Formaldehyde and tobacco smoke as alkylating agents: the formation of N-Methylenvaline in pathologists and in plastic laminate workers

Elsevier Editorial System(tm) for Science of the Total Environment
Manuscript Draft

Manuscript Number: STOTEN-D-11-00897

Title: Formaldehyde and tobacco smoke as alkylating agents: the formation of N-Methylenvaline in pathologists and in plastic laminate workers

Article Type: Full Length Article

Section/Category: Biomonitoring and Surveillance

Keywords: Formaldehyde, N-Methylenvaline, tobacco smoke, alkylating agents, occupational exposure

Corresponding Author: Prof. Roberto Bono, PhD

Corresponding Author's Institution: University of Turin

First Author: Roberto Bono, PhD

Order of Authors: Roberto Bono, PhD; Valeria Romanazzi, Dr.; Valentina Pirro, Dr.; Raffaella Degan, Dr.; Cristina Pignata, Dr.; Elisa Suppo, Dr.; Marco Pazzi, Ph.D.; Marco Vincenti, Ph.D.

Abstract: Objective. The aim of this study was to investigate the relationships between air-FA exposure and the N-Methylenvaline formation in three occupational contexts: a) the pathology wards, b) the industry of plastic laminates, c) a group of controls. All subjects recruited in this study were considered according to their smoking habits.
Methods. Formaldehyde was quantified with a HPLC equipped with an UV detector (360 nm), cotinine was quantified using an Agilent gas chromatograph, interfaced to an Agilent mass spectrometer. N-methylenvaline were measured by turbomass GC-MS Perkin Elmer equipped with a single quadrupole analyzer. Analysis of variance one-way ANOVA was performed to compare the biomarkers considered among the three groups.
Results. For air-FA and N-methylenvaline a difference between the three levels of exposure ($p < 0.0001$) and a significant higher concentration in the two professional exposures were proved. Mean values for FA (μg/m3): group a) 188.6, group b) 210.1, group c) 41.4; mean values for N-methylenvaline (nmol/gr of globin): group a) 377.9, group b) 342.8, group c) 144.8. Conversely, the comparison between the two professional exposures, group a) vs b), does not show significant differences highlighting similar exposure to FA and similar biological response. Tobacco smoke proves to have a minor impact on the formation of N-methylenvaline molecular adduct.
Conclusions. We can assume the positive relationship between professional exposure to air-FA and N-methylenvaline formation in the two considered type of occupational exposure. Tobacco smoke proves to have a minor impact on the formation of N-methylenvaline molecular adduct.

Suggested Reviewers: Fabio Barbone prof.
University of Udine, ITALY
fabio.barbone@uniud.it

Nino Künzli MD, Ph.D.
University of Basel Medical School, SWITZERLAND
nino.kuenzli@unibas.ch

Wieslaw Jedrychowsky prof.
University of Krakow, POLAND
myjedryc@cyf-kr.edu.pl

Opposed Reviewers:

Cover Letter

UNIVERSITÀ DEGLI STUDI DI TORINO
Dipartimento di Sanità Pubblica e di Microbiologia
Via Santena 5 bis
10126 TORINO, Italy

April 4st, 2011

Dear Editor of The Science of the Total Environment:
Please find enclosed the manuscript “Formaldehyde and tobacco smoke as alkylating agents: the formation of N-methylenvaline in pathologists and in plastic laminate workers" by R. Bono *et al.* In my view, the relevance of the results summarized in the manuscript is two-fold. Firstly, the evidence in humans that formaldehyde induces early biological effects which were monitored by measuring a protein adduct, the N-methylenvaline, in two groups of formaldehyde-exposed workers. Secondly, the evidence that also tobacco smoke can induce this response in this populations, independently from the occupational exposure to formaldehyde. Thus, data may hence represent a platform for designing a protective grid for workers and smokers.

Some information about the results presented:

- the manuscript is an original work, has not been previously published in whole or in part, and is not under consideration for publication elsewhere;
- the voluntary participation of all the human subjects did not occur until after informed consent was obtained;
- all authors have disclosed any potential competing interest regarding the submitted article and the nature of those interests;
- any permission is necessary from copyright holders
- all the authors have read the manuscript, agree that the work is ready for submission to a journal, and accept responsibility for the manuscript’s contents.

