariate analysis.

The sensor response was defined as the ratio between the sensor electrical resistance in air (R_{Air}) and the electrical resistance under VOCs exposure (R_{VOC}). The sensor responses (R_{Air}/R_{VOC}), corresponding to all the VOCs injections in the sensor chamber, were calculated and the first sensor response, that is always higher than the subsequent responses for all the sensors and all the types of considered biofluids, was used to compare the sensing performance of the 4-sensor array.

In a first descriptive statistics, we described the distribution of samples from the entire sample population of recruited guys living in LF

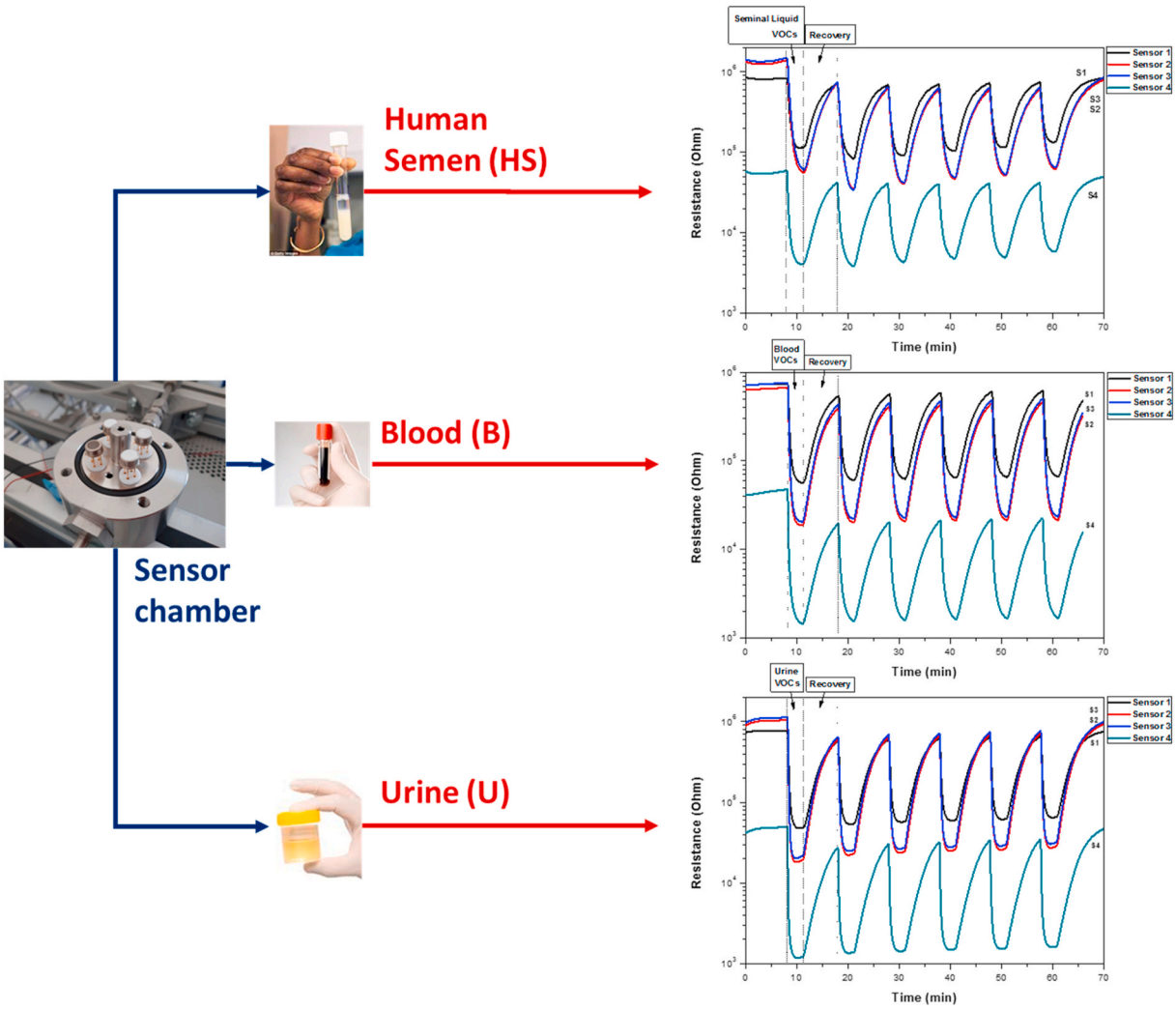

Fig. 2. Sensor chamber and typical dynamic responses from 4-gas sensors array for a human semen (HS), blood (B) and urine (U) samples from young men recruited in the experimental campaign.

and VSR by the graphical representation of Box-Whisker plots that use simple dispersion and position indices (Fig. 3). As displayed in the Box-Whisker plots, the sensor response patterns to the analyzed human semen, blood and urine samples showed a high between-subjects variability. These plots describe the central tendency of the variable sensor response (R_{Air}/R_{VOC}) from the different sensors of the array in terms of the median of the values (represented by the smallest box in the plot); the spread (variability) in the variable values is represented in this plot by the quartiles (the 25^{th} and 75^{th} percentiles, larger box in the plot) and the minimum and maximum values of the variable (the "whiskers" in the plot). As regards human semen, all the sensors showed a high between-subjects variability with higher responses values provided by S2 and S3; the sensor response S3 varied from a minimum of 4.7 to a 146.8 with a median of 27.8 (25% percentile: 20.2; 75% percentile: 87.1). The between-subjects variability shown by the sensors in their response to blood VOCs was less pronounced than that shown for human semen and urine with higher responses values provided by S2 and S3; the sensor response S2 varied from a minimum of 1 to a 154.1 with a median of 26.9 (25% percentile: 21.8; 75% percentile: 39.7). The between-subjects variability shown by the sensors in their response to urine VOCs was greater than that shown for blood but less than that shown for human semen with again higher responses values provided by S2 and S3; the sensor response S2 varied from a minimum of 1.1 to a 156.7 with a median of 40.7 (25% percentile: 19.1; 75% percentile: 60.5).

