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Occupational exposure to volatile organic compounds affects microRNA profiling: Towards the identification of novel biomarkers

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ABSTRACT

In the framework of a project aimed at finding novel predictive biomarkers of VOCs exposure-related diseases, the effect of exposure to ethylbenzene, toluene, and xylene has been analyzed in a group of painters (spray- and roller-painters) working in the shipyard industry. Airborne levels of solvents were higher in spray- than in roller-painters, and comparable to the Occupational Exposure Limits (OELs), particularly for toluene and xylene. The urinary concentration of each volatile organic compound (VOC) and of the corresponding metabolites were also concurrently measured. A set of oxidative stress biomarkers, i.e., the products of DNA and RNA oxidation, RNA methylation, and protein nitration, were measured, and found significantly higher at the end of the work shift. MicroRNA (MiRNA) expression was analyzed in the VOC-exposed workers and in a control group, finding 56 differentially expressed (DE) miRNAs at a statistically significant level (adjusted $p \leq 0.01$). The Receiver-Operating Characteristic curves, computed for each identified miRNA, showed high sensitivity and specificity. A pathway analysis in the Kyoto Encyclopedia of Genes and Genomes (KEGG) showed that miRNA-1, which was found downregulated in exposed workers, is involved in the lung cancer oncogenesis. A subset of 10 miRNAs (out of the 56 DE) was selected, including those with the highest correlation to the urinary dose biomarkers measured at the end of work-shift. Multivariate ANOVA analysis showed a statistically significant correlation between the urinary dose biomarkers (both the VOCs urinary concentration and the VOCs' metabolite concentration), and the identified miRNA subset, indicating that the exposure to low VOC doses may be sufficient to activate the miRNA response. Four miRNAs belonging to the subset strongly related to the VOCs and VOCs' metabolites concentration were individuated, miR-589-5p, miR-941, miR-146b-3p and miR-27a-3p, with well-known implications in oxidative stress and inflammation processes.

1. Introduction

Occupational exposure of painters has been classified Group 1 by IARC in 2010; it causes cancers of the lung and of the urinary bladder [1]. In the naval ship industry, the surface coating applications may release large quantities of dangerous substances representing a threat for the workers' health and safety. Most of the chemical compounds used in the shipyard painting activity are VOCs (Volatile Organic Compounds), some of them also with carcinogenic properties. Among them, benzene (IARC group 1), toluene, xylene (IARC group 3) and ethylbenzene (IARC group 2B) are worthy of note [2]. VOCs are also known to be neurotoxic and, as neurotoxicity mechanisms are still to be clarified, in vivo experiments have been performed [3] at the aim of deepening the knowledge about this issue. Such toxicants expose workers to chemical risk by inhalation and dermal absorption. Therefore, airborne concentration levels for VOCs must comply with Occupational Exposure Limits (OELs) established by the legislative regulation of each Country. To ensure individuals' health protection and to avoid the development of occupational diseases, if the exposure levels cannot be further reduced, personal protective equipment must be

Abbreviations: VOCs, volatile organic compounds; OELs, Occupational Exposure Limits; SPMA, S- Phenylmercapturic acid; SBMA, S-Benzylmercapturic acid; MHIPPA, methylhippuric acid; miRNA, MicroRNA; DE, differently expressed; KEGG, Kyoto Encyclopedia of Genes and Genomes

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worn. The dose effectively absorbed and metabolized by each subject can be assessed only by means of biological monitoring, which consists of measuring dose and/or effect biomarkers. The monitoring of both VOCs and VOCs' metabolites in biological fluids, such as urine, belongs to the first class. Urinary DNA and RNA oxidized nucleotides and nucleosides can be determined as effect biomarkers of oxidative damage. A different category of effect biomarkers, i.e., miRNAs, was recently included in occupational exposure studies [4]. MiRNAs were initially identified for the sensitivity of their expression profiles to neurological and cardiovascular diseases, pathogenesis of hearing disorders, development and progression of several types of cancer (brain, lung, breast, liver, colorectal), and they are now definitely recognized as novel diagnostic biomarkers in clinical medicine [5–9]. In that respect, many studies pointed out that, in the event of exposure to dangerous chemicals (e.g., VOCs), individual alterations in miRNA profile may occur, recognizing the use of circulating miRNAs, from full blood, serum or plasma [10], as novel and predictive biomarkers of risk evaluation at the workplace [11–14]. In a study conducted on 169 workers exposed to VOCs (toluene, xylene, and ethylbenzene), 467 miRNAs were identified for toluene, 211 miRNAs for xylene and 695 for xylene as exposure biomarkers to distinguish exposed from control subjects at higher level of sensitivity and specificity than urinary biomarkers. [12]. Another study investigated 91 subjects occupationally exposed to high levels of benzene. Two groups of miRNAs, one with higher expression and another with lower expression, were identified in exposed workers versus controls. Benzene exposure was found to relate to different levels of miRNA (upregulation of miR-638 and downregulation of miR-22 1–3 p and miR-122-5p in human plasma) [15]. The identification of specific miRNAs or of characteristic expression patterns, as biomarkers of exposure and effect, is particularly crucial when multiple exposure to low doses of a lot of different substances simultaneously present at workplace occur.

In this case, the combined effect of different risk agents can be worse than simply additive, and possibly synergistic interactions can be at work in inducing serious adverse health effects [16]. Methods have been proposed [17] to evaluate improved regulatory exposure limits, keeping into account all the different stressors. These methods are based on toxicological levels, capable of inducing adverse health effects, that are order of magnitude below the Occupational Exposure Limits. A recent review [18] proposes the alteration of miRNA profile as a mechanism for disease development in the case of exposure to metals present at low doses in the environment. The dysregulation of miRNA in neurological diseases was used as early biomarker, both for diagnostic purposes and for monitoring patients suffering from neurological degeneration. This study fits into this context, being focused on possible miRNA dysregulation related to VOCs exposure and consequent chronic inflammatory processes and oxidative stress.

Specific biomarkers of oxidative stress [19] have been evaluated: 1) the 8-Oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), the oxidized form of the nucleoside deoxy-guanosine, coming predominantly from DNA turnover and repair activity, 2) the 8-Oxo-7,8-guanosine (8-oxoGuo), the oxidized form of the nucleoside guanosine formed by guanine bound to a RNA ribose, coming from RNA turnover, and, 3) the 8-Oxo-7,8-dihydroguanine (8-oxoGua), the oxidized form of guanine coming from the total activity of DNA repair and both DNA and RNA turnover. Besides, the urinary concentration of 3-nitrotyrosine was also determined, as biomarker of nitrosative stress, produced by the nitration of Tyrosine residues in proteins. Nitration of proteins is a common process occurring under physiological conditions, but a significant increase of this process, induced by increased nitrosative stress, has been associated with a wide range of diseases [20]. Lastly, the urinary 5-methylcytidine was measured, a product of RNA methylation, consisting in a nucleoside molecule that is formed when cytosine is attached to a ribose ring, considered an epigenetic marker of RNA, whose aberrant levels were found to be associated with various cancers [21].