Hoping that the manuscript may fulfil the scientific standards of The Science of the Total Environment, my best regards.

Roberto Bono, Ph. D.

Contacts:
Phone: (+39) 011-670-5818
Fax: (+39) 011-236-5818
e-mail: roberto.bono@unito.it

HIGHLIGHTS

1. The formaldehyde exposure induces biological effects through the N-methylenvaline.

2. Also tobacco smoke can induce this similar biological response.

3. These findings have a primary prevention role for workers and smokers.

Formaldehyde and tobacco smoke as alkylating agents: the formation of N-Methylenvaline in pathologists and in plastic laminate workers

[1*]Bono R., [1]Romanazzi V., [2]Pirro V., [1]Degan R., [1]Pignata C., [1]Suppo E., [2]Pazzi M., [2]Vincenti M.

[1] Department of Public Health and Microbiology, University of Torino
Via Santena 5 bis, 10126, Torino, ITALY
[2] Department of Analytical Chemistry, University of Torino
Via Pietro Giuria 7, 10126, Torino, ITALY

***Corresponding author:**
Prof. Bono Roberto
Department of Public Health and Microbiology,
University of Torino, Italy
Via Santena 5 bis, 10126 Torino
e-mail: roberto.bono@unito.it
phone: +39 011 670 5818
fax: +39 011 670 5874

ABSTRACT:

Objective. The aim of this study was to investigate the relationships between air-FA exposure and the N-Methylenvaline formation in three occupational contexts: a) the pathology wards, b) the industry of plastic laminates, c) a group of controls. All subjects recruited in this study were considered according to their smoking habits.
Methods. Formaldehyde was quantified with a HPLC equipped with an UV detector (360 nm), cotinine was quantified using an Agilent gas chromatograph, interfaced to an Agilent mass spectrometer. N-methylenvaline were measured by turbomass GC-MS Perkin Elmer equipped with a single quadrupole analyzer. Analysis of variance one-way ANOVA was performed to compare the biomarkers considered among the three groups.
Results. For air-FA and N-methylenvaline a difference between the three levels of exposure ($p < 0.0001$) and a significant higher concentration in the two professional exposures were proved. Mean values for FA ($\mu g/m^3$): group a) 188.6, group b) 210.1, group c) 41.4; mean values for N-methylenvaline (nmol/gr of globin): group a) 377.9, group b) 342.8, group c) 144.8. Conversely, the comparison between the two professional exposures, group a) *vs* b), does not show significant differences highlighting similar exposure to FA and similar biological response. Tobacco smoke proves to have a minor impact on the formation of N-methylenvaline molecular adduct.
Conclusions. We can assume the positive relationship between professional exposure to air-FA and N-methylenvaline formation in the two considered type of occupational exposure. Tobacco smoke proves to have a minor impact on the formation of N-methylenvaline molecular adduct.

KEYWORDS
Formaldehyde, N-Methylenvaline, tobacco smoke, alkylating agents, occupational exposure

1. INTRODUCTION

Formaldehyde (FA) is a common environmental and occupational contaminant and it is considered the principal carbonyl in urban atmosphere. FA is emitted in air through various waste streams during the production of resins and the use disinfectants, preservatives, and a variety of other chemicals; it is present in tobacco smoke, paint, garments, diesel and gasoline exhaust, and in numerous medical and industrial products (Bono et al., 2010a; Saito et al., 2005). Exposition to air-FA can induce local irritations, acute and chronic toxicity, and genotoxic and carcinogenic activity (Schmid and Speit, 2007; Speit et al., 2007), as confirmed by an increased incidence of nasopharyngeal cancer in industrial workers, embalmers, and pathologist (Duhayon et al., 2008; Hauptmann et al., 2004), by the relationship demonstrated between FA and leukemia in a recent meta analysis (McGwin et al., 2010; Zhang et al., 2009), and by a significant positive association between FA exposure and childhood asthma.(McGwin et al., 2010) FA was re-evaluated for carcinogenic effects and reclassified as ‘‘Carcinogenic to humans (Group 1) (IARC, 2006). For these reasons FA is now considered a subject of major environmental and public health concern and its presence in occupational environments has been regulated several times by the American Conference Governmental Industrial Hygienists (ACGIH) until the actual ceiling limit value (TLV-C) of 0.3 ppm (0.370 mg/m^3) (Heck et al., 1990; Nielsen and Wolkoff, 2010).

