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Circulating microRNAs as potential biomarkers of occupational exposure to low dose organic solvents

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ABSTRACT

Circulating microRNAs (miRNAs) have been recently acknowledged as novel and non-invasive biomarkers of exposure to environmental and occupational hazardous substances. This preliminary study investigates the potential role of blood miRNAs as molecular biomarkers of exposure to the most common organic solvents (ethylbenzene, toluene, xylene) used in the shipyard painting activity. Despite the low number of recruited workers, a two-tail standard Students' test with Holm-Bonferroni adjusted p-value shows a significant up-regulation of two miRNAs (miR_6819_5p and miR_6778_5p) in exposed workers with respect to controls. A correlation analysis between miRNA, differentially expressed in exposed workers and in controls and urinary dose biomarkers i.e. methylhyppuric acid (xylenes metabolite), phenylglyoxylic and mandelic acid (ethylbenzene metabolites) S-benzyl mercapturic acid (toluene metabolite) and S-phenylmercapturic acid (benzene metabolite) measured at the end of the work-shift, allowed the identification of high correlation (0.80-0.99) of specific miRNAs with their respective urinary metabolites. MiRNA_671_5p correlated with methylhippuric, S-phenylmercapturic and S-benzyl mercapturic acid while the miRNA best correlating with the phenylglioxylic acid was miRNA_937_5p. These findings suggest miRNA as sensitive biomarkers of low dose exposure to organic chemicals used at workplace. Urinary DNA and RNA repair biomarkers coming from the oxidation product of guanine have been also associated to the different miRNAs. A significant negative association was found between 8-oxo-7,8-dihydroguanine (8-oxoGua) urinary concentration and miR_6778_5p. The findings of the present pilot study deserve to be tested on a larger population with the perspective of designing a miRNA based test of low dose exposure to organic solvents.

1. Introduction

MicroRNAs (miRNAs) are short-length, non-coding single strand RNA molecules acting as epigenetic factors on gene regulation. Similarly to other genes, miRNAs are highly conserved among different species [1]. Their complementary sequence targets mRNA to prevent protein production by two different mechanisms involving either mRNA degradation or inhibition of translation [2]. In the last ten years, aberrant expression of miRNAs has been intensively studied in the pathogenesis of diseases, including cancer, recognizing these small molecules as a new source of prognostic and diagnostic biomarkers with large applicability to the clinical medicine [3,4]. Nowadays, increasing evidence shows that alterations in miRNA expression may occur in response to environmental agents and pollutants, making miRNAs essential and specific also in the health risk assessment. Considering acute or chronic environmental exposure, clusters of miRNA differentially expressed in exposed and unexposed subjects represent a molecular signature associable to such exposures [5]. Also in the occupational setting, where strictly controlled exposure to toxicants or carcinogens may occur, miRNAs may be regarded as novel, alternative or complementary biomarkers to the most widely used in the biological monitoring (e.g. biomarkers of dose, of early effect and of susceptibility) [6]. Their stability and presence in body fluids as circulating molecules make miRNAs easy to isolate. They are ubiquitous and have been found in urine, blood plasma/serum, saliva, seminal fluid, pleural, peritoneal fluids, bronchial lavage, amniotic liquid, breast milk [7,8].

Very little is known about the role of miRNAs in the occupational setting and only few studies have been published so far. In a study

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conducted on farmworkers exposed to organophosphate pesticides significant differences in miRNA profiles were identified between workers and controls as well as between different seasons. Five miRNAs showed a positive dose-response relationship with organophosphate metabolites in the exposed workers [9]. In another study, plasma miRNA expression was investigated in ninety-one subjects occupationally exposed to high levels of benzene (12.1 mg/m^3 i.e. 3.7 ppm). 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 with high expression of miR-638 and decreased expression of miR-22 1-3 p and miR-122-5p in human plasma [10]. A further study reports on the effects of Trichloroethylene (TCE) exposure on workers employed in the lock industries. The authors found that TCE affected the pulmonary function and induced a significant decrease in the dehydroisoandrosterone sulphate (DHEAS) level of the exposed workers in comparison to the controls, indicating its potential effect for endocrine disruption. The effect of TCE on Let-7c miRNA expression was investigated in the plasma of exposed workers showing no changes following exposure. Further studies will be essential to clarify the mechanism of TCE toxicity [11].

Aberrant miRNA profiles were detected also in workers undergoing chronic benzene poisoning. Six miRNAs (miR-34a, miR-205, miR-10b, let-7d, miR-185 and miR-423-5p-2) were up-regulated and seven (miR-133a, miR-543, hsa-miR-130a, miR-27b, miR-223, miR-142-5p and miR-320b) were down-regulated when groups were compared to healthy controls ($p \leq 0.05$) [12]. Another recent study conducted on firefighters exposed to several toxicants and carcinogens (benzene, polycyclic aromatic hydrocarbons, formaldehyde, arsenic, 1–3 butadiene, cadmium, chromium, asbestos, flame retardants and particulates) reports nine miRNAs with at least a 1.5-fold significant difference between fifty-two incumbent firefighters and forty-five newly employed nonsmoking firefighters [13]. These few publications highlight miRNAs role as biomarkers of exposure or of early effect and their applicability to the biomonitoring at workplace [7,8].

In this work we present a preliminary study where a small group of workers exposed to organic solvents (ethylbenzene, toluene, xylene etc.) used in the shipyard painting activity and a small group of control subjects were bio-monitored for the respective metabolites, excreted in the urine. Urinary DNA and RNA oxidized nucleotides and nucleosides used as biomarkers of oxidative damage were also determined. In particular we measured 1) 8-Oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), the oxidized form of the nucleoside guanosine, formed by guanine attached to a deoxyribose from the 2′-deoxyribonucleotide pool [14]; 2) 8-Oxo-7,8-dihydroguanosine (8-oxoGuo), the oxidized form of the nucleoside guanosine formed by guanine bound to a RNA ribose; 3) 8-Oxo-7,8-dihydroguanine (8-oxoGua), the oxidized form of the guanine, coming predominantly from DNA [15–17]. Neither cell death nor diet contribute considerably to urinary 8-oxodGuo whose levels are not influenced by long-term storage of urine specimens at −20 °C [15]. The concentration of cotinine, urinary metabolite of nicotine, was also measured in these samples in order to classify the subjects as smokers or non-smokers. Smoke is a well known confounding factor for benzene exposure as it contains several carcinogenic products that can stimulate DNA repair activity [18,19]. Furthermore we analysed the circulating miRNome in exposed and control subjects in order to find possible associations between VOCs and urinary dose biomarkers and between miRNAs and dose/exposure biomarkers. Up-regulation of few miRNAs significantly associated to dose biomarkers in exposed workers with respect to controls was investigated in order to evaluate the role of miRNAs as potential biomarkers of exposure to VOCs.

Table 1
Subjects' characteristics.

	task	age	sex	smoke
N1	supervision	51	male	no
N2	spray painting	28	male	no
N3	spray painting	37	male	yes
N4	spray painting	34	male	yes
C1	control	33	male	no
C2	control	51	female	no
C3	control	50	female	no
C4	control	48	male	no

2. Materials and methods

2.1. Subjects and study design

For this study four high professional painters in a naval industry in the center of Italy were enrolled. All individuals were eligible and agreed to the methodological study after giving their informed consent. The data of this study were recorded as anonymous and can only be interpreted on a population level. All procedures performed in this study involving human participants (Declaration of Helsinki) were in accordance with the ethical standards of our Institutional committee and in accordance with the local ethics committee (ASL North West, Tuscany).

The exposed subjects were all males, identified as Ni with i from 1 to 4. They were compared to four unexposed control subjects indicated as Ci with the index running from 1 to 4. The characteristics of the subjects are shown in Table 1.