The data analyzed in this paper are part of a larger study aimed at assessing the early hearing dysfunctionality induced by the combined exposure to VOCs and noise in an occupational setting. The results of the audiological study, associating the level of hearing impairment to the VOCs exposure doses, was published in Sisto et al. 2020 [22].

In this study, biomonitoring of workers exposure to VOCs was performed in a ship industry, to identify the exposure levels and the corresponding dose and effect biomarkers, as well as the circulating miRNAs with potential biological role on VOC metabolism. In a pilot study (Sisto et al., 2019) [23], a very similar study design had been carried out on a small number of workers exposed to VOCs. Two DE miRNAs (miR-6819-5p and miR-6778-5p) were identified in exposed workers, with respect to controls. A correlation analysis between miRNA and VOCs' metabolites, allowed the identification of a set of miRNAs highly correlated to specific VOCs' metabolites. A significant negative association was found between the DE miR-6778-5p and the 8-oxoGua urinary concentration. The study design and the analysis technique [23] were replicated in the present study on a larger sample of workers exposed to VOCs. It must be stressed that this study is not just an extension of the previous pilot study. In fact, we hypothesize that the miRNA expression profile is crucially dependent also on the absorbed dose of the xenobiotic chemicals, and, in the present study, the workers were exposed to much higher doses of VOCs. The aim of this study was: i) to measure the airborne concentrations of VOCs to verify the compliance with the Occupational Exposure Limits (OELs) for some specific substances; ii) to quantify the workers' absorbed doses in terms of both VOCs and VOCs' metabolites urinary concentration iii) to identify novel effect biomarkers sensitive to the exposure to low VOCs concentrations. For the last purpose, biomarkers of nucleic acid oxidative stress and of protein nitrosative stress were evaluated, and the miRNome was analyzed in both exposed workers and controls, searching for significant differential expression patterns of biological relevance in the two groups.

2. Materials and methods

2.1. Subjects and study design

Seventeen shipyard painters were enrolled, exposed to organic solvents (toluene, xylene, etc.,) and to other substances, like diluents and additives (eptan-2-one, 2-butossiethyl acetate, 1-methyl-2metossiethylacetate, butanone, ethyl acetate, n-butyl acetate), with values of potential inhalation exposure to each solvent close to the relative Occupational Exposure Limits. The subjects, professional painters working in a naval industry in central Italy, were monitored during their work shift. All individuals were eligible and agreed to the study after having given their informed consent. All procedures performed in this study involving human participants were in accordance with the ethical standards of our Institutional Committee and in accordance with the local ethics committee (Health local agency, ASL, Regione Marche). Two main working tasks have been identified, roller- and spray-painting, the latter being associated to a higher airborne concentration of aromatic solvents of the mixture. Six spray- and 11 roller-painters, all using respirators with carbon filters, were included in the workers' sample. All subjects were males, two of them of Caucasian ethnicity, and the others of Bengalese ethnicity. The mean age was 39 years, ranging from a minimum age of 21 years to a maximum of 54 years. The urine samples were collected before and after the work-shift in June 2018. An anamnestic questionnaire was administered to the enrolled subjects, regarding the professional exposure to organic solvents. Information was collected also about the personal lifestyle and habits, the general health status, the cigarette smoke and use of drugs. Two smokers were identified in the group of roller painters and three in the group of spray painters. Questions were asked also about the handled materials and the protection equipment used at the workplace, as well as about previous exposure to solvents during the working life. The exposure to solvents was assessed by personal air sampling and urine

Table 1
Analytic description of workers and controls. The results of the statistical tests for comparison between roller and spray painters are also shown.

ID	Nationality	Sex	Age	Job task	Smoking habit
1C	Italy	Male	40	Research	no
2C	Italy	Male	37	Research	no
3C	Italy	Male	48	Research	no
1E	Bangladesh	Male	21	roller-painting	no
2E	Bangladesh	Male	35	roller-painting	yes
3E	Bangladesh	Male	39	roller-painting	no
4E	Bangladesh	Male	43	spray-painting	yes
5E	Bangladesh	Male	48	roller-painting	no
6E	Bangladesh	Male	40	spray-painting	no
7E	Bangladesh	Male	42	roller-painting	no
8E	Bangladesh	Male	38	spray-painting	no
9E	Bangladesh	Male	44	spray-painting	yes
10E	Bangladesh	Male	36	roller-painting	no
11E	Bangladesh	Male	43	roller-painting	no
12E	Iraq	Male	40	roller-painting	yes
13E	Bangladesh	Male	26	roller-painting	no
15E	Bangladesh	Male	32	roller-painting	N/A
16E	Bangladesh	Male	34	roller-painting	no
17E	Bangladesh	Male	54	spray-painting	yes
18E	Tunisia	Male	49	spray-painting	no
roller –spray comparison test	Pearson χ square		Students' *t* -test		Pearson
p value	n.s.		0.016		χ square
					n.s

sampling performed before and after the work-shift. The workers were monitored in an experimental campaign, on June 25th, 2018. Data relative to the hearing functionality and their association to the VOCs exposure doses have been object of another publication [22]. Genotoxic biomarkers of direct and oxidative damage to the DNA were also evaluated on the same workers and analyzed in another paper. (in preparation). Blood samples for miRNA isolation were taken during the routine medical surveillance. Three control subjects were also enrolled in the study, selected from the non-smoking members of the research team, matching sex and mean age of the exposed subjects. The controls were chosen among researchers to exclude any occupational exposure to VOCs, whereas non-smoking habit was requested because the urinary concentrations of some VOCs and VOCs' metabolites can be higher in smokers than in non-smokers [24], and the objective of this work was testing the hypothesis that there is a different gene expression in subjects exposed to VOCs with respect to the non-exposed subjects.

The description of the enrolled subjects in terms of age, ethnicity, smoking habit and job task are reported in Table 1.

2.2. miRNA extraction

The extraction and reading of the miRNA matrix was performed by Qiagen Genomic Services. The Next Generation Sequencing (NGS) analysis of the miRNAs was performed on samples of human blood plasma (200 μL). The NGS sequencing libraries were quantified and sequenced for the samples provided by our research team. The collected readings were subjected to quality control, unique molecular index-based correction (to remove PCR replicates), alignment and downstream analysis.

2.3. miRNA bio-informatic analysis

The miRNA data, normalized according to the Trimmed Mean of M values (TMM) method, were provided by QIAGEN, and analyzed by the author of this paper. The differentially expressed genes DE in exposed and control group were selected by means of the routine DESeq2 of Bioconductor (R Foundation for Statistical Computing, Vienna, Austria).

2.4. Personal air monitoring

The personal exposure to organic solvents was assessed by passive air sampling by means of Radiello® devices during the whole work-shift. Each Radiello was chemically extracted with carbon disulfide and the samples were analyzed by GC–MS with the internal standard method for the target VOCs, namely ethyl acetate, benzene, toluene, ethylbenzene, p-xylene, m-xylene, o-xylene.

2.5. Biological monitoring

The concentrations of ethyl acetate, benzene, toluene, ethylbenzene, p-xylene, m-xylene, o-xylene excreted unchanged were measured in the urine samples. Although benzene should not be present, as a substance or in solvent mixtures, in concentrations higher than 0.1 % by weight, according to the REACH regulation, it could be present in engine fuels, it is produced by smoking, and it is a class 1 carcinogen: for these reasons it was decided to include it in the biological monitoring [25].