FA is rapidly absorbed from the gastrointestinal and respiratory tract, but the exposure to this substance is not easily assessable through the direct measure in biological fluids, because the bio available portion in the site of contact is metabolized in few minutes; its metabolites are incorporated into macromolecules via the one-carbon pool pathway or eliminated with the expired air (CO_2) and urine (Goulding, 1991).

FA is an extremely reactive chemical, and reacts with monoamines or amides to form methylene bridges and produces covalently cross-linked complexes with protein and DNA

named DNA-protein cross-links (DPCs) that can be measured in blood or cells of exposed subjects (Chaw et al., 1980; Heck et al., 1990; Lu et al., 2010). Adducts to proteins are not subjected to repair mechanisms, thus they reflect exposure that corresponds to the life of the protein. Scientific interest is directed not only to major proteins, such as hemoglobin and albumin (half-lifetime in men, 120 and 23 days, respectively), but also to histones and collagen as potential indicators of long-term exposure (10-20 years) (Miraglia et al., 2004).

FA is able to bind to human serum albumin (HSA), forming the FA-HSA, a molecular adduct having antigenic properties; the antibody response against this adduct could also provide an indirect measure of FA exposure (Thrasher et al., 1988; Thrasher et al., 1990), even if the formation of IgG and IgE antibodies against FA is inconsistent (Doi et al., 2003; Kim et al., 1999). Nevertheless, the evaluation of IgG formation represents a useful indication of occupational exposure to air-FA, only for non-smokers (Carraro et al., 1997). DPCs were found in tissues in direct contact with formaldehyde, rarely away from them.(Goulding, 1991) At this concern, to monitoring air-FA exposure, DPCs level in blood of subjects professionally exposed to FA in pathology wards were measured and proved to be higher than in controls (Shaham et al., 1996; Shaham et al., 2003; Zhitkovich and Costa, 1992).

Kautiainen (Kautiainen et al., 1989) showed the formation of a molecular adduct through a covalent bond between formaldehyde and primary amino groups of the globin, forming the corresponding Schiff's base. The N-terminal valine of globin on 2α and 2β chains may undergo to an alkylation reaction, forming N-Methylenvaline. The formation of this early biological effect is dependent by exposure to some alkylating agents, of which FA is an example described in a previous study (Bono et al., 2006):

$$\text{R-CHO} + \text{H}_2\text{N-Val-Hb} \longleftrightarrow \text{R-CH = N-Val-Hb} + \text{H}_2\text{O}$$

$$\text{aldehydes} + \text{N-terminal(valine)} \longleftrightarrow \text{N-terminal (valine) adducts}$$

Pathologists and plastic laminate workers are two important risky professions, where workers may be exposed to high concentration of FA. The first use FA as a preservant and disinfectant in pathology laboratories. In particular, the higher levels of exposure to FA in pathology wards are recorded in reduction rooms, where FA is directly used to fix the biological tissues (Bono et al., 2010b). Plastic laminate workers are exposed to FA contained and released by pressed-wood products used in home construction, in furnishings containing urea-formaldehyde resins (e.g. particleboard, hardwood plywood, medium density fibreboard, and panelling) and phenol-formaldehyde resin (e.g. softwood plywood, oriented strand board) (Kelly et al., 1999). Therefore, we can assume that the widespread use of FA in many working and life contexts can represent a potential risk factor for human health when this pollutant is assumed by contact or breathed. Consequently, these two occupational exposures represent typical environments where workers can show biological answers due to FA exposure. Thus, the first aim of this study was to study the relationships between air-FA exposure and the N-Methylenvaline formation, taking into account the tobacco smoke habit as a possible confounding factor. Secondly, we intended to compare N-Methylenvaline formation in two different populations of workers exposed to air-FA: pathologists and plastic laminate workers.