A questionnaire was administered to the workers and control subjects to be enrolled in order to collect information on their life habits, cigarette smoking and use of drugs and to gather information on possible source of exposure to solvents outside the workplace. Questions were asked about the materials handled and the protection equipment used in the workplace. The exposure to solvents was assessed by personal air sampling and urine sampling performed at the end of the work-shift. The workers were monitored in two different experimental campaigns in July, July 1st and July 8th 2017. Blood samples for miRNA isolation were taken during the routine medical surveillance.

2.2. miRNA extraction

The extraction and reading of the miRNA matrix was performed by the Microgem Laboratory Research (Naples, Italy). The methodology described by Microgem is summarized in the following. Total RNA has been isolated from human blood plasma (200 µl) with miRNeasy Serum/Plasma kit (Qiagen). One µl of each sample has been analyzed by Bioanalyzer using small RNA chip. An aliquot of each RNA sample and the respective Spike-in controls have been dephosphorylated and labelled with Cy3-pCp in the presence of ligase. Samples have been purified and hybridized on Human miRNA microarray 8 x 60 K (miRBase database, Release 21.0) consisting of 2549 miRNA. The hybridization was performed at 55 °C for 20 h. After washing microarrays have been analyzed by laser scanner. Image analysis was performed by using the Feature Extraction 12.0 software (Agilent).

2.3. miRNA bio-informatic analysis

Data were normalized by means of quantile, probes filtering on the basis of expression values and on the basis of flag. A fold change analysis was performed with a cut-off of 1.5. The probes exceeding this fold change value were statistically analyzed. Once the expression values have been normalized and filtered, the downstream statistical analysis was performed by the authors of the paper.

2.4. Personal air monitoring

In order to avoid any interference during spray painting inside a tunnel under very uncomfortable working conditions in terms of postural and climatic conditions, it was decided to monitor personal exposure to organic solvents by passive air sampling. The Radiello devices performed personal air monitoring of the gaseous phase of the target VOCs ethylacetate, benzene, toluene, ethylbenzene, p-xylene, m-xylene, o-xylene and styrene: the sampling covered the whole work-shift. The Radiello was chemically extracted with carbon disulfide and the samples were analyzed by GC–MS with internal standard method.

2.5. Biological monitoring

The concentrations of ethylacetate, benzene, toluene, ethylbenzene, p-xylene, m-xylene, o-xylene, styrene were measured in different biological matrices: saliva and urine. The concentration of each VOC was correlated to its most common (specific) urinary metabolite. Not-metabolized (unmetabolized) VOCs in saliva were determined by GC–MS with the headspace analysis method [20] extended to urine analysis.

All the metabolites have been determined by HPLC-MS/MS with a Turbo Ion Spray (TIS) in the urine samples of workers, both before the start and at the end of the working shift, during two different sampling campaigns. The precursor→product ionic transitions monitored are in the negative ion mode for SPMA, MA, PGA, SBMA and MHIPP, while in the positive ion, MRM mode, (precursor→product) for the 8-oxoGua, 8-oxodGuo, for 8-oxoGuo, for cotinine and for their internal standards.

According to different analytical methods reported in Table 2 all values were divided by the concentration of urinary creatinine, in order to normalize 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 [21]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 [22].

2.6. Chemicals and supplies

Radiello passive air samplers were supplied by Supelco. All reagents were of high purity analytical grade.

All analytical reference standards were purchased from Chem-Service (Steinheim, Germany), except for styrene, which was purchased from Riedel-de Haën (Buchs, Switzerland), and for deuterium labelled styrene (d8) from Isotec, Inc. (Miamisburg, OH, USA).

Carbon disulfide was purchased from Sigma-Aldrich (Steinheim, Germany). A Milli-Q water purification system (Milli-pore, Bedford, MA, USA) was used to supply high purity de-ionized water. A polyethylene glycol capillary column DB-WAXetr 123–7334 (30 m × 0.32 mm i.d., 1.00 µm film thickness; J&W California, USA) was used for the chromatographic separation. Pure Helium (purity level 99.99%) was used as GC carrier gas (Air Liquid, Milan, Italy).

The analytical reference standards of 2 amino-6,8-dihydroxypurine hydrochloride (8oxoGua), 8-oxo-7,8 dihydro-2′deoxyguanosine (8-oxodGuo), 8oxo-7,8 dihydroguanosine (8-oxoGuo) and DL S-phenyl mercapturic acid (DL-SPMA) were purchased by Spectra 2000s.r.l (Rome, Italy). The analytical reference standards of DL mandelic acid, phenylglyoxylic acid and 2,3,4 methylhyppuric acid were purchased from Fluka (Sigma-Aldrich Milan, Italy) and cotinine (> 99.5%), benzylmercapturic acid (SBMA) 99% were supplied by Sigma-Aldrich (Milan, Italy).

The deuterium labeled internal standards 2-amino-6.8-dihydroxypurine-13C-hydrocloride, [^{13}C $^{15}N_2$] 8-oxodGuo and [^{13}C $^{15}N_2$] 8-oxoGuo, DL-SPMA-3,3-d2, ± mandelic-d5 acid (99.4%), sodium phenyl-d5- glyoxylate (99.8%) and cotinine-d3 (99%), were obtained from CDN Isotopes Inc (Pointe-Claire, Quebec, Canada). 6 N Hydrochloric acid, glacial acetic acid, 30% NH3 and ammonium acetate, dimethyl sulfoxide, sodium hydroxide solution (50–52% in water) and CHROMASOLV® gradient grade (≥99.9%) methanol and acetonitrile for LC/MS were obtained from Sigma Aldrich (Saint Louis, MO, USA). Ammonium acetate (98%; Merck, Darmstadt, Germany) buffer was in water, purified water was obtained from a Milli-Q Plus system (Millipore, Milford,MA, USA). The SPE cartridges, Sep-Pak Plus C18 (10 mL, 500 mg) were supplied by Waters. Anotop 10LC syringe filter device (0.2-µm pore size, 10-mm diameter) was purchased from Whatman Inc. (Maidstone, England). A Sinergi Fusion C18 column (150 × 4.6 mm, 4 µm) supplied by Phenomenex (USA) and Synergi 4U Polar RP C18 column (150 × 4.6 mm, 4 µm) supplied by Phenomenex (USA) were used in the study. Urinary creatinine has been determined by the method of Jaffè, using alkaline picrate test with UV/VIS detection at 490 nm.

2.7. 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 solvents metabolites (MA, PGA, MHIPP, SPMA, SBMA) were measured as continuous variables. Normality of the distributions was assessed according to the Curtosi and Kolmogorov-Smirnov tests. Spearman's ρ was used to assess the correlation between all the variables measured.

A two-tail standard Students' test was used to select miRNAs that are statistically different in the sample of exposed and control subjects. As the number of variables is large compared to the sample size, in order to control the multiplicity, the Holm Bonferroni criterion was used to define an adjusted p value.

The miRNAs were ordered according to an increasing p value and 35 miRNAs were identified. The 35 selected miRNAs were also ordered on the basis of the average up or down regulation of the miRNA of the exposed versus the control sample. The up-regulated group is followed by the down regulated one. The expression matrix M(i,j) (where i from 1 to 35 is the miRNA index and j from 1 to 8 is the subject index in which j = 1:4 for the controls and j = 5:8 for the exposed) was calculated as the difference between the intensity value for each subject for each miRNA and the average miRNA calculated on all the subjects (8

Table 2
Analytical methods for urinary metabolites.