In the same samples, for each VOC, the concentration of its most common and specific urinary metabolite was also determined. These are Methylhippuric acid (MHIPP, xylenes metabolite), Phenylglyoxylic and Mandelic acid (PGA, MA both ethylbenzene metabolites), S-Benzylmercapturic acid (SBMA, toluene metabolite) and S-Phenylmercapturic acid (SPMA, benzene metabolite).

Unmetabolized VOCs in the urine were determined by GC–MS with the headspace analysis method [26]. All the metabolites have been determined by HPLC-MS/MS (static headspace sampling devices G1888A, gas chromatograph 6890 N, mass spectrometry detector 5973 N, Agilent Technologies) with a Turbo Ion Spray (TIS) in the urine samples of workers, both before and after the working shift.

For all the different analytical methods reported in Table 2, the results were expressed as ratios to the concentration of urinary creatinine, in order to normalize the results for the dilution grade of urine. Urinary creatinine was determined by the method of Jaffè using alkaline picrate test with UV/Vis detection at 490 nm [27]. Samples with creatinine concentrations lower than 0.3 g/L or higher than 3.0 g/L were excluded from statistical analysis according to the American Conference of Governmental Industrial Hygienists (ACGIH) recommendation [28].

Table 2
Analytical methods and Limits of Detection for urinary metabolites.

Risk Agent	Biomarker	Limit of Detection (LoD)
Ethylbenzene	Phenylglyoxylic acid (PGA)	0.015 mg/L
Ethylbenzene	Mandelic acid (MA)	0.02 mg/L
Xylenes	Methylhyppuric acid (MHIPP)	1 μg/mL
Toluene	S-Benzylmercapturic acid (SBMA)	0.35 μg/L
Benzene	S-Phenylmercapturic acid (SPMA)	0.026 μg/L
Nicotine (active smoking)	Cotinine	12.41 μg/L
Oxidative stress on DNA	8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo)	0.5 μg/L
Oxidative stress on RNA	8-oxo-7,8-dihydroguanosine (8-oxoGuo)	0.7 μg/L
Oxidative stress on DNA and RNA	8-oxo-7,8-dihydroguanine (8-oxoGua)	0.5 μg/L
Nitrosative stress on proteins	3-nitrotyrosine	1.0 μg/L
Methylation of RNA	5-methylcytidine	0.01 μg/L

2.6. Statistical analysis

Analyses were carried out with SPSS/PC statistical software package 19.0. (Inc., Chicago, IL, USA) and Statistical software R (R Foundation for Statistical Computing, Vienna, Austria). The solvent metabolites (MA, PGA, MHIPP, SPMA, SBMA) were treated as continuous variables. Normality of the distributions was assessed according to the Kolmogorov-Smirnov test. Pearson correlation coefficient was used to measure the correlation between the miRNAs and the VOCs concentration as well as VOCs' metabolites.

The data normalized according to the Trimmed Mean of M values (TMM) provided by QIAGEN were analyzed using Bioconductor routines. The differentially expressed genes (DE) in exposed and control groups were selected by means of the routine DESeq2 of Bioconductor. The method used by the DESeq2 routine is based on the assumption that the reads counting for the gene i-th in the sample j-th (with j running on the subject index) is described by a GLM (generalized linear model) of the family of the Negative binomial distribution. The link function is a logarithmic one relating the model coefficients to the log2 of the fold change between exposed and control. The differentially expressed miRNAs were sorted by increasing p-value. A set of 56 genes was selected, with adjusted p-value ≤ 0.01.

The 56 selected miRNAs were also sorted by average up- or downregulation of the miRNA of the exposed versus the control sample, with the downregulated group followed by the upregulated one. The expression matrix M(i,j) (where i is the miRNA index and j is the subject index, from 1 to 3 for the controls and from 4 to 20 for the exposed workers) was calculated as the difference between the intensity value for each subject for each miRNA and the average miRNA calculated on all the subjects, divided by the square root of the pooled variance:

$$M(i,j) = \frac{miRNA(i,j) - mean(miRNA(i,1{:}\,20)}{\sqrt{\mathrm{var}(miRNA(i,1{:}\,3) + \mathrm{var}(miRNA(i,4{:}\,20)}} \quad (1)$$

A principal component analysis (PCA) was also performed. The correlations between the concentration of each solvent in urine, the solvent metabolites, MA, PGA, MHIPP, SBMA, SPMA, and between the oxidized guanine derivatives, 8-oxoGua, 8-oxoGuo, 8-oxodGuo and the selected 56 miRNAs, were evaluated.

A subset including the 10 miRNAs with the highest correlation ($R^2 > 0.6$) with VOCs and VOCs' metabolites was identified. A Multivariate ANOVA test was performed to test the statistical significance of the relation between dose and effect biomarkers and the miRNAs. For each of the 56 DE miRNAs the ROC curves were also computed.

Table 3
airborne concentration of the most important VOCs present in the mixture for the roller- and spray-painting activities. The ratio between the airborne concentration and the relative Occupational Exposure Limit (OEL) is also reported. The result of the *t*-test used for comparison between roller and spray painters is also shown.

	toluene	ethylbenzene	p-xylene mg/m^3	m-xylene	o-xylene	xylenes
roller						
mean	0.54	3.60	3.62	9.07	2.52	15.21
OEL ratio	0.00	0.01	0.01	0.04	0.01	0.06
median	0.17	1.00	0.69	2.14	0.41	3.17
SD	0.76	5.58	6.72	15.89	5.68	28.23
5th	0.02	0.10	0.08	0.23	0.07	0.43
25th	0.05	0.34	0.30	0.75	0.19	1.27
75th	0.68	4.79	3.46	9.45	1.16	14.07
95th	1.81	12.44	14.24	34.39	11.21	59.84
max	2.05	16.49	19.84	47.07	16.54	83.44
min	0.02	0.04	0.04	0.10	0.03	0.17
spray						
mean	78.81	25.25	26.22	64.92	22.82	113.96
OEL ratio	0.41	0.06	0.12	0.29	0.10	0.52
median	0.82	23.45	24.24	59.12	19.96	109.36
SD	121.51	17.15	21.35	48.37	19.95	88.43
5th	0.13	6.41	4.77	13.21	4.25	22.23
25th	0.23	13.67	9.01	29.83	5.51	44.37
75th	169.00	37.59	43.19	103.57	39.48	184.18
95th	240.73	45.67	50.11	121.02	45.17	210.61
max	246.02	47.83	51.99	124.98	46.50	218.15
min	0.1	4.04	3.38	7.83	3.83	15.04
t -test p value	0.044	0.003	0.007	0.005	0.009	0.006

3. Results

3.1. Detection of VOCs in biological samples

The airborne concentration of VOCs was about one order of magnitude higher in the case of spray-painters with respect to the roller-painters. The VOCs with the highest concentrations in the mixture were toluene and xylene; their airborne concentrations during the working shift are reported in Table 3, for the two groups of roller- and spray-painters.