2. MATERIALS AND METHODS

2.1 Epidemiological Sample.

44 pathologists and 51 workers of an industry of plastic laminates were recruited as subjects potentially exposed to air-FA. 78 subjects were enrolled as controls from some scientific labs and offices where FA is not used. All the 173 subjects were sampled during 2009 from 3 hospitals, 1 industry, 1 university, and several offices in Piedmont region (North-Western Italy). For each of 173 subjects, an air-FA sample was collected for an entire working shift (about 8 h). Information on personal medical history, smoking habits,

and drug intake were also collected through a questionnaire administered at the end of the working shift when a sample of venous blood and a spot of urine were collected as well. The description of smoking status of all of the subjects was *a priori* established, classifying as “non smokers”, the never smokers and the former smokers who had ceased smoking for at least 1 month, and “smokers” who smoked at least one cigarette per day. All subjects were informed about the objective of the study, and voluntarily, gave a written consent.

2.2 Personal Air-FA.

FA air samples were collected for a whole working shift (8 h) on Wednesday using passive, personal air samplers working with radial symmetry (Radiello), clipped near the breathing zone of the subject. Samplers were equipped with a specific sorbent tube containing florisil 35-50 mesh coated with 2,4-dinitrophenylhydrazine (DNPH). DNPH reacted with FA, and changes, by derivatization, to the specific 2,4-dinitrophenylhydrazone-FA which was quantified with a HPLC Perkin-Elmer equipped with an UV detector regulated at 360 nm: NIOSH Method no. 2016 (Eller, 1984). The instrument was set in according to the following specifications: pump Perkin-Elmer series 200, detector UV-vis Perkin-Elmer LC 295, dilutor model 401 Gilson, automatic sampler Gilson model 231, HPLC column, and cartridge 10 m LiChro CART 250-4. The instrumental conditions were as follows: mobile phase, 45% acetonitrile and 55% water; flux in column, 1 mL/min; and injection volume, 20 µL. The estimate limit of detection (LOD) was 0.05 µg/mL. The calibration curve was prepared using a calibration standard (2,4 dinitrophenilhydrazone-FA specific, having a certificated concentration of 3.83 µg/mL aliquoted with a range of concentration between 0.05 and 3 µg/mL.

2.3 Questionnaire.

A questionnaire was administered to each subject, at the end of shift, to acquire information concerning the individual and clinical features (age, sex, place of residence,

hobbies, and therapies), smoking habits, profession (qualifications, seniority, and job-specific work), and the presence and use of environmental and personal devices to prevent air exposure and health risks.

2.4 Blood Sampling and N-methylenvaline analysis.

For each subject, 10 mL of venous blood were collected at the end of the working shift in a eparinized tubes, and immediately processed for the quantification of N-Methylenvaline, as described in our previous study (Bono et al., 2006). Briefly, the collected blood was centrifuged and washed with NaCl after that ultra pure water was added to lise erythrocytes. The emolysate was centrifuged and dialyzed in water; 2-propanol acidified, ethyl-acetate, and pentane were added to precipitate the globin which was subsequently dried and stored at -20°C. Through the application of Edman degradation method, modified by Törnqvist (Tornqvist et al., 1986), globin was derivatized with the reduction performed by Kautiainen (Kautiainen et al., 1989).

For each sample the procedure was applied twice. In the first application, 50 mg of dry globin was diluted in formammide and buffered with NaOH to obtain the N-methylvaline (N-methylvaline background). In the second application of the procedure, the globin diluted with formammide was followed by addition of $NaBH_4$ in buffered water (Tornqvist and Kautiainen, 1993):

$$\text{R-CH = N-Val-Hb} \xrightarrow{\text{Reduction } (NaBH_4)} \text{R-CH}_2\text{NH-Val-Hb}$$

N-terminal (valine) adducts — reduced N-terminal (valine) adducts

This procedure achieved the reduction of the soughed N-methylenvaline to stable N-methylvaline which adds up to background N-methylvaline (N-methylvaline background + N-methylenvaline reduced to N-methylvaline). Therefore, the N-methylenvaline, produced

by the alkylating activity of FA (the dependent variable that we intend to study), is obtained by subtracting the endogenous N-methylvaline from the total amount of N-methylvaline arising from the reduction procedure. In each sample and for both procedures, N-methyl-L-isoleucine as internal standard was dissolved in the formammide solution. Subsequently, each sample was derivatized with pentafluorophenyl isothiocyanate (PFPITC) and products were separated from globin by extraction with diethyl ether. The extract was dried, re-dissolved in toluene and washed with ultra pure water and Na_2CO_3. Lastly, N-methylvaline-pentafluorophenyl thiohydantoin (N-methylvaline-PFPTH) was isolated together with the internal standard by evaporation. The purification of samples was done with C-18 cartridges, before the GC/MS analysis.