Such distribution of sensor responses reflects the inherent biological variability also found in sample population by HS-SPME-GC/MS analysis. Moreover, the responses of the sensors array reflect the different multitude of chemical compounds coexisting in the considered biofluids and the variety of chemical classes corresponding to a different expression of a given chemical class in a biofluid (Longo et al., 2021a).

3.2. Data analysis

In a next dataset processing, we applied multivariate data analysis aiming to explore any differences in pollution characteristics that could lead to discrimination between the two groups of vulnerable population living in the contaminated areas of *Land of Fires* (LF) and *Valley of Sacco river* (VSR). For this objective, we used the sub dataset of sensor responses towards blood VOCs due to a greater number of blood samples belonging to both classes. In such response submatrix, sensor array responses (R_{Air}/R_{VOC}) to the first exposure to blood VOCs were considered as variables; the mean value was subtracted from each response value and the difference was divided by the standard deviation to transform the curve to the standard normal distribution. The standardized matrix was processed by Principal Component Analysis (PCA), a simple unsupervised dimensionality-reduction method that is often used to reduce the dimensionality of large data sets, by transforming a large set of variables into a smaller one that still contains most of the information in

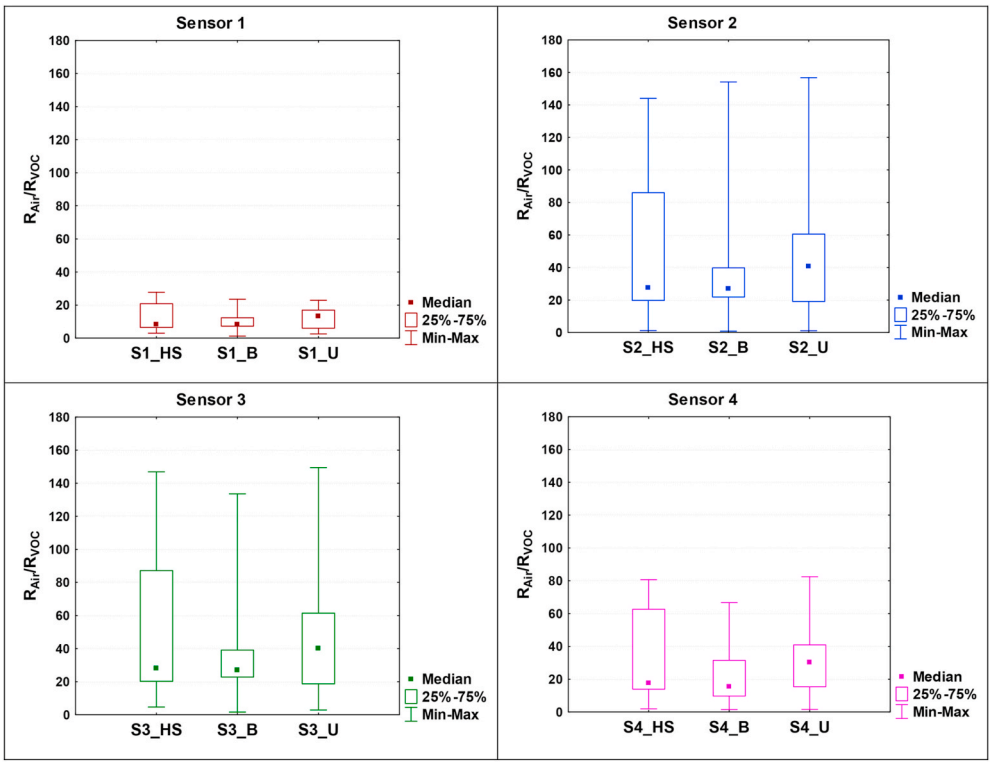

Fig. 3. Box & Whisker plot for each sensor of the array displaying the sensor responses distribution in the sample population.

the large set. From a geometric point of view, the goal of PCA is to identify directions (or principal components) along which the variation in the data is maximal. Fig. 4 shows the PCA score plot in the 3-D space of first three Principal Components PC1-PC2-PC3, and in the projection planes of PC1-PC2 and PC1-PC3. It can be observed that, the points related to the group VSR are more aggregated than those of the group LF, which are more dispersed.