The mean value of the personal airborne concentration for each VOC, calculated over all the subjects, is also reported normalized to its corresponding Occupational Exposure Limit Value (OEL), according to the Italian Legislation [29]. In terms of airborne concentration of the volatile organic solvents considered, it is worth noting that the spray-painting exposure is about one order of magnitude higher than that found in the roller-painting activity. For the spray-painters, an airborne concentration approximately equal to 40 % of the OEL (192 mg/m^3) and to 50 % of the OEL (221 mg/m^3) was found, respectively, for toluene and for the xylenes mixed isomers. In Table 4 the urinary concentrations of the aromatic solvents are reported at the beginning and the end of the work-shift for the groups of roller- and spray-painters.

The mean urinary concentration of the metabolites of the solvents present in the mixture at the end of the work-shifts have been reported in Table 5. In particular, MHIPPA is the metabolite of the xylene isomers, PGA and MA were identified as metabolites of ethylbenzene (in the absence of styrene), SBMA is the metabolite of toluene, and SPMA was used as metabolite of benzene. The urinary cotinine concentration was used as biomarker for assessing the effect of the smoking habit, as cotinine is the metabolite of nicotine. All the metabolite concentrations analyzed here were found below the ACGIH BEIs (Biological Exposure Index). The BEI of the ACGIH for the considered metabolites are: 1.5 g/

Table 4
Urinary concentration of the solvents present in the mixture at the beginning and at the end of the work-shift in the groups of roller- and spray-painters. The p value of the Students' *t*-test for the comparison between roller and spray painters at the end of the woks-shift is also shown.

	ethylacetate	benzene	toluene	ethylbenzene	p-xylene	m-xylene	o-xylene	Total xylenes
				ng/mL roller				
Before Work-shift								
Mean	na*	4.9	na	6.2	2.7	12.1	na	6.8
Median	na	3.6	na	7.5	2.3	13.9	na	3.3
SD	na	4.3	na	2.6	1.2	6.4	na	7.6
5th	na	1.6	na	2.7	1.1	5.8	na	1.5
25th	na	1.7	na	3.4	2.1	9.4	na	2.1
75th	na	6.8	na	7.7	3.7	15.6	na	6.8
95th	na	9.9	na	9.0	4.3	17.0	na	20.2
max	na	10.7	na	9.3	4.4	17.4	na	21.5
min	na	1.5	na	2.5	1.0	4.9	na	1.2
After work-shift								
Mean	76.1	25.9	7.6	8.3	5.3	43.1	46.6	52.7
Median	76.1	2.2	7.6	5.5	2.6	15.5	46.6	19.3
SD	56.1	59.5	0.3	9.5	8.0	49.5	20.7	77.0
5th	40.4	1.6	7.3	3.4	1.5	13.9	33.4	6.8
25th	56.3	1.9	7.4	4.0	2.0	14.6	39.3	18.1
75th	95.9	7.8	7.7	7.3	3.9	57.9	53.9	33.1
95th	111.8	119.7	7.8	22.8	17.3	91.8	59.7	158.0
max	115.8	172.0	7.8	34.9	27.8	100.2	61.2	189.2
min	36.4	1.5	7.2	3.3	1.2	13.7	32.0	3.9
				spray				
Before work-shift								
Mean	na	4.4	na	4.4	2.4	na	na	9.6
Median	na	3.8	na	3.4	1.7	na	na	1.7
SD	na	2.0	na	3.3	2.1	na	na	19.5
5th	na	2.8	na	2.2	1.1	na	na	1.1
25th	na	2.9	na	2.8	1.3	na	na	1.3
75th	na	5.2	na	3.8	2.1	na	na	2.1
95th	na	6.8	na	9.2	5.6	na	na	37.6
max	na	7.1	na	11.0	6.7	na	na	49.4
min	na	2.7	na	2.0	1.0	na	na	1.0
After work-shift								
Mean	447.7	21.9	186.6	29.2	27.7	86.7	52.3	149.3
Median	312.9	11.1	159.9	16.3	15.3	50.0	33.3	90.7
SD	531.0	26.9	182.1	27.9	34.1	86.0	44.9	163.5
5th	28.9	4.4	10.9	14.4	7.4	34.0	24.2	41.9
25th	30.0	4.8	21.0	14.7	9.0	38.3	26.3	52.5
75th	730.5	28.2	326.8	25.8	23.1	84.8	59.4	144.3
95th	1055.3	54.7	398.8	70.9	78.1	215.6	106.9	392.8
max	1136.5	61.3	416.8	85.0	96.1	256.4	118.8	471.2
min	28.6	4.2	8.4	14.4	7.2	32.9	23.7	41.0
t -test p value	0.042	n.s.	0.043	0.023	0.018	0.029	0.05	0.031

* na means that the value is below the detection limit.

g Cr for the MHIPP, 25 μg/g Cr for the SPMA, 300 μg/g Cr for the SBMA and 400 mg/g Cr for the sum of PGA and MA. For the spray painters, who are characterized by higher doses than the roller painters, the average end-shift concentration of MHIPP is about 6% of the BEI, whilst the maximum value is about 10 %, the average concentration of SPMA is 8% of the BEI and maximum end-shift concentration is 38 %, the average concentration of SBMA is 5% of the BEI and the maximum 10 %, and, finally, the average end–shift concentration of the sum of PGA and MA is 3% of the BEI whilst the maximum is about 7%. Although also in the case of spray painters the MHIPP concentration is well below the BEI, it is about 3 order of magnitude, on average, higher than the concentration due to the smoking habit [24]. (see Lorkiewicz et al. [24], Table 1) The concentration of the sum of PGA and MA is typically about 30 times lower in smokers than in the painters under investigation. On the other hand, the average concentrations of SPMA and SBMA are comparable with the concentrations found in smokers in Lorkiewicz. Although the range of doses explored in this work for xylenes, is a low dose range, it is still much higher than the typical range of exposure to VOCs due to smoking only. A Student's *t*-test was used to verify the hypothesis that the concentrations were increased by the exposure to the painting activity. The test was statistically significant for SPMA, MHIPPA, SBMA and cotinine.

The VOCs' metabolites concentrations of controls were 315 μg/g Cr for the sum of MA and PGA, 0.02 μg/g Cr for SPMA, 35.7 μg /g Cr for the sum of the 2nd and 4th peak of MHIPP and 3.7 μg/g Cr for SBMA.

In Table 6, the urinary concentrations of DNA and RNA oxidation, RNA methylation and protein nitration products, measured at the end of the work-shift, are shown for both groups of roller- and spray-painters. In the comparison between the beginning and the end of the work shift, a statistically significant increase was observed in the urinary concentration of 8-oxoGuo, 8-oxodGuo and 5-methylcytidine. The same comparison was not statistically significant for the 8-oxoGua and the $3NO_2$ tyrosine.

3.2. Differentially expressed miRNAs

Adopting the significance criterion $p \leq 0.01$ for the adjusted p-value, 56 miRNA were found significantly differentially expressed in

Table 5
concentrations at the beginning and at the end of the work-shift of the different urinary solvent metabolites present in the mixture in the groups of roller- and spray-painters. The 2nd and 4th peak of the MHIPP acid are both reported as MHIPP2 and MHIPP4 respectively. The p value of the comparison between roller and spray painters at the end of the work-shift is also reported.