VOC	Biomarker	Method	CV	LOD	Reference
Ethylbenzene	Phenylglyoxylic acid (PGA)	HPLC-MS/MS	11%	0.015 mg/l	Paci et al., 2013 [23]
Ethylbenzene	Mandelic acid (MA)	HPLC-MS/MS	11%	0.02 mg/l	
Xylenes	Methylhyppuric acid (MHIPP)	HPLC-MS/MS	15%	1 µg/mL	This paper
Toluene	S-benzyl mercapturic acid (SBMA)	HPLC-MS/MS	15%	0.35 µg/L	Sabatini et al., 2008 [24]
Benzene	S-phenylmercapturic acid (SPMA)	HPLC-MS/MS	10%	0.026 µg/L	SPMA and cotinine were determined according to
Nicotine (active smoking)	cotinine	HPLC-MS/MS	10%	12.41 µg/L	Tranfo et al., 2017 [25]
DNA repair activity	8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo)	HPLC-MS/MS	20%	1.69 nmol/L	Andreoli et al., 2010 [26]
RNA repair activity	8-oxo-7,8-dihydroguanosine (8-oxoGuo)	HPLC-MS/MS	20%	2.34 nmol/L	
DNA and RNA repair activity	8-oxo-7,8-dihydroguanine (8-oxoGua)	HPLC-MS/MS	20%	2.99 nmol/L	

Table 3
Urinary metabolites measured in the two experimental campaigns at the beginning and the end of the work-shift.

A Campaign	Metabolites expressed in μg /g Creatinine						
	Before shift						
Subject code	MA	PGA	MA + PGA	SBMA	MHIPP	SPMA	Cotinine
N1	5299.65	2143.46	7443.10	< LOD	< LOD	0.18	< LOD
N2	3199.99	291.02	3491.01	< LOD	< LOD	0.18	< LOD
N3	2856.87	2229.66	5086.52	< LOD	< LOD	0.41	< LOD
N4	1356.23	708.25	2064.48	0.00	1335.13	0.34	2616.01
	End shift						
N1	4609.75	2731.45	7341.19	< LOD	< LOD	< LOD	< LOD
N2	3901.52	2564.39	6465.91	0.197	897.73	0.13	< LOD
N3	1419.88	4326.67	5746.55	< LOD	2078	0.63	588.67
N4	3339.34	2839.78	6179.12	< LOD	1706.6	0.77	1914.42
Paired t-test	**ns**	**0.01**	**0.07**	**ns**	**0.08**	**ns**	**ns**
B Campaign	Metabolites expressed in μg /g Creatinine						
	Before shift						
Subject code	MA	PGA	MA + PGA	SBMA	MHIPP	SPMA	Cotinine
N1	1239.16	706.58	1945.74	< LOD	< LOD	< LOD	< LOD
N2	754.63	2421.58	3176.21	3.28	< LOD	< LOD	< LOD
N3	706.70	2406.92	3113.62	6.55	32.45	0.27	386.22
N4	1746.60	2205.08	3951.68	3.19	4768.84	0.72	1849.52
	End shift						
N1	940.59	816.53	1757.12	< LOD	219.86	0.18	< LOD
N2	1022.30	1659.64	2681.94	3.34	3722.39	0.12	< LOD
N3	312.84	2576.83	2889.66	5.40	8559.29	0.46	468.05
N4	1558.08	1550.51	3108.59	7.90	5252.53	1.31	1363.64
Paired t-test	**ns**	**ns**	**0.03**	**ns**	**0.096**	**0.044**	**ns**

Table 4
Unmetabolized VOCs in saliva and in urine.

Subjects	ethylacetate	benzene	toluene	ethylbenzene	p-xylene	m-xylene	o-xylene	ΣXylenes	styrene
Saliva beginning of work-shift (ng/ml)									
N1	34.42	0.85	1.19	0.18	0.26	0.47	0.34	1.07	1.29
N2	83.37	0.78	1.39	0.26	0.30	0.57	0.38	1.24	1.26
N3	25.52	2.76	3.69	0.46	0.41	1.03	0.47	1.90	2.08
N4	18.66	1.62	4.96	0.90	0.75	2.03	1.07	3.86	2.73
Saliva end of work-shift (ng/ml)									
N1	27.10	0.84	3.40	0.32	0.34	0.85	0.58	1.77	1.61
N2	83.40	0.83	14.65	0.84	0.80	2.11	0.86	3.78	2.02
N3	108.83	0.76	18.40	0.92	1.05	2.34	0.85	4.23	1.33
N4	71.23	0.81	3.63	0.33	0.37	0.88	0.56	1.80	1.14
Paired t-test saliva	n.s	0.08	n.s	n.s	n.s	n.s	n.s	n.s	n.s
Urine beginning of work-shift (ng/ml)									
N1	0.07	0.16	0.10	0.02	0.03	0.07	0.03	0.14	0.05
N2	0.04	0.14	0.12	0.02	0.03	0.05	0.04	0.12	0.07
N3	0.00	0.16	0.11	0.02	0.03	0.05	0.04	0.12	0.06
N4	0.08	0.28	0.46	0.04	0.05	0.11	0.08	0.24	0.08
Urine end of work-shift (ng/ml)									
N1	0.14	0.14	0.31	0.03	0.04	0.09	0.05	0.18	0.11
N2	0.32	0.16	2.24	0.08	0.09	0.19	0.08	0.37	0.11
N3	0.40	0.17	4.11	0.13	0.12	0.31	0.10	0.53	0.10
N4	0.07	0.24	0.89	0.08	0.10	0.23	0.11	0.44	0.12
Paired t-test urine	0.07	n.s.	0.07	0.04	0.03	0.04	0.01	0.03	0.00
Correlation urine - saliva									
beginning	−0.18	0.18	0.79	0.76	0.86	0.83	1.00	0.93	0.64
end	0.74	−0.22	0.96	0.78	0.68	0.65	0.12	0.60	−0.13

subjects, 4 exposed and 4 controls), divided by the square root of the pooled variance

$$M(i,j) = \frac{miRNA(i,j) - mean(miRNA(i,\,1{:}\,8))}{\sqrt{\mathrm{var}(miRNA(i,\,1{:}\,4)) + \mathrm{var}(miRNA(i,\,5{:}\,8))}} \quad (1)$$

Hierarchical clustering analysis, with similarity measure and complete linkage algorithm, and

principal component analysis, PCA, were also performed.

The correlations between the concentration of each solvent metabolite, MA, PGA, MHIPP, SBMA, SPMA, and between the oxidized guanine derivatives, 8oxoGua, 8oxoGuo, 8oxodGuo and the selected miRNA were measured. Only correlations higher than 0.8 were considered. Univariate linear regression models were studied between each urinary biomarkers and the set of miRNA selected through the high correlation criterion, previously described. The regression coefficients and an adjusted p-value for the linear regression significance were calculated.

3. Results

3.1. Personal environmental monitoring

During the painting work-shift the personal exposure to solvents, due to airborne concentration without considering the personal

Table 5
Correlation between each organic solvent, measured in saliva and in urine at the beginning and the end of the work-shift, and its main urinary metabolite.

VOC-metabolite pair	Work-shift	Correlation	
		Urine matrix	Saliva matrix
Xylenes - Methylhyppuric acid	beginning	0.99	0.96
	end	0.98	0.65
p-xylene - Methylhyppuric acid	beginning	0.96	0.96
	end	0.95	0.73
m-xylene - Methylhyppuric acid	beginning	0.95	0.94
	end	1.00	0.64
o-xylene - Methylhyppuric acid	beginning	0.99	0.99
	end	0.83	0.50
Ethylbenzene - PGA	beginning	0.07	0.46
	end	1.00	0.80
Ethylbenzene - MA	beginning	0.90	0.67
	end	−0.51	−0.71
Ethylbenzene - MA + PGA	beginning	0.60	0.85
	end	0.77	0.36
Benzene - SPMA	beginning	0.96	0.45
	end	0.98	−0.24
Toluene - SBMA	beginning	0.01	0.54
	end	0.3	0.13

protective equipment.67 resulted (mean values): ethylbenzene 17.5 mg/m^3, toluene 208.6 mg/m^3, styrene 0.16 mg/m^3, p-xylene 16.3 mg/m^3, m-xylene 36.5 mg/m^3, o-xylene 17.6 mg/m^3. It is worth stressing that these values refer to gaseous phase of solvents collected during spray painting and do not consider the aerosol components. It is also worth highlighting that styrene was not included in the solvent painting mixture. In the case of toluene air concentration resulted comparable with the Italian Limit Value for Personal Exposure 192 mg/m^3, while for the other solvents the gaseous concentration is well below the relative Italian Limit Values (Ethylbenzene 442 mg/m3; Xylenes - sum of the isomers- 200 mg/m^3).