To evaluate the ability of the 4-sensor array to classify the two known populations LF and VSR, both canonical Linear Discriminant Analysis (LDA), that is a linear classifier, and Quadratic Discriminant Analysis (QDA), that uses a quadratic decision surface to separate measurements of two or more classes of objects, were performed on the data matrix of

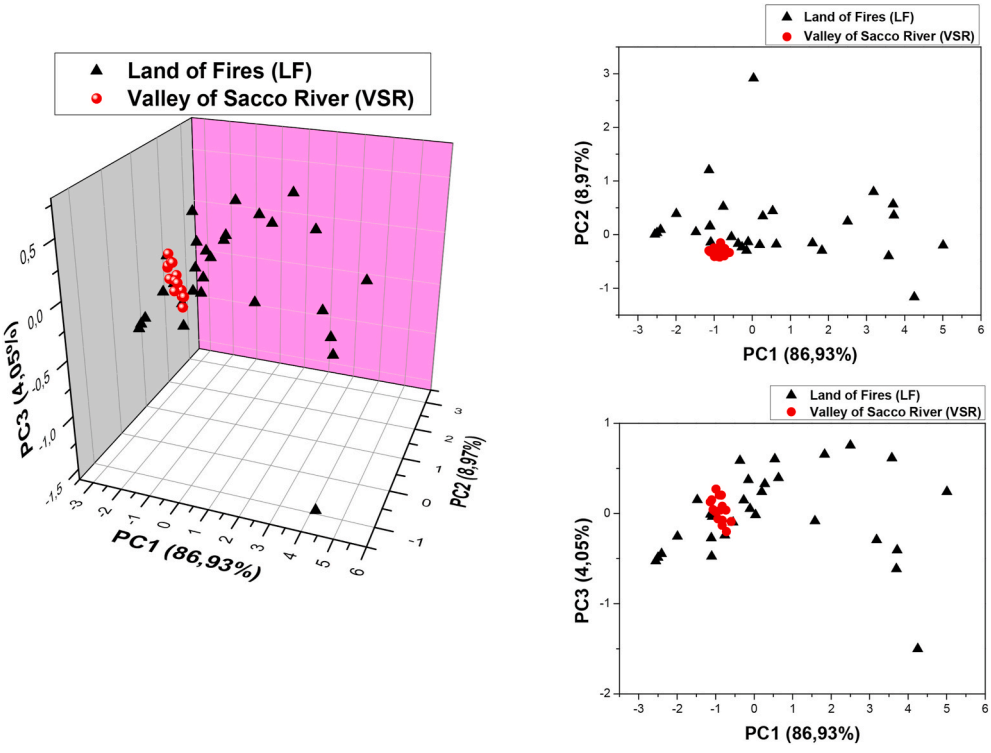

Fig. 4. PCA score plots in the space of PC1-PC2-PC3 based on sensor responses data for data subset related to blood.

sensor array responses to blood VOCs. Proportional to group size for Prior Probabilities option was selected. Since there are only two classes, only one canonical root (100% variance, canonical correlation = 0.54) results both from LDA and QDA. The results of Discriminant Analysis algorithms LDA and QDA are reported in Table 2 where the confusion matrices provided by LDA (Table 2a) and QDA (Table 2b) show the performance of the classifiers in classifying the two classes (LF and VSR); the tables report the number of false positives, false negatives, true positives, and true negatives. The classifier parameters (Sensitivity, Specificity, False Negative Rate, False Positive Rate, and Accuracy) indicate that the two contaminated areas LF and VSR can be separated with a better discrimination power for QDA compared to LDA.

This is in line with the different VOC profiles for the two contaminated areas obtained by the GC/MS analysis on the different biological samples analyzed which showed that the two contaminated areas can be discriminated based on the VOC profiling of the biofluids collected from the sample population living in the neighbourhoods of these contaminated sites (Longo et al., 2021a).

The joint use of e-nose and GC/MS gave the opportunity to relate the fingerprinting of the considered biosamples in terms of the response patterns of the sensor array to the corresponding fingerprinting in terms of volatile composition of the sample headspace. In our previous work, VOC patterns of all the human semen, blood and urine samples from the sample population were analyzed by the complementary HS-SPME-GC/MS analysis; the VOCs were searched by non-target analysis, identified by comparing mass spectra with those of the data system library (NIST14, P>60%) and quantified by internal standard method (Longo et al., 2021a). In this work, the statically relevant VOCs were extracted and combined to sensor array responses. First, the correlation matrices between VOC variables and sensor responses for all the subgroups listed in Table 1 (HS; B; U; HS ∩ B ∩ U; HS ∩ B; HS ∩ U; B ∩ U) were calculated. As example, in Table S1 the correlation matrix (Pearson correlation coefficients) for the HS ∩ B ∩ U group is reported. It can be observed that some VOCs variables are positively correlated to sensor response variables (i.e. as one variable increases so does the other, and vice versa), whereas others are negatively correlated (i.e. as one variable increases the other decreases, and vice versa); this may be related to oxidative/reductive characteristics of volatile chemical species and to the chemical surface reactions between the adsorbed species at the sensor surfaces that lead to increase or decrease of sensor electrical resistance. Moreover, some sensor variables are slightly correlated with the considered VOCs variables, some ones showed medium/high correlations coefficients. In order to better investigate if such correlations can indicate a predictive relationship that can be exploited in practice, we moved on to a subsequent analysis step by applying statistical regression techniques.

The concentration (in ng/mL) of the significant VOCs determined by HS-SPME-GC/MS were thus used as reference data in multivariate regression modelling, building a predictive model from the set of independent or predictor variables (gas sensor responses) to the set of continuous dependent variables (VOC concentrations). In particular, Multiple Linear Regression (MLR) was used as multiple regression technique; it models the linear relationship between several explanatory (independent) variables and a dependent variable to predict its outcome.