		MA (μg/g Cr)	PGA (μg/g Cr)	SPMA (μg/g Cr)	2 MHIPP (μg/g Cr)	4 MHIPP (μg/g Cr)	SBMA (μg/g Cr)	Cotinine (μg/g Cr)
	Beginning of work-shift							
roller	Mean	9031.8	24537.3	0.39	1470.3	7274.7	4.5	234.9
	Median	530.0	660.0	0.03	455.0	2558.8	3.1	16.8
	SD	26766.6	78229.5	0.79	2318.6	11909.0	3.2	331.2
	5th perc	320.0	340.0	0.01	149.9	1287.7	1.8	3.5
	25th perc	360.0	520.0	0.02	308.5	1637.7	2.6	6.0
	75th perc	1490.0	1540.0	0.15	1141.7	6613.9	4.9	457.0
	95th perc	46670.0	131395.0	1.94	5678.0	26840.1	10.4	790.5
	max	89680.0	260400.0	2.34	7824.6	41660.0	12.6	877.7
	min	310.0	310.0	0.01	114.3	1167.9	1.4	3.4
	End of work-shift							
	Mean	6690.9	4342.7	1.23	9647.5	39859.0	14.1	638.4
	Median	3600.0	2250.0	0.33	4217.8	26151.6	15.9	25.5
	SD	8589.5	3939.5	2.36	16188.1	36540.5	5.7	969.1
	5th perc	1450.0	1410.0	0.00	1436.3	11230.6	5.9	8.5
	25th perc	1655.0	1675.0	0.04	2215.1	13136.0	9.9	16.4
	75th perc	6940.0	6145.0	0.49	9020.5	50926.9	17.0	1175.5
	95th perc	21490.0	11230.0	5.75	33850.7	101510.3	21.9	2263.2
	max	30390.0	13320.0	7.39	57472.0	129540.6	22.0	2592.5
	min	1290.0	1250.0	0.00	1399.6	9973.3	5.8	8.1
spray	**Beginning of work-shift**							
	Mean	1130.0	1076.7	1.83	1287.1	4741.8	7.3	1418.0
	Median	810.0	1210.0	0.42	1311.6	4800.8	7.2	412.6
	SD	963.2	595.1	2.83	547.4	2507.5	4.5	2229.1
	5th perc	332.5	370.0	0.03	668.7	1991.6	2.3	3.2
	25th perc	472.5	555.0	0.06	851.4	2550.2	3.5	4.5
	75th perc	1425.0	1520.0	2.38	1718.0	6819.9	10.6	1702.7
	95th perc	2542.5	1680.0	6.08	1880.1	7498.7	12.6	4758.2
	max	2850.0	1730.0	7.13	1924.8	7643.1	12.9	5678.7
	min	320.0	350.0	0.02	623.2	1913.0	2.2	2.8
	End of work-shift							
	Mean	8503.3	4495.0	2.65	17233.5	89310.8	15.9	2132.0
	Median	5420.0	4985.0	1.11	19421.7	81611.6	12.7	583.4
	SD	7928.5	2268.5	3.56	11200.1	36247.4	8.5	3819.2
	5th perc	1732.5	1615.0	0.27	3960.1	55822.3	8.1	7.8
	25th perc	3982.5	2832.5	0.86	7562.2	68674.5	10.8	12.3
	75th perc	11140.0	5877.5	2.69	27055.3	95805.3	19.9	1662.9
	95th perc	20127.5	6990.0	8.00	27853.5	141209.6	28.0	7788.7
	max	22560.0	7280.0	9.60	27917.9	155537.3	30.2	9773.5
	min	1070.0	1430.0	0.10	3430.7	52205.5	7.2	7.0
	t -test p value	n.s	n.s	n.s	n.s.	0.009	n.s	n.s.

the exposed and control groups. The list of miRNA differentially expressed in exposed and control subjects is reported in Table 7, along with the parameters of the ROC curve for each miRNA. The sensitivity and specificity of each miRNA in discriminating exposed and control subjects are always high.

The expression matrix calculated accordingly to Eq. (1), relative to the selected 56 miRNAs is reported in Fig. 1, clearly showing that, despite the small number of controls, the selected DE miRNAs are able to effectively discriminate exposed from control subjects.

This result is in agreement with the exposure biomarker profile as seen in Fig. 2, where the PCA analysis carried out on the exposure biomarkers variables is shown.

The results of multivariate ANOVA analysis testing the association between the urinary solvent metabolites, MHIPP, PGA, MA, SBMA, and a set of 10 specific miRNAs chosen among the miRNA that differed most between exposed and controls is shown in Table 8. The statistical association between this subset of miRNAs and the VOCs' metabolites themselves was significant in the case of the MHIPP ($p = 0.009$), of the SBMA ($p = 0.04$) and the SPMA ($p = 0.014$).

The same analysis was applied to the case of the urinary concentration of the unmetabolized VOCs. In this case, a set of seven miRNA strongly correlated to xylenes was selected. A significant association between the selected miRNA set and the unmetabolized VOCs was found in the case of the xylenes ($p = 0.003$) and of toluene ($p = 0.023$). When the isomers of xylene were separately considered, the association was significant for the p-xylene isomer. The set of miRNAs with the highest correlation with solvent metabolites and the set of miRNAs with the highest correlation with the urinary concentration of solvents partially overlap. The MANOVA test was also applied to the effect biomarkers. This test gave a significant result for the association between the 8-oxodGuo concentration ($p = 0.025$), coming from the DNA repair and turnover, and the set of miRNA associated to the xylenes urinary concentration, as it can be seen in Table 8. The MANOVA test did not give a significant association between the selected miRNA and cotinine. This result corroborates our hypothesis that the association between the selected group of miRNAs and the VOCs' metabolites was not affected by the smoking habit of some subjects of the exposed workers group.

4. Discussion

In this study, environmental and biological monitoring were carried out in a group of workers exposed to solvents. The workers' results were compared to those of a small control group of non-exposed and non-smoker subjects. The aim of the work was to study the effect of VOCs' exposure on the human metabolism and to find out relevant miRNAs as

Table 6
Concentrations at the beginning and at the end of the work- shift of the DNA and RNA oxidation products, 8-oxoGuo, 8-oxodGuo and 8-oxoGua, $3NO_2$tyrosine and 5-methylcytidine in the roller- and spray-painters. The result of the *t*-test, relative to the comparison between roller and spray painters, is also reported.