3.2. Biological monitoring

Two different experimental campaigns were conducted in this study with the same workers; A campaign in the first week of July and B campaign in the second week of July. The urine monitoring results of exposed subjects were obtained in two different campaigns at the beginning and at the end of the work-shift. Table 3 reports the results obtained for the urinary metabolites of the selected VOCs. The *t*-test at the bottom of each campaign shows the difference between start and end of the shift.

Despite the Entry 5 of Annex XVII to the REACH Regulation 1907/2006 (03/10/2017) [27] prohibits the presence of benzene in mixtures at or above a concentration of 0.1% by weight (w/w), we found the presence of the benzene metabolite SPMA in most of the samples since this compound is a ubiquitous environmental pollutant produced, for example, by active smoking. Accordingly, cotinine values allowed to characterize the smoking habit of the four exposed workers: subjects N1 and N2 are non-smokers, subject N3 is a very light smoker, subject N4 is a smoker.

A paired Students' test (Table 3) between the beginning and the end of the work-shift shows that the increase of metabolite concentration due to the working activity is significant only in the case of PGA (A campaign) and for SPMA and the sum of PGA and MA (B campaign). The methylhyppuric acid concentration increases on average in both campaigns although the statistical test result is not significant. These findings reveal that active and passive smoking behavior of the subjects heavily affects the concentrations of the VOC and their urinary metabolites.

Table 4 instead reports the concentration of the most abundant unmetabolized VOCs measured in the headspace of the samples, both for saliva and urine samples, measured at the beginning and the end of the work-shift.

In Table 4, a paired two-tails Students' test shows a significant increase of the concentrations of ethylbenzene, p-xylene, m-xylene, o-xylene measured in urine at the end of the work-shift. The same test has been applied to saliva but it is not statistically significant. A good correlation was found between the concentrations of toluene in saliva and in urine particularly at the end of the work-shift (0.96 compared to 0.79 at the beginning). Differently, in the case p-xylene, m-xylene and o-xylene the correlation between the concentration measured in urine and in saliva is particularly good at the beginning of the work-shift, 0.86, 0.83 and 1.00 respectively whilst at the end of the work-shift it is reduced especially for the o-xylene. The correlation between the concentration in urine and in saliva is acceptable, 0.76, 0.78 respectively for the ethylbenzene at the beginning and the end of the work-shift. These findings show that saliva is a much more difficult matrix to be managed likely due to the volatility of the VOCs. The correlation between the VOC directly measured in biological fluids (saliva, urine) and the corresponding urinary metabolites was studied. The results are summarized in Table 5.

Methylhyppuric acid is well correlated to the different kind of xylene, p-xylene, o-xylene, m-xylene and their sum both at the beginning and the end of the work-shift, especially as it regards the concentrations measured in urine (for the sum, 0.99 at the beginning and 0.98 at the end of the work-shift). These findings confirm that the methylhyppuric acid concentration can be used as a good biomarker of the xylene absorption. Ethylbenzene and PGA are well correlated, both in saliva and in urine at the end of the work-shift. Differently, ethylbenzene and MA show high correlation, both in saliva and in urine only at the beginning of the work-shift. These findings suggest that, whilst the PGA seems to be a good biomarker of the ethylbenzene absorption during the working activity, the MA at the end of the work-shift is influenced by other VOCs, in particular by the presence of styrene, as it can be seen by the concentration data found in both urine and saliva. Although styrene should not be present in the paints, it can be a residual pollutant coming from other working tasks. Benzene and SPMA are very well correlated but only in the case in which the urine concentration of benzene is considered. As previously pointed out, the saliva sampling is much more affected by the alteration due to the high volatility of the organic compounds. The correlation between toluene concentrations, in both urine and saliva and the SBMA was found very poor both at the beginning and the end of the work-shift.

3.3. miRNA

Adopting the adjusted p-value, only two miRNA were found statistically different in the group of exposed and control subjects, i.e.: hsa_miRNA_6819_5p, hsa_miRNA_6778_5p.

In particular, these miRNA were both up-regulated in the group of exposed subjects in comparison to the controls. The hierarchical cluster analysis of the selected miRNA is shown in Fig.1.

The expression matrix, calculated accordingly to Eq. (1), relative to the selected 35 miRNAs is reported in Fig. 2. As shown in this figure, miRNA profile of N1 subject is much more similar to the miRNA profile of the controls than to that of the exposed subjects. This result is not surprising as the subject N1 was involved in a task very different from that of the other exposed workers. Indeed N1 subject was not engaged in the spray painting as the others, being his task mainly the supervision of the other activities. This result is confirmed by PCA analysis, performed with respect to the miRNA, as shown in Fig. 3.

This occurrence is in agreement with the exposure biomarker profile as seen in Fig. 4 where the PCA analysis carried out on the exposure biomarkers variables is shown.

The results of the Linear regression analysis of the association between the urinary biomarkers of exposure, MHIPP, PGA, MA, SBMA, SPMA and oxidized guanine derivatives and the exponential function of