We performed different MLR analysis for each of the subgroups listed in Table 1 (HS; B; U; HS ∩ B ∩ U; HS ∩ B; HS ∩ U; B ∩ U), considering in the different analysis the effective samples for each subgroup and the VOCs found in them. According to the VOC distribution in human semen, blood and urine, some VOCs are common to all the three biofluids (HS, B and U), others to two of them, whereas others are present only in one biofluid, thus the 42 statistical relevant VOCs selected by HS-SPME-GC/MS give rise to 22 VOC variables for HS, 29 for B, and 26 for U. The different MLR analysis were hence applied to all these VOCs used as dependent variables; the number of the predictor variables was 12 (4 sensors x 3 biofluids), or 8 (4 sensors x 2 biofluids), or 4 (4 sensors x 1 biofluid) depending on whether all 3 or 2 or 1 biofluid are included.

The overall fit of the MLR model can be described by the test of SS Whole Model vs. SS Residual shown in Table 3 for HS ∩ B ∩ U group and in Table S2 for the other groups (Table S2a for subgroup HS ∩ B; Table S2b for subgroup HS ∩ U; Table S2c for the subgroup B ∩ U; Table S2d for the subgroup HS; Table S2e for the subgroup B; Table S2f for the subgroup U). In such spreadsheets (Table 3 and Tables S2) the multiple R, R-square, adjusted R-square, and overall F-test results, for all the dependent variables that were predicted with a significant p-value ($p < 0.05$) were displayed. The *Multiple R* is the coefficient of multiple correlation, which is the positive square root of R-square (the coefficient of multiple determination); the R-square value is an indicator of how well the model fits the data (e.g., an R-square close to *1.0* indicates that we have accounted for almost all of the variability with the variables specified in the model); the *adjusted* R^2 is interpreted similarly to the R^2 value except the adjusted R^2 takes into consideration the number of degrees of freedom. The overall F-test determines whether the relationship between the dependent variable and the set of independent variables is statistically significant; if the p value for the overall F-test is less than your significance level, you can conclude that the R-squared

Table 2
Confusion matrix based on sensor array responses to blood VOCs for: a) Canonical Linear Discriminant Analysis (LDA), b) Canonical Quadratic Discriminant Analysis (QDA).

a)		Predicted Group	
		Land of Fires (TF)	Valley of Sacco River (VSR)
True Group	**Land of Fires (TF)**	TP = 25 **Sensitivity** or True Positive rate (TPR) = TP/(TP + FN) = **86.21%**	FN = 4 **False Negative Rate** (FNR) = FN/(FN + TP) = **13.79%**
	Valley of Sacco River (VSR)	FP = 2 **False Positive Rate** (FPR) = 1-TNR = FP/(FP + TN) = **13.33%**	TN = 13 **Specificity** or True Negative Rate (TNR) = TN/(TN + FP) = **86.67%**
		Accuracy (ACC) = TP + TN/Total (TP + TN + FP + FN) = **86.36%**	

b)		Predicted Group	
		Land of Fires (TF)	Valley of Sacco River (VSR)
True Group	**Land of Fires (TF)**	TP = 28 **Sensitivity** or True Positive rate (TPR) = TP/(TP + FN) = **96.55%**	FN = 1 **False Negative Rate** (FNR) = FN/(FN + TP) = **3.45%**
	Valley of Sacco River (VSR)	FP = 0 **False Positive Rate** (FPR) = 1-TNR = FP/(FP + TN) = **0%**	TN = 15 **Specificity** or True Negative Rate (TNR) = TN/(TN + FP) = **100%**
		Accuracy (ACC) = TP + TN/Total (TP + TN + FP + FN) = **97.73%**	

Table 3
Parameters of Multiple Linear Regression based on sensors and VOC molecule concentrations (subgroup HS ∩ B ∩ U).