Work-shift beginning		8-oxoGua	8-oxoGuo µg/g Creatinine	8-oxodGuo	$3NO_2$tyrosine	5-methylcytidine
roller	Mean	7.70	8.85	2.80	3.55	4.40
	Median	6.31	7.00	2.78	1.11	3.27
	SD	7.52	5.00	0.90	6.79	3.70
	5th perc	0.22	5.30	1.61	0.52	1.52
	25th perc	2.31	5.57	2.25	0.80	1.85
	75th perc	9.27	8.82	3.09	2.53	5.15
	95th perc	21.29	18.59	4.29	14.00	10.55
	max	21.99	18.69	4.42	23.68	14.20
	min	0.03	5.11	1.52	0.47	1.37
Work-shift end						
roller	Mean	9.97	14.51	5.23	3.55	7.92
Work-shift beginning	Median	5.76	14.72	5.59	3.47	6.89
	SD	12.26	4.10	1.57	1.82	3.56
	5th perc	0.01	10.00	3.38	1.46	4.22
	25th perc	0.45	10.91	3.92	2.42	5.17
	75th perc	18.85	17.61	6.24	4.32	10.19
	95th perc	28.92	20.22	7.50	6.13	13.39
	max	29.00	21.77	7.53	7.80	13.53
	min	0.01	9.94	2.99	1.06	3.58
spray	Mean	25.09	12.86	2.97	3.23	7.81
	Median	18.97	9.51	2.74	1.75	5.37
	SD	20.29	9.42	0.95	3.20	7.56
	5th perc	10.77	4.88	2.06	0.70	1.95
	25th perc	12.02	6.10	2.18	0.82	3.05
	75th perc	25.64	17.12	3.76	5.45	8.83
	95th perc	54.89	26.43	4.16	7.63	19.00
	max	64.37	28.83	4.21	8.01	22.10
	min	10.66	4.71	2.05	0.70	1.74
Work-shift end						
spray	Mean	13.47	19.10	6.09	4.05	10.71
	Median	8.09	15.80	5.72	2.75	5.96
	SD	17.01	8.35	2.46	3.44	11.26
	5th perc	0.90	11.37	3.78	1.64	4.75
	25th perc	4.04	13.68	5.20	1.93	5.73
	75th perc	13.35	25.13	5.96	4.35	8.07
	95th perc	38.68	30.27	9.53	9.20	27.31
	max	46.63	31.16	10.71	10.65	33.51
	min	0.22	10.62	3.35	1.62	4.42
	t -test p value	n.s.	n.s.	n.s.	n.s.	n.s.

biological indicators of the response to VOCs. Since the VOCs airborne concentrations might be higher than expected, they should be measured with accuracy to confirm the compliance with the OELs established by the legislative regulation of each Country.

As the workers wear personal protective equipment (PPE), the VOCs concentration effectively taken and metabolized can be assessed only by means of biological monitoring. In this work, it was assessed that the spray-painters exposure was about one order of magnitude higher than the exposure of roller-painters. In the case of spray-painting, the concentration of xylenes was found comparable to the 50 % of the OEL, and the concentration of toluene amounted to about the 40 % of its own OEL, according to the Italian legislation. This finding confirms that the extent of the inhalation exposure to the compounds present in the paint in the shipyard factory is strongly dependent on the job task. The biological monitoring was performed by measuring both the VOCs and the VOCs' metabolites urinary concentrations at the beginning and the end of the work-shift. The end-shift concentrations were found significantly increased due to the daily working activity.

All the urine metabolite concentrations analyzed here were found below the ACGIH BEIs (Biological Exposure Index), indicating the conditions of the environmental exposure found in this shipyard factory do not represent a strong danger for the personnel from the point of view of safety standards. Similarly, the significant rise in DNA and RNA oxidation, detected between the start and end of the work-shift, demonstrate the oxidative stress is caused by VOCs exposure. However, the human nucleic acid repair and turnover activity is efficient in removing these products, except for the case of defective human metabolism [30]. The miRNA analysis performed on all the subjects allowed us to identify 56 miRNA differently expressed in controls with respect to exposed subjects ($p < 0.01$). A pathway analysis was performed by means of the KEGG, and the miRNA-1, significantly downregulated in exposed workers with respect to the controls, was found to be downregulated also in the oncogenesis process of lung cancer. The downregulated miRNA and their target genes in lung cancer are shown at https://www.genome.jp/kegg-bin/show_pathway?hsa05206. Four target genes are known to be associated to the downregulation of miRNA-1 in lung cancer tumorigenesis, HDAC4, FoxP1, Pim-1, c-Met [31]. A correlation analysis between miRNA and exposure biomarkers allowed the individuation of two subsets of 10 and 7 significant miRNAs, partially overlapping. The first set was significantly associated to the VOCs' metabolites and the other one was significantly associated to the unmetabolized VOCs urine concentration. The non-significant association between the selected miRNAs and cotinine, the smoking specific biomarker, corroborates the hypothesis that the selected miRNAs are not significantly affected by the smoking habit.

Based upon these results, four miRNAs out of ten, miR-589-5p and miR-941, miR-146b-3p and miR-27a-3p are particularly worth noting, since they are implicated in oxidative stress and inflammatory processes.

In a previous pilot study [23] by the authors of the present paper,

Table 7
List of miRNA differentially expressed (p-value ≤ 0.01) in exposed and control subjects. The area under the ROC curve and the sensitivity and specificity of each miRNA in discriminating exposed from control subjects is also reported.