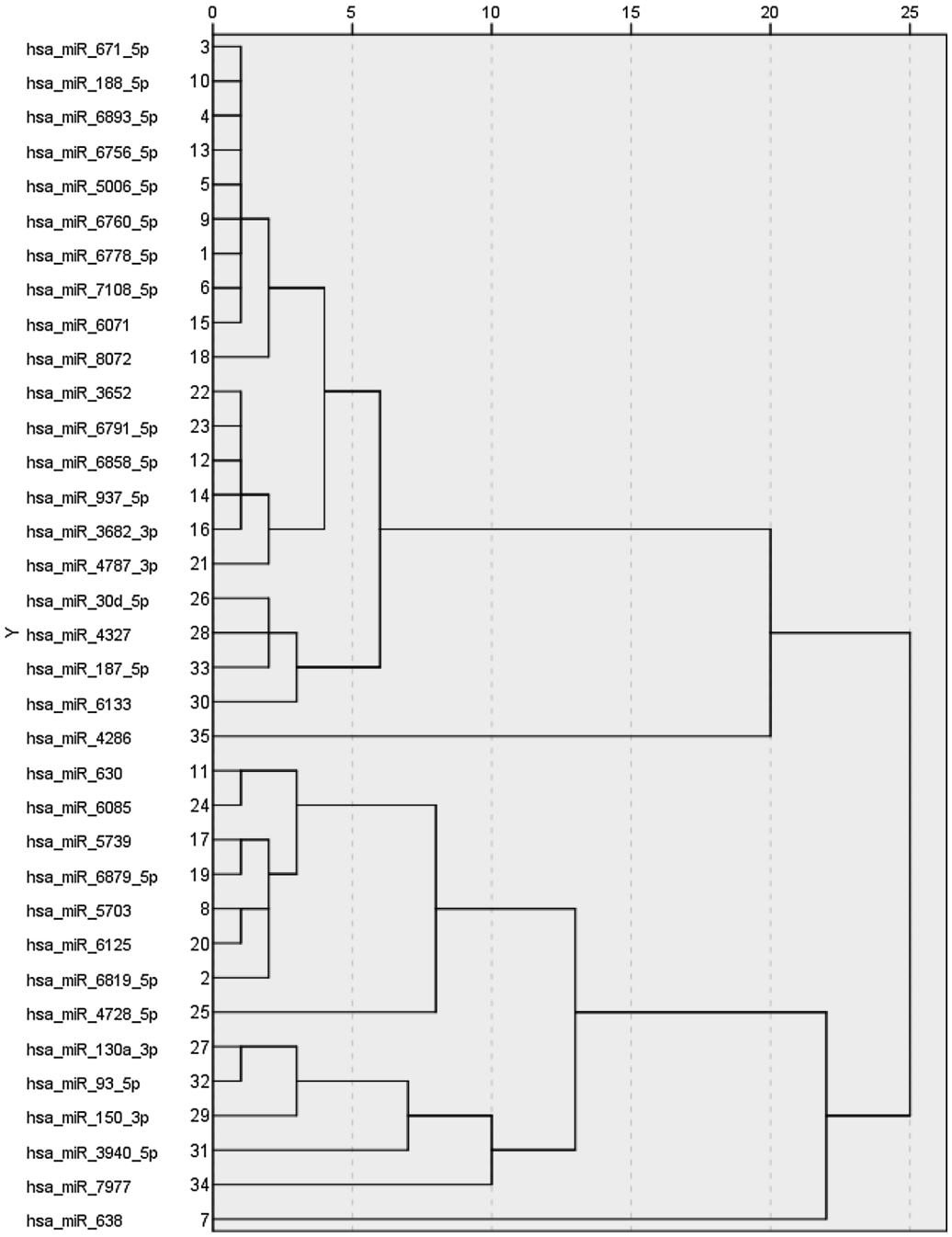

Fig. 1. Hierarchical clustering analysis with respect to the miRNA most differently expressed between exposed and control.

the specific miRNAs chosen among the miRNA that most differed in exposed and controls is shown in Table 6. The determination coefficient R2, the slope of the linear regression and its statistical significance (adjusted with an Holm Bonferroni criterion in order to control the multiplicity) are also shown.

Here miRNA_671_5p is significantly correlated to the Methylhyppuric acid concentration. Fig. 5 describes the exponential function of the miRNA expression value (miRNA_671_5p) versus the urinary concentration of Methylhyppuric acid (creatinine adjusted) measured at the end of the work-shift, averaged on the two different experimental campaigns. The data and the best fitting linear regression model are shown.

The same miRNA is also correlated to SPMA and SBMA. The regression model is not statistically significant in these cases as the p-value significance does not survive to the adjustment. A negative correlation was found between the creatinine adjusted 8oxoGua concentration, measured in the end shift urine and averaged on two different experimental campaigns, and miRNA_6778_5p as shown in Fig.6. Although the linear regression model does not reach the statistical significance, this finding indicates that the up-regulation of miRNA_6778_5p occurs in exposed subjects with respect to the controls and might be related to the oxidative stress control in chronically exposed workers.

4. Discussion

The study here presented on the biomonitoring of a group of individuals working in the ship painting activity aims to the following purposes:

1) to investigate the association between exposure to VOCs and

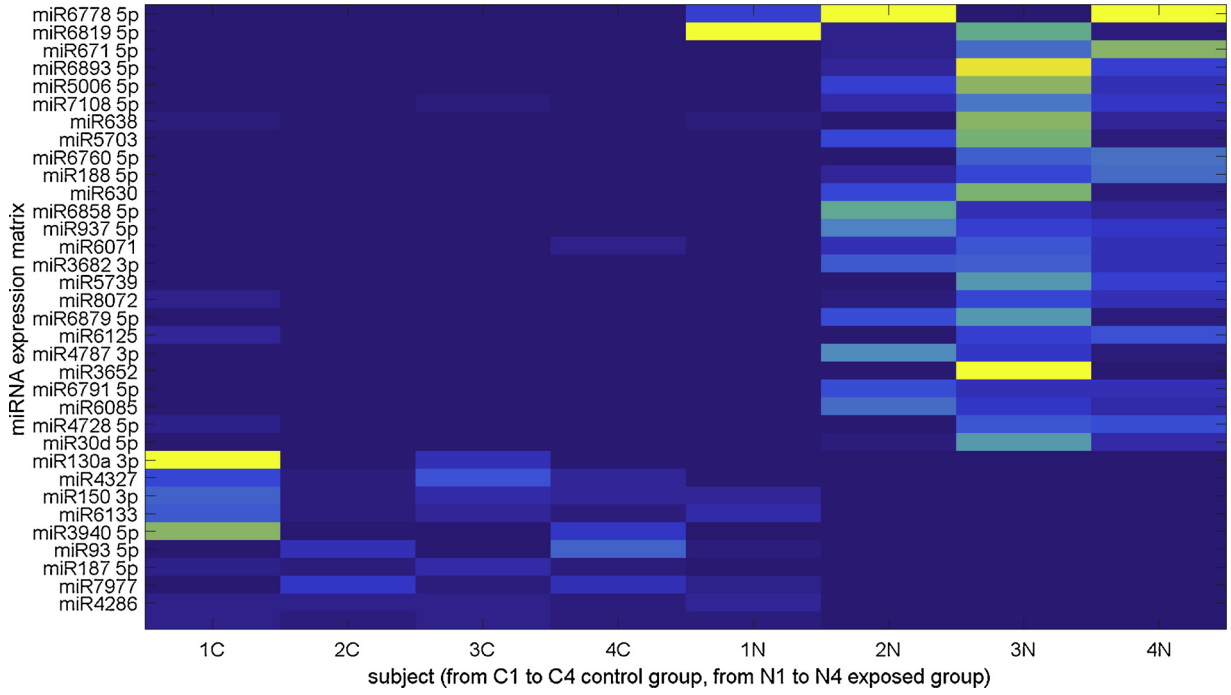

Fig. 2. Expression matrix for the selected miRNA. The miRNAs are grouped as up and down-regulated in exposed with respect to control subjects.

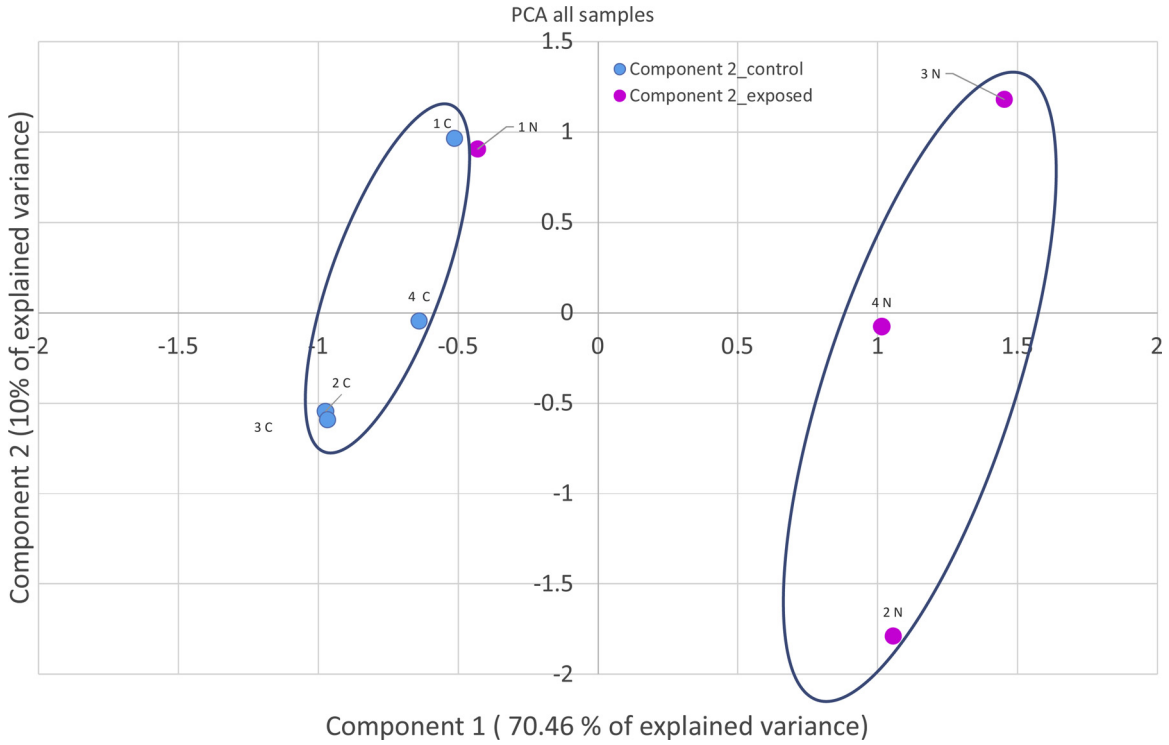

Fig. 3. Principal component analysis (PCA) performed on the miRNA of all samples of the dataset. The N1 subject is much more similar to the controls than to the exposed workers.

their respective urinary and saliva metabolites during the work-shift;

2) to characterize the miRNome in controls and exposed subjects and find differences in miRNA profile;

3) to identify miRNAs differentially expressed in the two groups and associated to VOCs' and relative metabolites as potential biomarkers of exposure.