		Multiple R	Multiple R2	Adjusted R2	SS Model	df Model	MS Model	SS Residual	df Residual	MS Residual	F	p
Human Semen (HS)	2-Anthracenamine_HS	0.999942	0.999884	0.998490	34947.73	12	2912.310	4.059970	1	4.059970	717.3231	0.029170
	Acetone_HS	0.999815	0.999629	0.995183	1139061	12	94921.77	422.2416	1	422.2416	224.8044	0.052078
	n-Hexane_HS	0.999838	0.999676	0.995792	1261008	12	105084.0	408.2985	1	408.2985	257.3705	0.048676
	Butanal_HS	0.999943	0.999887	0.998526	15387.68	12	1282.306	1.744799	1	1.744799	734.9308	0.028819
	D-Limonene_HS	0.999955	0.999910	0.998827	1691774	12	140981.1	152.6333	1	152.6333	923.6592	0.025708
	Pentanal_HS	0.998667	0.997336	0.982681	29043.55	11	2640.323	77.59027	2	38.79513	68.05809	0.014567
	Pyrrole_HS	0.999953	0.999907	0.998786	36503.34	12	3041.945	3.408565	1	3.408565	892.4415	0.026153
Blood (B)	(3-Methoxy-phenyl)-(6-methyl-4-phenyl-quinazolin-2-yl)-amine_B	0.534392	0.285574	0.226039	1923.614	1	1923.614	4812.334	12	401.0278	4.796711	0.048996
	1-(6-Methyl-benzothiazol-2-yl)-3-(4-methyl-benzoyl)-thiourea_B	0.554402	0.307361	0.249642	190.0528	1	190.0528	428.2836	12	35.69030	5.325055	0.039648
	11H-Dibenzo[b,e] [1,4] diazepin-11-one, 5,10-dihydro-5-[3-(methylamino)propyl] -_B	0.771298	0.594900	0.473371	123.6646	3	41.22154	84.20986	10	8.420986	4.895097	0.024015
	4-(4-Chlorophenyl)-2,6-diphenylpyridine_B	0.769838	0.592651	0.470446	2969.933	3	989.9778	2041.335	10	204.1335	4.849658	0.024654
	Acetic acid, sodium salt_B	0.999866	0.999732	0.996511	30734.17	12	2561.181	8.249683	1	8.249683	310.4581	0.044325
	Butane, 2-methyl-_B	0.998816	0.997633	0.984617	181.0509	11	16.45917	0.429482	2	0.214741	76.64669	0.012947
	Heptanal_B	0.999413	0.998826	0.992371	125.2326	11	11.38478	0.147166	2	0.073583	154.7200	0.006439
	n-Hexane_B	0.999476	0.998952	0.993186	660296.9	11	60026.99	692.9446	2	346.4723	173.2519	0.005752
	Oxime-, methoxy-phenyl-__B	0.997405	0.994818	0.966315	975.0672	11	88.64248	5.079390	2	2.539695	34.90280	0.028172
	Propane, 2-(ethenyloxy)-_B	0.556497	0.309689	0.252163	448.1593	1	448.1593	998.9682	12	83.24735	5.383466	0.038751
	2-Chloro-4-(4-methoxyphenyl)-6-(4-nitrophenyl) pyrimidine_B	0.999910	0.999820	0.997661	1055.005	12	87.91711	0.189886	1	0.189886	463.0006	0.036303
	Cyclohexane_B	0.998540	0.997083	0.981038	13778.23	11	1252.566	40.31095	2	20.15547	62.14522	0.015939
	Cyclopentane, methyl-_B	0.991132	0.982343	0.923486	252444.2	10	25244.42	4537.568	3	1512.523	16.69027	0.020291
	Octane_B	0.999961	0.999922	0.998981	2844.838	12	237.0698	0.222950	1	0.222950	1063.334	0.023960
	Pentane_B	0.996613	0.993237	0.956040	166988.0	11	15180.73	1137.033	2	568.5163	26.70236	0.036635
	Benzaldehyde, 2-nitro-, diaminomethylidenhydrazone_B	0.998570	0.997141	0.981418	98.03987	11	8.912716	0.281076	2	0.140538	63.41864	0.015622
Urine (U)	1H-Indole, 5-methyl-2-phenyl-_U	0.998630	0.997262	0.982206	7.468983	11	0.678998	0.020503	2	0.010251	66.23497	0.014964
	11H-Dibenzo[b,e] [1,4] diazepin-11-one, 5,10-dihydro-5-[3-(methylamino)propyl] -_U	0.999413	0.998826	0.992371	55.41971	11	5.038155	0.065126	2	0.032563	154.7200	0.006439
	2,4,5-Trioxoimidazolidine_U	0.995926	0.991869	0.947149	52.40240	11	4.763855	0.429571	2	0.214785	22.17960	0.043909
	2-Anthracenamine_U	0.920201	0.846769	0.668001	355.0324	7	50.71892	64.24630	6	10.70772	4.736670	0.038362
	2-Butanone_U	0.995456	0.990933	0.941062	2823.679	11	256.6980	25.83783	2	12.91891	19.86994	0.048864
	2-Ethyl-oxetane_U	0.986863	0.973899	0.886895	29.07337	10	2.907337	0.779184	3	0.259728	11.19378	0.035733
	2H-Pyrrol-2-one, 1,5-dihydro-1-(4-methoxyphenyl)-5,5-diphenyl-_U	0.999856	0.999713	0.996266	82.78334	12	6.898611	0.023786	1	0.023786	290.0325	0.045857
	2-Pentanone_U	0.988100	0.976342	0.897481	34117.63	10	3411.763	826.7174	3	275.5725	12.38064	0.031018
	3-Hexanone_U	0.997770	0.995544	0.971037	55.32891	11	5.029901	0.247641	2	0.123820	40.62254	0.024263
	4-(4-Chlorophenyl)-2,6-diphenylpyridine_U	0.986554	0.973289	0.884253	1093.172	10	109.3172	30.00095	3	10.00032	10.93138	0.036938
	4-Heptanone_U	0.899609	0.809296	0.645835	439936.7	6	73322.79	103667.8	7	14809.68	4.951002	0.027229
	5,9-Dodecadien-2-one, 6,10-dimethyl-, (E,E))-_U	0.995729	0.991475	0.944589	718.1994	11	65.29085	6.175091	2	3.087545	21.14652	0.045996
	Acetic acid, sodium salt_U	0.989862	0.979827	0.912585	27.99185	10	2.799185	0.576295	3	0.192098	14.57162	0.024628
	Acetone_U	0.999148	0.998298	0.988934	723198.2	11	65745.29	1233.321	2	616.6604	106.6151	0.009328
	Disulfide, dimethyl_U	0.997041	0.994091	0.961589	8395.598	11	763.2362	49.90772	2	24.95386	30.58590	0.032072
	Fluoren-9-ol, 3,6-dimethoxy-9-(2-phenylethynyl)-_U	0.997669	0.995343	0.969728	101.1546	11	9.195875	0.473301	2	0.236651	38.85846	0.025348
	Hexanal_U	0.988280	0.976697	0.899020	17.24043	10	1.724043	0.411339	3	0.137113	12.57389	0.030348
	Oxime-, methoxy-phenyl-__U	0.999934	0.999869	0.998291	429.6851	12	35.80710	0.056493	1	0.056493	633.8329	0.031031
	Pyrrole_U	0.999897	0.999794	0.997319	649.6194	12	54.13495	0.133984	1	0.133984	404.0406	0.038859
	Benzaldehyde, 2-nitro-, diaminomethylidenhydrazone_U	0.998570	0.997141	0.981418	10.45323	11	0.950294	0.029969	2	0.014984	63.41864	0.015622

value is significantly different from zero.