To test linearity and reproducibility of analysis, a calibration curve was prepared with 50 mg of globin dissolved in formammide and added of calibration and internal standard (Bono et al., 1999; Bono et al., 2005).

2.5 GC-MS analysis.

Dried samples were dissolved in 100 µl of toluene and immediately analyzed. Analysis was performed using a Turbomass GC-MS by Perkin Elmer (Norwalk, CT, USA), equipped with a single quadrupole analyzer, with sources for electron impact and chemical ionization. The injection volume was 1 µl in pulsed splitless mode. The capillary column used was a Phenomenex, Zebron ZB-5, 30 m X 0,25 mm X 0,25 µm film thickness. Initial column temperature was 80°C, and increased by 22°C/min to 190°C, then 5°C/min to 235°C and lastly by 20°C/min to 300°C; isothermal at 300°C for 7 min. The transfert-line temperature was set at 200°C. Carrier gas was ultrapure He (1.0 ml/min).

The mass analyzer is a quadrupole type, setted in SIM mode (Selected Ion Monitoring), with a programmed selection of masses m/z as follows: selected ions for N-methylvaline: 290-310Th; selected ions for N-methyl-L-isoleucine: 334-314Th. The resulting chromatographic peaks were integrated to give the quantitative results. Coefficients of

variation (CV, %) calculated to test repeatability were 4,8%, for the internal standard and 4,3% for samples.

2.6 Urine collection and cotinine analysis.

A spot of fresh urine were collected the sampling day at the same time (the first of the morning) from each volunteer and stored at -80 °C to measure cotinine within the next 20 days. Cotinine measurement was carried out taking into account the possible role played by tobacco smoke in the human intake of FA, or of other independent variables. To 10 ml of urine transferred into a glass tube for extraction, 4 g of NaCl, 500 µl of NaOH (5M) and 10 µl of cotinine-d3 ($[^2H_3]$-Cotinine) as internal standard, were added. Subsequently, to extract cotinine from urine the follow procedure was executed twice: addiction of 2 ml of trichloromethane ($CHCl_3$), liquid-liquid extraction in a shaking wheel for 15 minutes, and a centrifugation for 10 min at 1000 g. The two resulting organic phase were collected and joined in a new glass tube and evaporated to dryness in a rotary evaporator. Calibration curve was performed by introducing increasing quantity of cotinine (2,5 µg/ml) in a urine pool of non smoking subjects, to obtain a concentration range from 10 to 100 ng/ml. The points of the calibration curve were then treated as the samples.

The dry residue was reconstituted by addiction of 200 µl of $CHCl_3$ and transferred into a vial for the GC-MS analysis as described below. Analysis was performed using an Agilent Technologies 6890 gas chromatograph, interfaced to an 5973 MSD Inert Agilent mass spectrometer. The injection system used was a Gerstel CIS4 PTV. Initial injection temperature was 50°C programmed at 10°C/s; final temperature was 300°C, held for 10 min. The injection volume was 1 µl in split mode. The capillary column used was a HP-5MS 30mx0.25mmx0.25µm film thickness. Initial column temperature was 50°C, and increased by 15°C/min to 300°C. Carrier gas was ultrapure Helium (1.0 ml/min). The transfer-line temperature was set at 280°C. The ionization source worked in electronic impact mode and the mass spectrometer operated in SIM mode. The monitored masses

for cotinine were: 98.00; 118.00; 176.00 Th. The monitored masses for the internal standard were: 101.00; 121.00; 179.00 Th. The resulting chromatographic peaks were integrated to give the quantitative results. Coefficients of variation (CV%) calculated to test repeatability were below 5% for cotinine and its internal standard.