Although the study is preliminary and involves few workers, the data collected here indicate interesting relations between miRNAs and exposure to VOCs.

The quality of the data relative to the biological monitoring of the solvent metabolites has been assessed by means of simultaneous measure of the same unmetabolized solvents in different biological fluids. Urinary biomarkers of oxidative stress were also measured, *i.e,* the oxidized form of the nucleoside guanosine which was associated to the miRNA profile. Since some of the VOCs studied are also present, both unchanged and in the form of metabolites, in the urine of smokers, it

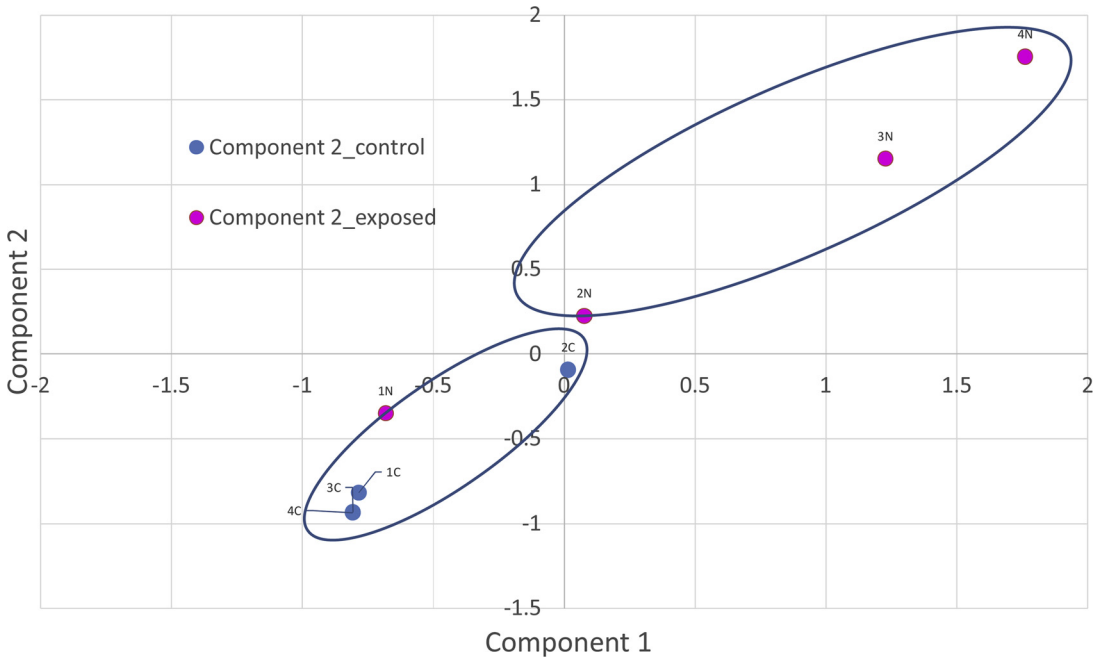

Fig. 4. Principal component analysis (PCA) performed on the exposure biomarkers of all samples of the dataset. The N1 subject is much more similar to the controls than to the exposed subjects.

Table 6
Linear regression analysis of the association between urinary biomarkers and selected miRNAs.

Urinary biomarker	miRNA	R2	β coeff	Stat sign Adjusted p value
MHIPP (μg/g Creatinine)	**hsa_miR_671_5p**	0.98	0.039	0.001071
	hsa_miR_6893_5p	0.75	0.04	n.s.
	hsa_miR_5006_5p	0.69	0.061	n.s.
	hsa_miR_7108_5p	0.7	0.017	n.s
	hsa_miR_6760_5p	0.83	0.029	0.0443
	hsa_miR_188_5p	0.85	0.034	0.0342
	hsa_miR_937_5p	0.78	0.006	n.s.
	hsa_miR_6071	0.68	0.025	n.s.
	hsa_miR_3682_3p	0.68	0.016	n.s.
	hsa_miR_6879_5p	0.65	0.160	n.s.
PGA (μg/g Creatinine)	hsa_miR_6893_5p	0.77	0.053	n.s.
	hsa_miR_5006_5p	0.76	0.0838	n.s
	hsa_miR_7108_5p	0.64	0.0214	n.s.
	hsa_miR_638	0.72	0.783	n.s.
	hsa_miR_5703	0.73	0.342	n.s
	hsa_miR_630	0.72	0.731	n.s.
	hsa_miR_937_5p	0.83	0.008	0.0415
	hsa_miR_8072	0.7	0.137	n.s.
MA (μg/g Creatinine)	hsa_miR_6819_5p	0.64	0.283	n.s.
SBMA (μg/g Creatinine)	hsa_miR_671_5p	0.65	34.04	n.s.
SPMA (μg/g Creatinine)	hsa_miR_671_5p	0.76	168.63	n.s.
8oxoGua (μg/g Creatinine)	hsa_miR_6778_5p	0.72	−3.76	n.s.

was not possible identify the amount of VOCs coming from the painting activity from those related to the smoking habit. In any case, as specific metabolites have been identified for each VOC, it was possible to find specific association between VOCs and miRNAs.

The analysis of the miRNome in the eight subjects demonstrated differences in miRNA expression between exposed and unexposed workers. Two miRNAs in particular, miR_6819_5p and miR_6778_5p were significantly up-regulated in the exposed group in comparison to controls, with the exception of one single individual (N1) which the PCA analysis classified in the control group.

When all the miRNAs were analyzed against each of the VOC metabolites (MHIPP, PGA, MA, SPMA, SBMA) including 8oxoGua we found interesting associations.

MHIPP, the metabolite of all xylenes, strongly associated to miRNA_671_5p. It also represented a reliable biomarker of xylene absorption, mostly in urine. MiRNA_671_5p was associated, though not significantly, to the SPMA and the SBMA, the metabolites of benzene and toluene, while PGA, a metabolite of both ethylbenzene and/or styrene, was found significantly associated to miRNA_937_5p. Between 8oxoGua and miRNA_6778_5p a high anti-correlation was observed which did not reach the statistical significance. However, this is the first association involving miRNA and an oxidized nucleoside in biomonitoring studies.

Although it is difficult to infer the biological meaning standing behind the miRNA pattern found by this analysis, we believe the difference in miRNAs expressed in exposed workers and controls is worth of further investigation and should be confirmed on a larger number of individuals, provided that similar environmental and occupational conditions are reproduced.