In general, all the MLR analysis for the different subgroups gave good results, since for several VOCs the regression algorithm based on the sensor array responses succeeded in predicting the concentration value. The multiple regression modelling which allowed the prediction of a higher number of VOCs with high quality of fitting was that related to HS ∩ B ∩ U group; 43 VOCs (7 from HS, 16 from B and 20 from U) listed in Table 3 were predicted with multiple R value closed to 1. By comparing with the other MLR analysis, a smaller number of VOCs from each biofluid was in general predicted (17 VOCs for HS ∩ B: 6 from HS, 11 from B; 14 VOCs for HS ∩ U: 6 from HS, 8 from U; 17 VOCs for B ∩ U: 10 from B, 7 from U; 4 VOCs for HS; 11 VOC for B; 9 VOCs for U). This is related to the greater starting number of independent (predictive) variables for the model that considers all the 3 biofluids compared to the models that consider only two of them or those that consider one biofluid. In all the models and for each VOC variable a Pareto chart of t-value for coefficients was anyway used as guide for selecting the optimal predictors. It can be argued that a combined predictive analysis based on ternary set of biofluids is better than a binary one that in turn is better than one. However, although predictive results based on two matrices of body fluids or single body fluid have lower predictive power, they are significant as they show a good R-value for some VOCs. It should also be considered that MLR results for binary or single body fluid matrices could be improved with a larger number of samples collected from a larger sample population. In particular, the results for the B ∩ U group can be considered promising for next application to a sample population including both male and female.

As example we report the observed *vs* predicted plots obtained by the MLR analysis for the subgroup HS ∩ B ∩ U for some of the VOCs found in each biofluids; in particular, Fig. 5 shows the regression plot for some VOC in human semen (HS), Fig. 6 that related to some VOC in blood (B) and Fig. 7 for some VOCs in urine (U). These results are promising since they demonstrated that a gas sensor system even with a reduced number of sensors and a small sample population is a powerful approach to analyze complex mixtures of VOCs as those in the headspace of biological samples. Indeed, our gas sensor system once trained by the data of compound concentrations obtained by a combined HS-SPME-GC/MS analysis was able to assess the concentration of many VOCs present in the considered biofluids. In perspective for HBM initiatives, an enose based on a gas sensor array suitably trained by a GC/MS analysis could represent a simple, rapid, and reliable method to predict some patterns of VOCs quantified by GC/MS and investigated as candidate markers of environmental exposure in human body fluids that is an essential goal.

The presence of these VOCs in the considered biofluids was discussed from a physiological point of view in our previous work and for some compounds the possible health risk effects were mentioned (Longo et al., 2021a). Many volatilomics studies have yet to be done looking forward to the definition of significant VOC patterns that can be considered as candidate biomarkers of chemical exposure. In the perspective of this challenging volatilomics approach in human biomonitoring studies, the specific role of gas sensors-based systems is a novelty. The technological innovation brought about by these electronic devices offers a different way of biomonitoring of the volatile markers of chemical exposure.

It goes beyond the scope of the work to go into the detail of the specific VOCs predicted by the sensor system developed, but we would like to highlight that this study is inserted in a broader context of studies in the sector, in particular to those that attribute to seminal fluid a name of environmental risk sentinel.

Indeed, there is a lot of work showing the correlation between certain heavy metals and oxidative damage to DNA. In addition, it has been demonstrated that some heavy metals can change the properties of sperm nuclear basic proteins. In fact, in areas of high environmental impact sperm nuclear basic proteins can change their canonical protective role towards DNA and be involved in oxidative DNA damage (Lettieri et al., 2020b). In addition, it has been showed that there is a lower seminal antioxidant activity in the spermatozoa in polluted areas (Lettieri et al., 2020a). The development of environmental pollution biosensors is also motivated by the fact that altered environmental conditions, together with the direct and indirect short- and long-term effects of viral infection could cause a worsening of semen quality with important consequences for male fertility, especially in those areas with higher environmental impact (Montano et al., 2021b). Current observations emphasize the weight that environmental pollution has on the sensitivity of a given population to several diseases and how semen quality, may be a potential indicator of sensitivity for virus insults in high polluted areas, and help to predict the risk for harmful effects of