Na Name	Regulation	Log2 fold change	p -value	ROC curve: area under the curve	Sensitivity; Specificity
hsa-miR-1	downregulated	−8.34	9.52E-28	0.98	94.1%; 100 %
hsa-miR-483 − 3p	downregulated	−4.77	5.78E-10	1	100 %; 100 %
hsa-miR-1249	downregulated	−4.33	8.77E-09	1	100 %; 100 %
hsa-miR-4646 − 3p	downregulated	−4.38	2.52E-08	1	100 %; 100 %
hsa-miR-215 − 5p	downregulated	−2.63	9.43E-08	1	100 %; 100 %
hsa-miR-885 − 3p	downregulated	−3.72	1.09E-06	0.86	100 %; 86.7 %
hsa-miR-34a-5p	downregulated	−1.99	5.63E-06	1	100 %; 100 %
hsa-miR-497 − 5p	downregulated	−2.64	7.56E-06	1	100 %; 100 %
hsa-miR-122 − 5p	downregulated	−3.14	1.16E-05	0.96	88.2%; 100 %
hsa-miR-195 − 5p	downregulated	−1.82	3.19E-05	1	100 %; 100 %
hsa-miR-206	downregulated	−3.01	3.28E-05	1	100 %; 100 %
hsa-miR-3613 − 5p	downregulated	−1.92	5.04E-05	0.98	94.1%;100 %
hsa-miR-203a	downregulated	−2.87	7.36E-05	0.98	94.1%;100 %
hsa-miR-375	downregulated	−2.72	8.34E-05	1	100 %; 100 %
hsa-miR-141 − 3p	downregulated	−2.04	1.08E-04	1	100 %; 100 %
hsa-miR-92b-3p	downregulated	−1.96	1.39E-04	1	100 %; 100 %
hsa-miR-218 − 5p	downregulated	−2.64	2.01E-04	0.80	100 % ; 66.7 %
hsa-miR-200a-3p	downregulated	−1.95	2.76E-04	1	100 %; 100 %
hsa-miR-486 − 3p	downregulated	−1.71	6.46E-04	1	100 %; 100 %
hsa-miR-6803 − 3p	downregulated	−2.41	8.72E-04	1	100 %; 100 %
hsa-miR-429	downregulated	−2.06	1.69E-03	1	100 %; 100 %
hsa-miR-486 − 5p	downregulated	−1.39	2.24E-03	0.98	94.1%; 100 %
hsa-miR-483 − 5p	downregulated	−2.20	2.88E-03	1	100 %; 100 %
hsa-miR-4732 − 3p	downregulated	−1.53	3.83E-03	0.98	94.1%; 100 %
hsa-miR-30a-5p	downregulated	−1.34	4.16E-03	0.98	94.1%; 100 %
hsa-miR-125b-5p	downregulated	−1.49	5.41E-03	0.94	82.4%; 100 %
hsa-miR-92a-3p	downregulated	−1.24	5.79E-03	0.98	94.1%; 100 %
hsa-miR-92b-5p	downregulated	−1.41	6.41E-03	1	100 %; 100 %
hsa-miR-1908 − 5p	downregulated	−1.78	7.62E-03	1	100 %; 100 %
hsa-miR-193b-5p	downregulated	−1.81	8.91E-03	0.94	88.2%; 100 %
hsa-miR-4772 − 3p	upregulated	10.76	4.64E-06	1	100 %; 100 %
hsa-miR-551a	upregulated	10.08	6.72E-05	1	100 %; 100 %
hsa-miR-425 − 3p	upregulated	2.36	5.13E-04	1	100 %; 100 %
hsa-miR-1304 − 3p	upregulated	2.94	5.31E-04	0.96	94.1%; 100 %
hsa-miR-345 − 5p	upregulated	2.72	7.68E-04	1	100 %; 100 %
hsa-miR-223 − 3p	upregulated	3.68	8.28E-04	0.92	88.2%; 100 %
hsa-miR-199b-5p	upregulated	3.45	9.99E-04	1	100 %; 100 %
hsa-miR-30c-5p	upregulated	1.82	1.54E-03	1	100 %; 100 %
hsa-miR-181a-3p	upregulated	2.34	1.70E-03	0.98	94.1%; 100 %
hsa-miR-2355 − 5p	upregulated	7.77	3.18E-03	0.97	94.1%; 100 %
hsa-miR-145 − 5p	upregulated	3.09	3.22E-03	0.92	88.2%; 100 %
hsa-miR-26b-3p	upregulated	2.25	3.32E-03	0.96	94.1%; 100 %
hsa-miR-425 − 5p	upregulated	1.50	4.09E-03	1	100 %; 100 %
hsa-miR-146b-3p	upregulated	2.07	4.13E-03	0.96	94.1%; 100 %
hsa-miR-454 − 5p	upregulated	1.87	4.13E-03	0.94	94.1%; 100 %
hsa-miR-181a-2 − 3p	upregulated	1.57	5.06E-03	1	100 %; 100 %
hsa-miR-361 − 3p	upregulated	1.93	5.59E-03	0.96	88.2%; 100 %
hsa-miR-873 − 3p	upregulated	7.74	6.09E-03	1	100 %; 100 %
hsa-miR-2115 − 5p	upregulated	7.72	6.30E-03	0.97	94.1%; 100 %
hsa-miR-27a-3p	upregulated	2.04	6.60E-03	1	100 %; 100 %
hsa-miR-339 − 5p	upregulated	1.80	6.71E-03	0.90	82.4%; 100 %
hsa-miR-589 − 5p	upregulated	2.23	7.08E-03	0.98	94.1%; 100 %
hsa-miR-23a-5p	upregulated	3.57	8.15E-03	0.94	88.2%; 100 %
hsa-miR-941	upregulated	2.12	8.41E-03	0.94	88.2%; 100 %
hsa-miR-23a-3p	upregulated	1.62	9.63E-03	0.94	94.1%; 100 %
hsa-miR-93 − 3p	upregulated	1.46	1.02E-02	1	100 %; 100 %

only two DE miRNA were identified in a small sample of workers exposed to VOCs in a naval industry compared to a control group. The correlation analysis between the VOCs' metabolites measured at the end of the work shift and the miRNAs, selected specific miRNAs, strongly correlated to specific metabolites. However, it is noteworthy the miRNAs found in the first paper [23] did not match those identified in the present work. This is not surprising as the exposure to the different chemicals were quite different in the two experimental campaigns, as regards both the absolute and the relative concentrations of the different VOCs. In particular, the concentration of MHIPP was about one order of magnitude higher in this study than in the previous work [23] and it is not unexpected that the expression profiles could be a function of the dose range. Another explanation for this difference is the different ethnicity, being the workers of the present paper prevalently bengalese whilst the workers of the previous work were all caucasian. Ethnicity is known to affect the gene expression profiles, and miRNAs significantly differ among ethnic groups [32–35]. Ethnicity can play a role in the response to environmental and occupational exposure to VOCs, affecting the susceptibility to the potentially adverse effect of the different chemicals. Indeed, the presence of different ethnicities among worker populations results in a plurality of detoxification pathways of dangerous substances, with variable effects on workers' health [30].

A recent study [36]. showed the first two miRNAs (miR-589-5p and miR-941) were regulated by exposure to substances (including organic

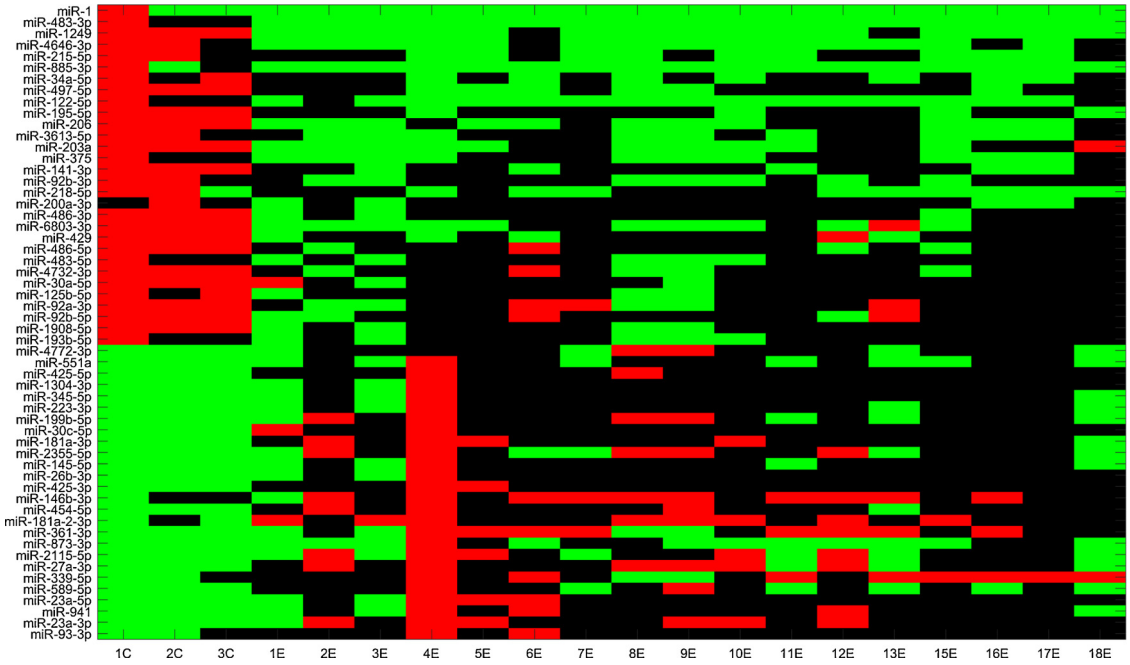

Fig. 1. Expression matrix for the selected miRNA. The miRNAs are grouped as down- and upregulated in exposed with respect to control subjects.