According to EU-OSHA principles regarding the health and safety at work [28] it would be useful to find further biomarkers which may possibly act as “sentinels” of exposure or of early effect in the exposure to dangerous substances such as the carcinogenic VOCs analyzed here. In this context, miRNAs look as candidates with great potential, as they are present in any human biological fluid and can be isolated with commercial kits with no risk for the worker. As far as we know there are few studies investigating the role of miRNAs as novel biomarkers of occupational exposure, most of which have already been described in the introduction [9,10, 12,13]. One of these studies found in the recent scientific literature is similar to our investigation. It was carried out on 169 workers exposed to three VOCs (toluene, xylene, and ethylbenzene) and by microarray analysis the authors were able identified 467 miRNAs for toluene, 211 miRNAs for xylene and 695 for xylene as exposure biomarkers which could distinguish each VOC in the exposed and control groups [29]. This study together with our investigation confirms the potential role of miRNAs in the biological response induced by occupational carcinogens. It also highlights the importance to find miRNA signatures specific of single or multiple exposures which, once validated, will contribute to increase the safety conditions at

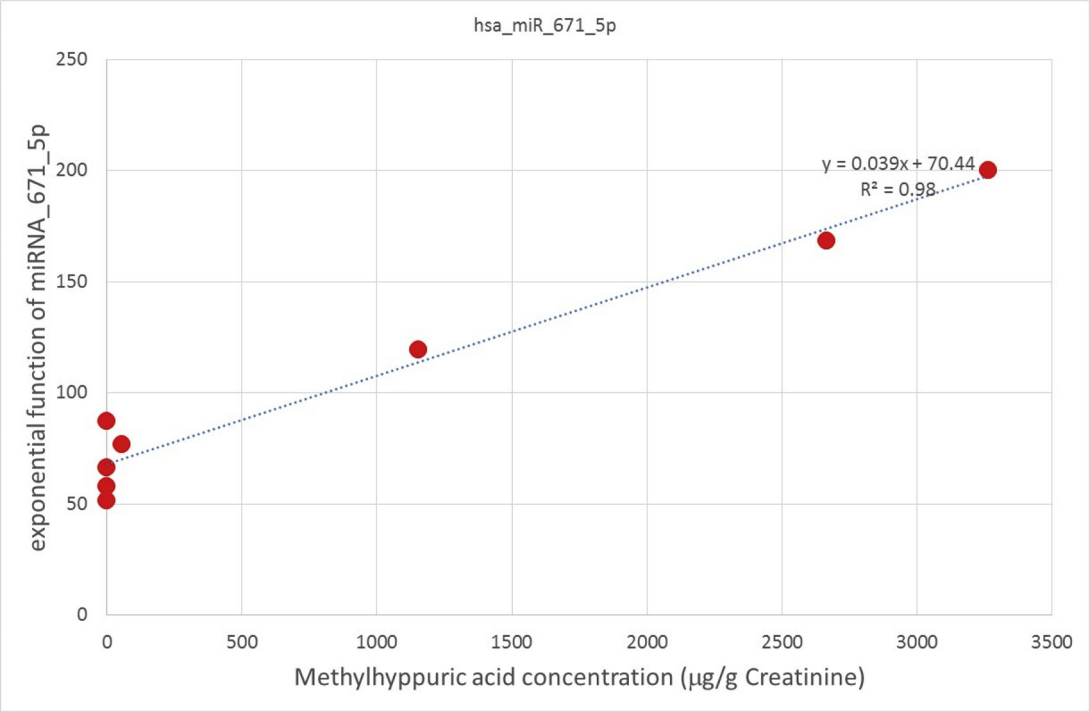

Fig. 5. Exponential function of the (miRNA_671_5p) expression value versus the end shift concentration of Methylhyppuric acid.

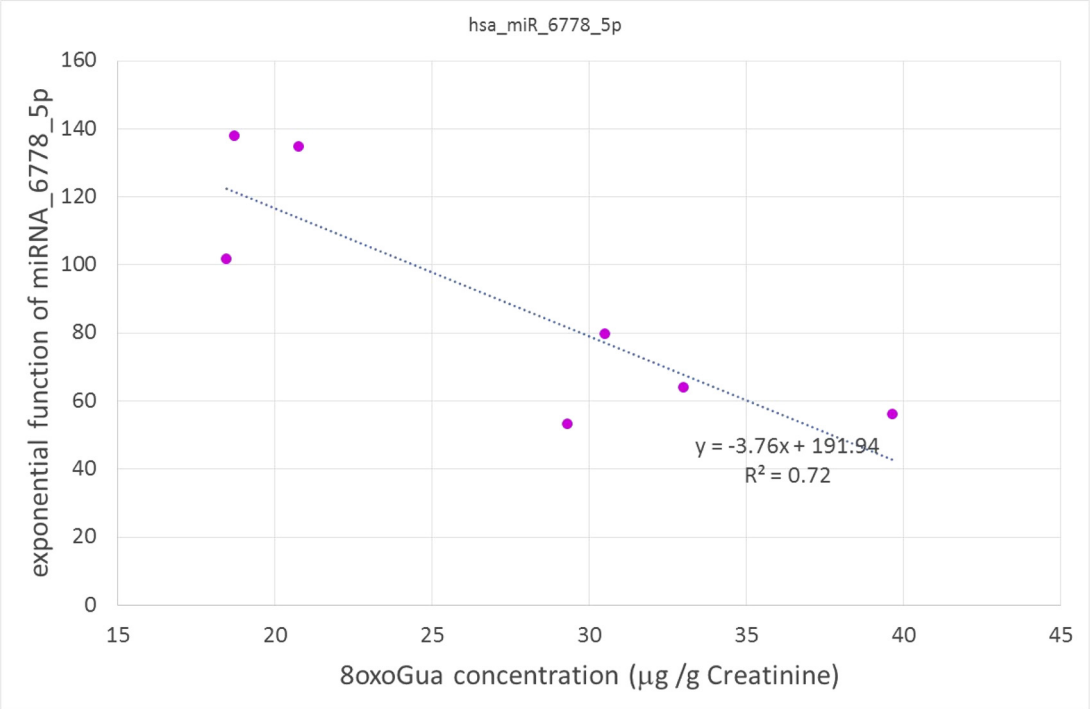

Fig. 6. Exponential function of the (miRNA_6778_5p) expression value versus the concentration of the 8oxoGua. The best fitting linear regression is also shown.

work.

5. Conclusions

Two miRNAs, specifically miR_6819_5p and miR_6778_5p, were found significantly up-regulated in a group of 4 workers assigned to a shipyard painting activity with respect to a control group. Significant associations were found between miRNAs and organic solvents absorbed dose, evaluated through the VOC urinary metabolites. In particular, the methylhippuric acid significantly correlated to miRNA_671_5p, miRNA_6760_5p and to miRNA_188_5p, whilst the phenylglioxylic acid was significantly associated to miRNA_937_5p. An interesting, although not significant anticorrelation was found between the 8-oxo-7,8-dihydroguanine (8-oxoGua) urinary concentration, interpreted as biomarker of oxidative stress, and miR_6778_5p. As proven in the clinical medicine miRNAs are the novel molecular biomarkers of disease. Similarly, in the occupational medicine field, the use of miRNAs to identify molecular signature associated to a specific

exposure would be advantageous for disease prevention and health promotion at workplace. Although the small number of subjects included in the present investigation can be seen as a limitation, this can be considered a pilot study, from which some important information can be drawn that could drive future and more robust studies: specific miRNAs could be used as biomarkers of exposure to match with specific VOCs at defined concentrations. Target genes of miRNA differentially expressed in exposed subjects can be identified and their polymorphisms could be possibly characterized to add further information on the genetic susceptibility of workers.

The Transparency document associated with this article can be found in the online version.

