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

Renata Sisto⁎, Pasquale Capone, Luigi Cerini, Enrico Paci, Daniela Pigini, Monica Gherardi, Andrea Gordiani, Nunziata L’Episcopo, Giovanna Tranfo, Pieranna Chiarella

Italian Workers Compensation Authority (INAIL), Department of Occupational and Environmental Medicine, Epidemiology and Hygiene, via di Fontana Candida 1, 00078, Monte Porzio Catone, Rome, Italy

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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 ≤ 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-Benzymercapturic acid; MHIPPA, methylhippuric acid; miRNA, MicroRNA; DE, differently expressed; KEGG, Kyoto Encyclopedia of Genes and Genomes

⁎ Corresponding author.

E-mail addresses: r.sisto@inail.it (R. Sisto), p.capone@inail.it (P. Capone), l.cerini@inail.it (L. Cerini), e.paci@inail.it (E. Paci), d.pigini@inail.it (D. Pigini), m.gherardi@inail.it (M. Gherardi), a.gordiani@inail.it (A. Gordiani), n.lepiscopo@inail.it (N. L’Episcopo), g.tranfo@inail.it (G. Tranfo), p.chiarella@inail.it (P. Chiarella).

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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-221–3p 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 of 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 deoxyguanosine, 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 miRNAome 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 dilsents and additives (eptan-2-one, 2-butoxiethyl acetate, 1-methyl-2metoxiyeth acetate, butanol, ethyl acetate, n-buyl 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
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 (N, Agilent Technologies) with a Turbo Ion Spray (TIS) 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 acid 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 Jaffe 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 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            |

**p value**

| Students' $t$ -test | Pearson $\chi^2$ test | $\chi^2$ square | n.s. |
|--------------------|------------------------|-----------------|------|
| 0.016              | 0.008                  | 0.016           | n.s. |

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 acid 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.

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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).
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 | Methylhypurric acid (MHP) | 1 µg/mL |
| Toluene | S-Benzylmercuricapturic acid (SBMA) | 0.35 µg/L |
| Benzene | S-Phenylmercuricapturic acid (SPMA) | 0.026 µg/L |
| Nicotine (active smoking) | Cotinine | 12.41 µg/L |
| Oxidative stress on DNA | 8-oxo-7,8-dihydro-2′,3′-deoxyguanosine (8-oxoGuo) | 0.5 µg/L |
| Oxidative stress on RNA | 8-oxo-7,8-dihydroguanosine (8-oxoGuo) | 0.7 µg/L |
| Oxidative stress on RNA and proteins | 8-oxo-7,8-dihydroguanine (8-oxoGu) | 0.5 µg/L |
| Nitrosative stress on proteins | 3-nitrotyrosine | 1.0 µg/L |
| Methylolation 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, MHP, 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 down-regulation 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:

\[
M(i, j) = \frac{\text{miRNA}(i, j) - \text{mean(miRNA)}(i, \text{1:20})}{\sqrt{\text{var(miRNA)}(i, \text{1:3}) + \text{var(miRNA)}(i, \text{4:20})}}
\]

A principal component analysis (PCA) was also performed. The correlations between the concentration of each solvent in urine, the solvent metabolites, MA, PGA, MHP, SBMA, SPMA, and between the oxidized guanine derivatives, 8-oxoGuo, 8-oxoGuo, 8-oxoGuo 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.

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/m³) and to 50 % of the OEL (221 mg/m³) 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, MHPPA 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 AGIHI BEIs (Biological Exposure Index). The BEI of the AGIHI for the considered metabolites are: 1.5 g/
g Cr for the MHIPP, 25 μg/g Cr for the SPMA, 300 μg/g Cr for the SBMA and 400 μg/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, MHIPP, 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 3NO2 tyrosine.

### 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 work-shift is also shown.

| solvent       | Before Work-shift | After work-shift |
|---------------|-------------------|------------------|
| ethylacetate  | ng/mL             | p-value          |
| benzene       | na                | 0.042            |
| toluene       | na                | 0.043            |
| ethylbenzene  | na                | 0.023            |
| p-xylene      | na                | 0.018            |
| m-xylene      | na                | 0.029            |
| o-xylene      | na                | 0.05             |
| Total xylenes | na                | 0.031            |