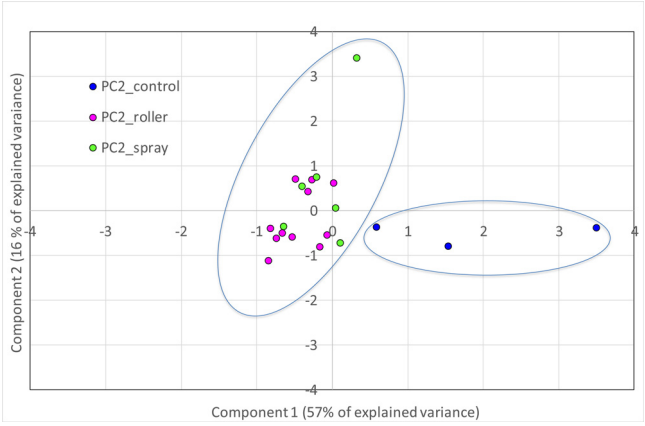

Fig. 2. Principal component analysis (PCA) performed on the miRNA of all samples of the dataset.

solvents) present in the electronic cigarette smoke, inducing oxidative stress in human bronchial epithelial cells. Of particular interest is miR-589-5p, as it was found to bind the RNA promoter and to activate the transcription of Cyclo-oxygenase 2 (COX-2), an inducible protein regulating inflammation in normal physiology and disease [37]. The cited in vitro study showed that treatment with the anti-miR complementary to miR-589-5p resulted in reduced basal expression of COX-2.

Also, miR-146b-3p was recognized to impact on activation of host defense pathways, which are linked to the control of immunity and inflammation [38]. Other papers showed that miR-27a-3p upregulation may directly downregulate expression of the transcription factor Nuclear factor erythroid 2-related factor 2 (Nrf2), involved in many physiological and cellular processes (antioxidant system defense, immune response), as shown in SH-SY5Y neuroblastoma cells [39–42]. The level of MiR-27a expression may negatively affect some genes implicated in the oxidative stress response as in the case of PINK1, a mitochondrial serine/threonine-protein kinase, resulting in an increase of reactive oxygen species (ROS) [42]. Lastly, different studies showed also a link between dysregulation of miR-27a-3p and inflammatory response, resulting in a variation of proinflammatory cytokines (TNF-α and IL-6) as

Table 8
MANOVA test results. The Wilks test measures the statistical significance of the association between the selected miRNAs and the urinary biomarkers of dose and effect.

miRNA	Urinary biomarker	Wilks test
(group of miRNA highly correlated to MHIPP)	MHIPP (μg/g Creatinine)	0.009 **
hsa-miR-4772-3p	SBMA (μg/g Creatinine)	0.04
hsa-miR-425-3p	SPMA (μg/g Creatinine)	0.014 *
hsa-miR-223-3p	Cotinine (μg/g Creatinine)	n.s
hsa-miR-199b-5p		
hsa-miR-2355-5p		
hsa-miR-146b-3p		
hsa-miR-454-5p		
hsa-miR-27a-3p		
hsa-miR-589-5p		
hsa-miR-941		
(group of miRNA highly correlated to Xylenes)	Total Xylenes (ng/mL)	0.0003 ***
hsa-miR-4772-3p	p-xylene(ng/mL)	0.0003 ***
hsa-miR-141-3p	Toluene (ng/mL)	0.0226 *
hsa-miR-92b-3p	8-oxodGuo (μg/g Creatinine)	0.0254 *
hsa-miR-199b-5p		
hsa-miR-2355-5p		
hsa-miR-146b-3p		
hsa-miR-27a-3p		

demonstrated in mice with sepsis [43,44]. Further studies involving a larger sample size should investigate the possible correlation between the miRNAs associated to the VOC metabolite concentrations and the 3-nitrotyrosine used as a biomarker of nitro-oxidation of proteins.

To summarize the findings of the present paper, the up-regulation of the four miRNAs discussed here might be of significant biological interest in the human response to VOCs exposure and it should be further investigated on a larger number of controls and workers involved in the same job task.

5. Conclusions

This study was aimed at the individuation of novel early biomarkers of effect, quantitatively related to the exposure doses to VOCs in an

occupational setting. The reduction of exposure risk to toxicants, most of which are also carcinogenic, is ensured not only by the use of personal protective equipment (PPE) but also by scheduled sanitary surveillance and biomonitoring campaigns finalized to the prevention of potential diseases occurring as consequence of prolonged exposures over time. VOCs are commonly used in several jobs and reducing their concentration indoors and outdoors is an important health occupational and environmental goal. Some common inflammation, lung and blood disorders are potentially associated with VOCs exposure [45] and several new and old epidemiological studies indicate exposure to toluene, xylene and benzene represent a risk factor for haematological malignancies [46,47]. Biomarkers of dose and effect are therefore essential for the risk evaluation associated to the VOC exposure. This study shows that also if the Occupational Exposure limits for different VOCs are not exceeded, thanks to the personal protective equipment, the chronic long-term exposure to mild VOCs doses in a sample of painters causes an alteration in the miRNAs expression profile. In particular, 56 miRNAs were identified as significantly differentially expressed in the workers exposed to a mixture of VOCs, with respect to a group of control subjects. A pathway analysis showed that the miRNA-1, significantly downregulated in exposed workers with respect to the controls in this study, is involved in the lung cancer tumorgenesis. This finding is worth further investigation, as it reinforces the hypothesis that the miRNAs could be used as very early and predictive biomarkers of effect also in case of a low dose scenario. In this scenario of mild doses of exposure to VOCs, a subset of miRNAs, among the differentially expressed ones, were found significantly correlated to the dominant VOCs of the mixture and to their specific metabolites. This finding shows that it is possible to assess quantitative relations between dose biomarkers and specific miRNAs, as early effect biomarkers, in order to monitor the health status of workers and prevent the development of exposure-related diseases.

A statistically significant association was found also between the selected miRNAs and the 8-oxodGuo, a biomarker of oxidative damage to the DNA, coming from DNA turnover and repair. In the group of the differentially expressed miRNAs highly correlated to the dose biomarkers, a subset of four miRNAs, hsa-miR-589-5p, hsa-miR-941, miR-146b-3p and miR-27a-3p were individuated, with well-known implications in oxidative stress and inflammation processes. The miRNAs identified in our study might therefore serve as promising biomarkers to predict the health risk caused by mild exposure to the occupational VOCs. The findings of this work seem to be an encouraging strategy for the controlling the risk coming from chronic exposure to VOCs. The proposed method can be particularly useful when complex exposure scenarios, in which different risk agents act simultaneously at low doses but with possible synergistic interactions. The miRNA expression profiling can be an early and predictive tool for the identification of exposure-related disease particularly when multiple stressors are combined.

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.

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