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References

[1] E. Berezikov, Evolution of microRNA diversity and regulation in animals, Nat. Rev. Genet. 12 (2011) 846–860, https://doi.org/10.1038/nrg3079.2011.
[2] R.S. Pillai, MicroRNA function: multiple mechanisms for a tiny RNA? RNA 11 (12) (2005) 1753–1761, https://doi.org/10.1261/rna.2248605.
[3] H.S. Liu, H.S. Xiao, MicroRNAs as potential biomarkers for gastric cancer, World J. Gastroenterol. 20 (34) (2014) 12007–12017, https://doi.org/10.3748/wjg.v20.i34.12007.
[4] J.C. Zhang, Z. Xu, T.F. Zhang, Y.L. Wang, Circulating microRNAs as diagnostic and prognostic tools for hepatocellular carcinoma, World J. Gastroenterol. 21 (34) (2015) 9853–9862, https://doi.org/10.3748/wjg.v21.i34.9853.
[5] K. Vrijens, V. Bollati, T.S. Nawrot, MicroRNAs as potential signatures of environmental exposure or effect: a systematic review, Environ. Health Perspect. 123 (5) (2015) 399–411, https://doi.org/10.1289/ehp.1408459.
[6] E. Schraml, M. Hackl, J. Grillari, MicroRNAs and toxicology: a love marriage, Toxicol. Rep. 4 (2017) 634–636, https://doi.org/10.1016/j.toxrep.2017.11.001.
[7] A. Etheridge, I. Lee, L. Hood, D. Galas, K. Wang, Extracellular microRNA: a new source of biomarkers, Mutat. Res. 717 (1-2) (2011) 85–90, https://doi.org/10.1016/j.mrfmmm.2011.03.004.
[8] J.A. Weber, D.H. Baxter, S. Zhang, D.Y. Huang, K.H. Huang, M.J. Lee, D.J. Galas, K. Wang, The MicroRNA Spectrum in 12 Body Fluids, Clin. Chem. 56 (11) (2010) 1733–1741, https://doi.org/10.1373/clinchem.2010.147405.
[9] B.A. Weldon, S.P. Shubin, M.N. Smith, T. Workman, A. Artemenko, W.C. Griffith, B. Thompson, E.M. Faustman, Urinary microRNAs as potential biomarkers of pesticide exposure, Toxicol. Appl. Pharmacol. 312 (2016) 19–25, https://doi.org/10.1016/j.taap.2016.01.018.
[10] Y. Liu, X. Chen, Y. Shi, J. Zhang, H. Zhang, F. Zhang, B. Zhu, Plasma microRNA expression in workers occupationally exposed to benzene, J. Public. Health. Emerg. 1 (11) (2017), https://doi.org/10.21037/jphe.2016.12.03.
[11] M. Varshney, A. Chandra, R. Jain, R. Ahmad, V. Bihari, C.K. Chandran, M.K.R. Mudiam, S. Patnaik, S.K. Goel, Occupational health hazards of trichloroethylene among workers in relation to altered mRNA expression of cell cycle regulating genes (p53, p21, bax and bcl-2) and PPARA, Toxicol. Rep. 2 (2015) 748–757, https://doi.org/10.1016/j.toxrep.2015.04.002.
[12] W. Bai, Y. Chen, J. Yang, P. Niu, L. Tian, A. Gao, Aberrant miRNA profiles associated with chronic benzene poisoning, Exp. Mol. Pathol. 96 (2014) 426–430, https://doi.org/10.1016/j.yexmp.2014.04.011.
[13] K.S. Jeong, J. Zhou, S.C. Griffin, E.T. Jacobs, D. Dearmon-More, J. Zhai, S.R. Littau, J. Gulotta, P. Moore, W.F. Peate, C.M. Richt, J.L. Burgess, MicroRNA changes in firefighters, J. Occup. Environ. Med. 60 (5) (2018) 469–474, https://doi.org/10.1097/JOM.0000000000001307.
[14] M.D. Evans, M. Saparbaev, M.S. Cooke, DNA repair and the origins of urinary oxidized 2'-deoxyribonucleosides, Mutagenesis. 25 (5) (2010) 433–442, https://doi.org/10.1093/mutage/geq031.
[15] D. Il'yasova, P. Scarbrough, I. Spasojevic, Urinary biomarkers of oxidative status, Clin. Chim. Acta 413 (19-20) (2012) 1446–1453, https://doi.org/10.1016/j.cca.2012.06.012.
[16] K.D. Jacob, N. Noren Hooten, A.R. Trzeciak, M.K. Evans, Markers of oxidant stress that are clinically relevant in aging and age-related disease, Mech. Ageing Dev. 134 (2013) 139–157, https://doi.org/10.1016/j.mad.2013.02.008.
[17] A. Valavanidis, i T. Vlachogiann, C. Fiotakis, 8-Hydroxy-2' -deoxyguanosine (8-OHdG): a critical biomarker of oxidative stress and carcinogenesis, J. Environ. Sci. Health C Environ. Carcinog. Ecotoxicol. Rev. 27 (2) (2009) 120–139, https://doi.org/10.1080/10590500902885684.
[18] A. Pilger, H.W. Rüdiger, 8-Hydroxy-2'-deoxyguanosine as a marker of oxidative DNA damage related to occupational and environmental exposures, Int. Arch. Occup. Environ. Health 80 (1) (2006) 1–15, https://doi.org/10.1007/s00420-006-0106-7.
[19] T. Dziaman, Z. Banaszkiewicz, K. Roszkowski, D. Gackowski, E. Wisniewska, R. Rozalski, M. Foksinski, A. Siomek, E. Speina, A. Winczura, A. Marszalek, B. Tudek, R. Olinski, 8-Oxo-7,8-dihydroguanine and uric acid as efficient predictors of survival in colon cancer patients, Int. J. Cancer 134 (2014) 376–383, https://doi.org/10.1002/ijc.28374.
[20] M. Gherardi, A. Gordiani, M.P. Gatto, Development and validation of method for analysis of some ototoxic solvents in saliva matrix by headspace gas chromatography/mass spectrometry, J Chromatogr. B 878 (2010) 2391–2396, https://doi.org/10.1016/j.jchromb.2010.07.007.
[21] M.H. Kroll, R. Chesler, C. Hagengruber, D.W. Blank, J. Kestner, M. Rawe, Automated determination of urinary creatinine without sample dilution: theory and practice, Clin. Chem. 32 (3) (1986) 446–452.
[22] ACGIH, Threshold limit values (TLVs) for chemical substances and physical agents and biological exposure indices (BEIs), American Conference of Governmental Industrial Hygienists (2014).
[23] E. Paci, D. Pigini, L. Caporossi, M. De Rosa, A. Santoro, R. Sisto, B. Papaleo, G. Tranfo, Matrix effect in the quantitative determination of mandelic and phenylglyoxylic acid in urine samples by HPLC-MS/MS with isotopic dilution, Current. Anal. Chem. 9 (3) (2013) 439–446.
[24] L. Sabatini, A. Barbieri, P. Indiveri, S. Mattioli, F.S. Violante, Validation of an HPLC–MS/MS method for the simultaneous determination of phenylmercapturic acid, benzylmercapturic acid and o-methylbenzyl mercapturic acid in urine as biomarkers of exposure to benzene, toluene and xylenes, J. Chromatogr. B. 863 (1) (2008) 115–122, https://doi.org/10.1016/j.jchromb.2008.01.022.
[25] G. Tranfo, D. Pigini, E. Paci, F. Marini, R.C. Bonanni, Association of exposure to benzene and smoking with oxidative damage to nucleic acids by means of biological monitoring of general population volunteers, Environ. Sci. Pollut. Res. 24 (16) (2017) 13885–13894, https://doi.org/10.1007/s11356-016-6366-1.
[26] R. Andreoli, P. Manini, Palma. G De, R. Alinovi, M. Goldoni, W.M. Niessen, A. Mutti, Quantitative determination of urinary 8-oxo-7,8-dihydro-2′-deoxyguanosine, 8-oxo-7,8-dihydroguanine, 8-oxo-7,8-dihydroguanosine, and their non-oxidized forms: daily concentration profile in healthy volunteers, Biomarkers 15 (3) (2010) 221–231, https://doi.org/10.3109/13547500903434501.
[27] ECHA, European Chemical Agency, Entry 5 of Annex XVII to the REACH Regulation (2006), (1907/2006).
[28] EU-OSHA, European Agency for Safety and Health at Work, Available at (2016) https://osha.europa.eu/en/themes/dangerous-substances/roadmap-to-carcinogens).
[29] M.K. Song, J.C. Ryu, Blood miRNAs as sensitive and specific biological indicators of environmental and occupational exposure to volatile organic compound (VOC), Int. J. Hyg. Environ. Health 218 (7) (2015) 590–602, https://doi.org/10.1016/j.ijheh.2015.06.002.