| solvent       | Mean  | Median | SD | 5th | 25th | 75th | max | min | Mean  | Median | SD | 5th | 25th | 75th | max | min | t -test | p value |
|---------------|-------|--------|----|-----|------|------|-----|-----|-------|--------|----|-----|------|------|-----|-----|---------|---------|
| ethylacetate  | 4.9   | 3.6    | 4.3| 1.6 | 1.7  | 6.8  | 9.9 | 10.7| 1.5   | 11.8   | 56.1| 40.4 | 56.3 | 95.9 | 115.8| 36.4| 0.042   | 0.031   |
| benzene       | na    | na     | na | na  | na   | na   | na  | na  | na    | na     | na  | na  | na   | na   | na  | na  | n.s.    | n.s.    |
| toluene       | na    | na     | na | na  | na   | na   | na  | na  | na    | na     | na  | na  | na   | na   | na  | na  | n.s.    | n.s.    |
| ethylbenzene  | 6.2   | 7.5    | 2.6| 2.7 | 3.4  | 7.7  | 9.0 | 10.7| 2.5   | 76.1   | 76.1| 40.4 | 56.3 | 95.9 | 115.8| 36.4| 0.042   | 0.031   |
| p-xylene      | 2.7   | 1.2    | 1.1| 2.1 | 2.1  | 3.7  | 4.3 | 4.4  | 1.0   | 25.9   | 2.2  | 1.5  | 1.9  | 7.8  | 7.8  | 1.5  | 0.042   | 0.031   |
| m-xylene      | 12.1  | 2.3    | 1.1| 9.4 | 3.7  | 15.6 | 4.3 | 4.4  | 4.9   | 8.3    | 5.5  | 3.4  | 4.0  | 7.3  | 7.7  | 3.3  | 0.042   | 0.031   |
| o-xylene      | na    | na     | na | na  | na   | na   | na  | na  | na    | 43.1   | 2.6  | 1.5  | 2.0  | 3.9  | 17.3 | 1.2  | 0.042   | 0.031   |
| Total xylenes | na    | na     | na | na  | na   | na   | na  | na  | na    | 46.6   | 26.6 | 33.4 | 39.3 | 57.9 | 91.8 | 32.0 | 0.042   | 0.031   |
| n/s.          | n/s.  | n/s.   | n/s.| n/s.| n/s.  | n/s. | n/s.| n/s.| n/s.  | n/s.   | n/s.| n/s.| n/s. | n/s. | n/s.| n/s.| n/s.    | n/s.    |

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

|          | Beginning of work-shift |          |          |          |          |          |
|----------|-------------------------|----------|----------|----------|----------|----------|
|          | Mean (μg/g Cr)          | Median   | SD       | 5th perc | max      | min      |
| roller   | 9031.8                  | 24537.3  | 660.0    | 0.03     | 9647.5   | 310.0    |
|          | 2537.3                  | 4217.8   | 399.5    | 0.26     | 5188.8   | 310.0    |
|          | 8589.5                  | 2456.0   | 1410.0   | 0.00     | 16188.1  | 900.0    |
|          | 360.0                  | 4221.5   | 615.0    | 0.04     | 13136.0  | 360.0    |
|          | 6940.0                  | 9020.5   | 11230.0  | 0.75     | 50926.9  | 6940.0   |
|          | 360.0                  | 57472.0  | 6139.0   | 0.57     | 26840.4  | 360.0    |
|          | 750.0                  | 1290.0   | 5045.0   | 0.00     | 11376.0  | 750.0    |
|          | 1130.0                 | 1287.1   | 710.0    | 0.13     | 15187.0  | 1130.0   |
|          | 1250.0                 | 4714.8   | 710.0    | 0.13     | 15187.0  | 1250.0   |
|          | 6940.0                 | 11376.0  | 710.0    | 0.13     | 15187.0  | 6940.0   |
|          | 1130.0                 | 4714.8   | 710.0    | 0.13     | 15187.0  | 1130.0   |
|          | 1250.0                 | 15187.0  | 710.0    | 0.13     | 15187.0  | 1250.0   |

### Table 7

Concentrations 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.

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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, 3NO₂tyrosine 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-oxoGuo µg/g Creatinine | 8-oxodGuo µg/g Creatinine | 8-oxoGua µg/g Creatinine | 3NO₂tyrosine g/g Creatinine | 5-methylcytidine g/g Creatinine |
|----------------------|---------------------------|---------------------------|---------------------------|-----------------------------|---------------------------------|
| 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                      | 5.60                      | 0.90                        | 6.79                            | 3.70                            |
|                      | 5th perc                  | 0.22                      | 10.30                     | 1.61                        | 1.52                            | 1.52                            |
|                      | 25th perc                 | 2.31                      | 3.57                      | 2.25                        | 0.80                            | 1.85                            |
|                      | 75th perc                 | 9.27                      | 8.52                      | 3.09                        | 2.53                            | 5.15                            |
|                      | max                       | 21.29                     | 18.59                     | 4.29                        | 14.00                           | 10.55                           |
|                      | min                       | 21.99                     | 18.69                     | 4.42                        | 23.68                           | 14.20                           |
|                      | t-test p value            | n.s.                      | n.s.                      | n.s.                        | n.s.                            | n.s.                            |
| Work-shift end       |                           |                           |                           |                             |                                 |
| roller               | Mean                      | 9.97                      | 14.51                     | 5.23                        | 3.55                            | 7.92                            |
|                      | Median                    | 5.76                      | 14.72                     | 5.59                        | 3.47                            | 6.89                            |
|                      | SD                        | 12.26                     | 4.16                      | 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                           |
|                      | max                       | 28.92                     | 20.22                     | 7.50                        | 6.13                            | 13.39                           |
|                      | 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                            |
|                      | max                       | 54.89                     | 28.43                     | 4.16                        | 7.63                            | 19.00                           |
|                      | min                       | 64.37                     | 28.83                     | 4.21                        | 8.01                            | 22.10                           |
| 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                           | 32.51                           |
|                      | 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.                            |
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 posed to VOCs in a naval industry compared to a control group. The work shift and the miRNAs, selected specifically, were correlated to specific VOCs, a different ethnicity, being the workers of the previous paper prevalently Bengalese whilst the workers of the previous work were all caucasian. 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.

A recent study [36], showed the first two miRNAs (miR-589-5p and miR-941) were regulated by exposure to substances (including organic dangerous substances, with variable effects on workers’ health).
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 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 di(2015) 599–609), https://doi.org/10.1016/j.toxrep.2015.05.002.

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