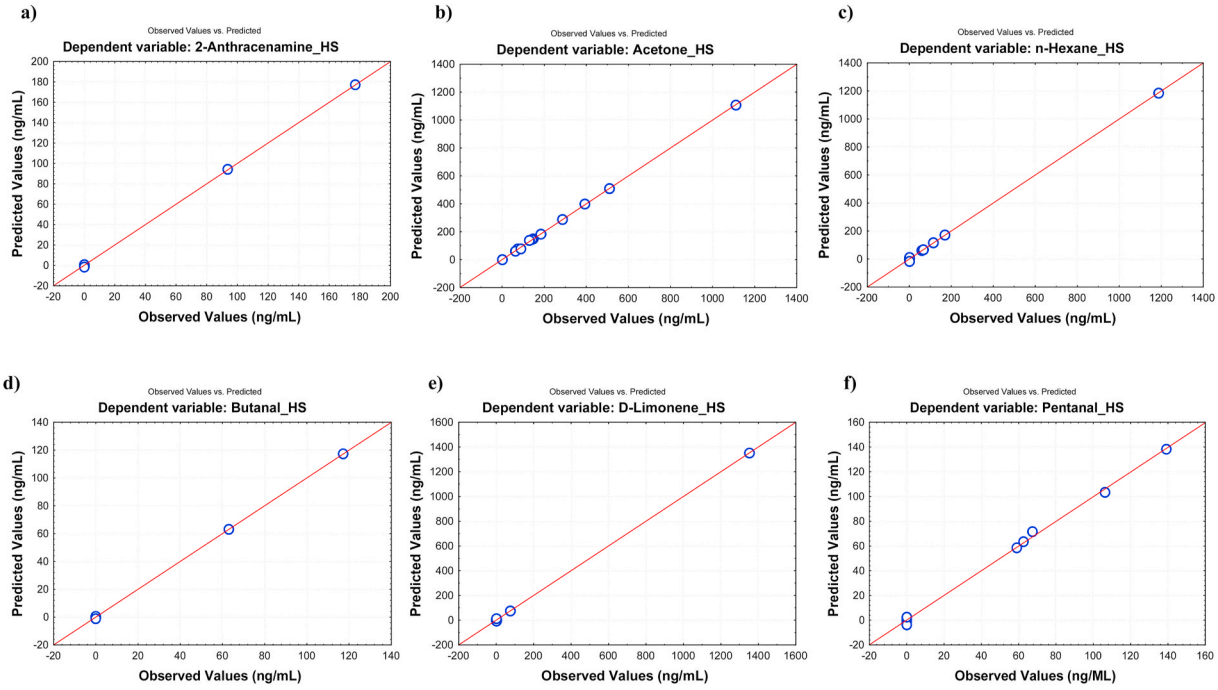

Fig. 5. Observed vs. Predicted values for some VOC molecule concentrations found in human semen (HS) as result of the MLR analysis for the subgroup HS ∩ B ∩ U.

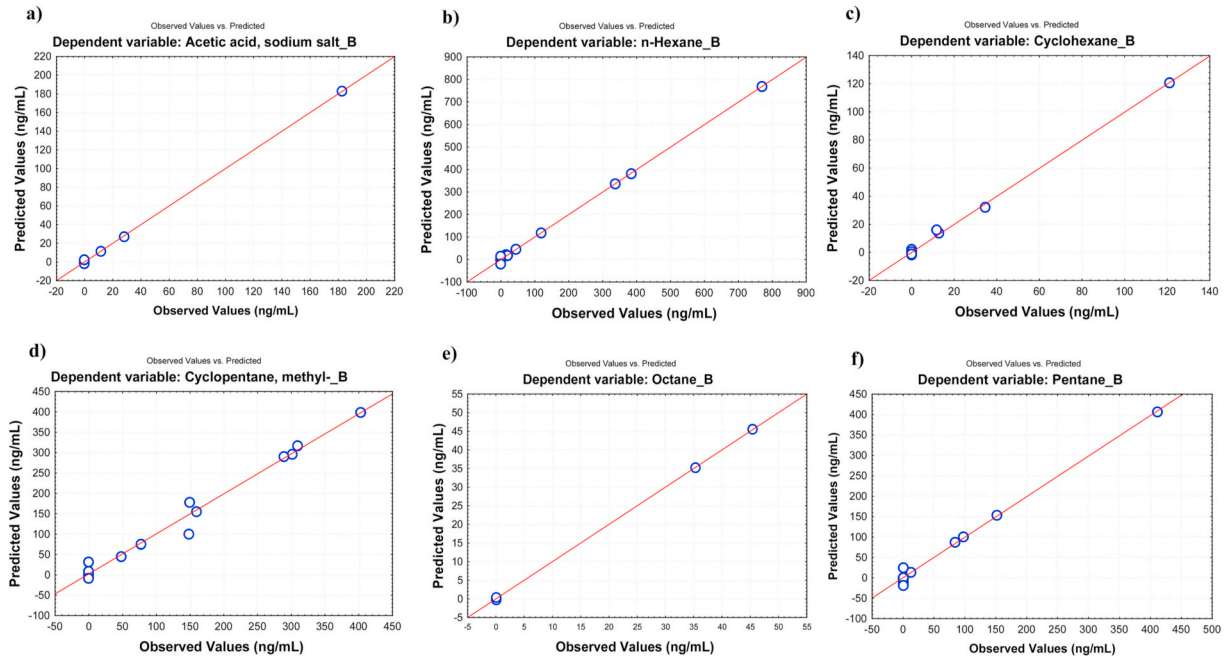

Fig. 6. Observed vs. Predicted values for some VOC molecule concentrations found in blood (B) as result of the MLR analysis for the subgroup HS ∩ B ∩ U.

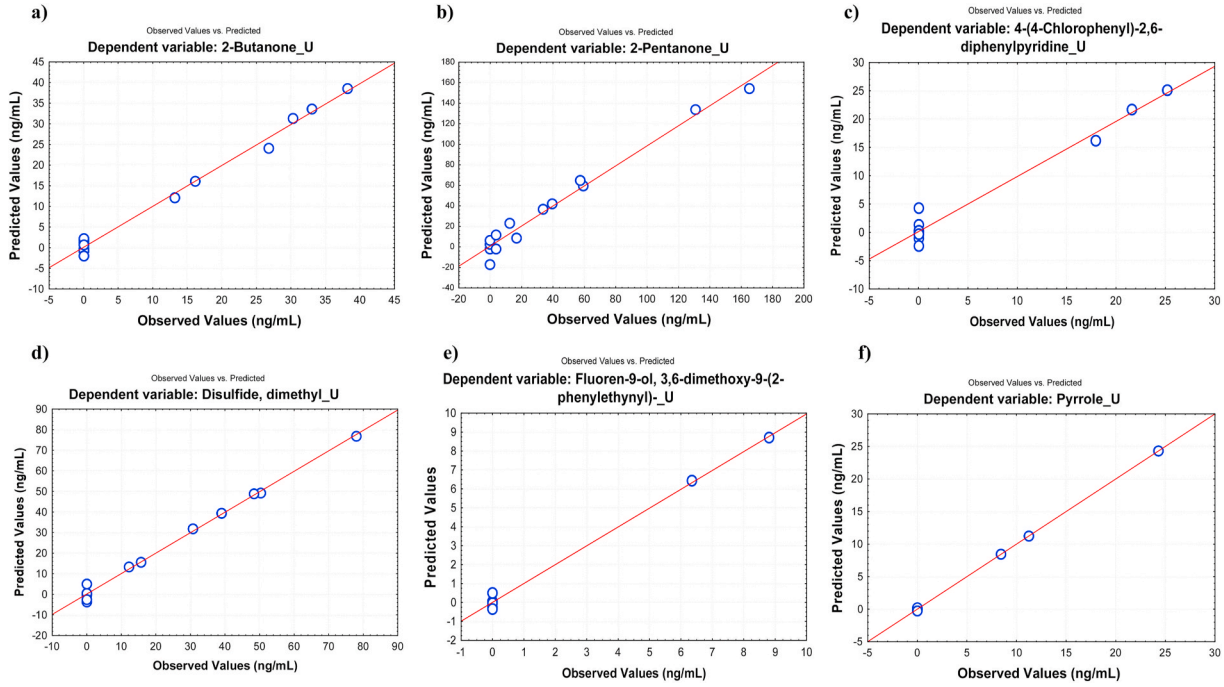

Fig. 7. Observed vs. Predicted values for some VOC molecule concentrations found in urine (U) as result of the MLR analysis for the subgroup HS ∩ B ∩ U.

viral epidemic (Montano et al., 2021c). In addition, very recently it has also been shown that kallikrein-related serine peptidase 3 could be an early biomarker of environmental exposure in young women (Raimondo et al., 2021). Finally, heavy metals, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls, dioxins, pesticides, ultrafine particles, produced by human activities put a strain on the body's entire defence system. Evidence from pre-clinical and clinical studies showing that the impairment of male fertility and gonadal development, as well as cancers of reproductive system, due to the exposure of organic and inorganic pollutants, may be counteracted by flavonoids (Montano et al., 2021c; Montano et al., 2022).

4. Conclusions

This preliminary work points how an easy-to-use gas sensors-based system, supported and trained by SPME-GC/MS, is able to extract fundamental information from the human Volatilome of body fluids. Volatilomics as novel omic approach offers new opportunities for enhanced understanding of the exposure-response continuum in health risk assessment and permitting the observation and measurement of biological response modulation at a volatilomic scale. In this context, the enose and GC/MS methodologies, working in tandem in the training mode described in the work, are proposed as a new analytical tool that

can contribute to the crucial and delicate question of health risk assessment in populations in the neighbourhoods of two contaminated sites (*Land of Fires* and *Valley of Sacco river*, Italy), characterized by different and peculiar sources of pollution of the territory but all linked to illegal waste management. The major advantage of Volatilomic research based on joint use of e-nose and GC/MS in Exposomics by human biomonitoring is that Volatilomics provides a snapshot view of a biological sample and enables capture of information about the interactions of an individual with his environment, that are particularly significant when the chemical environmental exposure is continuous and prolonged as occurs for residents in contaminated sites. This approach provides an untargeted but complete profile of the characteristic VOCs present in any biofluids by a standard GC/MS-based analytical analysis as well as a global fingerprint of the volatile fraction of any biofluid by a modern analytical tool as the e-nose based on gas microsensors, the latter offering the opportunity for more extensive biomonitoring studies due to its ease of use, its compactness and reduced cost. Such synergy between e-nose and GC-MS implements an advanced training of the former by applying statistical regression models and provides a more complete information about the biochemical status or biochemical phenotype of subjects than many other possible approaches.

The gas sensor system was able to assess some differences in the two groups living in LF and VSR based on the gas sensor responses to VOC blood classifying the two classes. We established also mathematical models by Multiple Linear Regression (MLR) between data obtained by sensors and data obtained by gas chromatography, and interesting results demonstrated the capability of the sensor system to predict the presence of specific VOCs in human semen, blood, and urine.

The general perspective on which this work was conducted is a long-range focus on exploiting human biomonitoring approaches to discover unknown biomarkers and develop individual health risk predictors for humans.

Author contributions

Valentina Longo: Conceptualization; Methodology; Validation; Formal analysis; Writing – review & editing. Angiola Forleo: Conceptualization; Methodology; Validation; Writing – review & editing. Antonio Vincenzo Radogna: Conceptualization; Software. Pietro Siciliano: Conceptualization; Funding acquisition. Tiziana Notari: Methodology. Sebastiana Pappalardo: Conceptualization; Methodology. Marina Piscopo: Conceptualization; Methodology; Writing – review & editing. Luigi Montano: Conceptualization; Methodology; Writing – review & editing. Simonetta Capone: Conceptualization; Methodology; Validation; Writing - Original Draft; Writing – review & editing; Supervision; Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2022.119119.

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