2.7 Statistical analysis:

The non-normal distribution of data of airborne chemicals and urinary biomarkers tested through Kolmogorov-Smirnov test for each of the 3 populations (pathologists, workers in the plastic laminates industry, and controls) suggested a log-transformation of data to stabilize the variance and normalized the distributions. Student's t-test or the analysis of variance one-way ANOVA were then applied to compare two or more groups of independent samples, and Pearson's correlations were used to test the associations between variables. For ANOVA test, we assumed as *post-hoc* multiple comparison the equal variance of Tukey. Lastly, a p value of ≤0.05 (two-tailed) was considered significant for all of the tests. All the statistical analysis were performed using SPSS Package, version 17.0 (www.spss.com).

3. RESULTS

Within the 44 pathologists, subjects who work in reduction rooms (n. 19), where FA is directly used, show a statistically significant higher level of FA ($p < 0.0001$) than who works in other services (n. 25). Conversely, air-FA levels of these 25 other pathologists show similar concentrations to that of subjects recruited as controls, where they were therefore included. Thus, the resulting final groups were constituted by 19 pathologists (exposed to FA), 51 plastic laminates workers (exposed to FA), and 103 controls. **Table 1** shows some epidemiological general aspects of the three resulting groups of volunteers. At this concern, to excluding a possible confounding factor, the three age groups were compared and any statistical differences were recorded.

Tobacco smoke is a known source of several air pollutants, including FA, and many of them possess alkylating properties (Bono et al., 1997; Bono et al., 2005). To quantify the level of exposure to this important confounding factor, we have measured the urinary cotinine, a metabolite of nicotine, sensitive and specific biomarker of passive and active tobacco smoke intake. To test the epidemiological behaviour of this biomarker of exposure, correlation between numbers of cigarettes smoked *per* day and cotinine levels among all the 173 subjects was calculated by means of Pearson test. A strong and highly significant correlation was found: r = 0.814, $p < 0.0001$ **(figure 1)**.

To compare the biomarkers considered in this study among the three groups, analysis of variance one-way ANOVA was performed. Results show in **table 2** underline, for air-FA and N-methylenvaline, a general difference between the three levels of exposure investigated ($p < 0.0001$). A significant higher concentration in the two types of professional exposures (19 and 51) were demonstrated with the Tukey *post-hoc* test, in comparison to controls (n. 103). Conversely, the comparison between the two different professional exposures (19 *versus* 51) does not show significant differences, neither considering air-FA nor N-methylenvaline. This aspect highlights similar levels of exposure to FA and of this biological response in these two so different professions. Finally, by means of t-test, the merged data of the two professions (51 + 19 = 70) showed a significant higher levels of FA and N-methylenvaline than controls. Statistical analysis of cotinine of the whole population, underlines a general difference between the three levels of exposure investigated ($p < 0.002$) (**table 2**). In detail, comparing the 19 and the 51 exposed workers, the *post-hoc* Tukey test does not show difference in the tobacco smoke habit, as well as between the 19 pathologist and the 103 controls. Conversely, the 51 plastic laminate workers show a significant higher tobacco smoke habit with respect to controls. This finding is probably due to sociological reasons that leads to a greater use of tobacco in blue collars (the plastic laminate workers) than white collars (the pathologist

and controls). Lastly, t-test confirms a general higher level of tobacco assumption in exposed (n. 70) than controls (n. 103) ($p < 0.001$).

In order to excluding the tobacco smoke in the formation of N-methylenvaline due to the FA professional exposure, analysis of table 2 was repeated excluding active smokers and considering only the 120 non-smokers **(Table 3).** These last show a negligible and significantly lower levels of cotinine (10.6 ng/ml) in relation to that of active smokers (1093.1 ng/ml), $p < 0,0001$. Also in this case the whole population of non-smokers shows a general difference between the three levels of exposure investigated for air-FA ($p < 0.0001$) and for N-methylenvaline ($p < 0.0001$). In detail, *post-hoc* Tukey test highlights for FA a higher level of air-FA in the plastic laminated workers than pathologists ($p = 0.025$) and a higher exposure in the two professional exposed group than controls: 13 *versus* 79 and 28 *versus* 79 ($p < 0.0001$). *Post-hoc* Tukey test shows for N-methylenvaline any differences between the two type of professional exposure to FA for N-methylenvaline but significant differences comparing the two groups of exposed to FA with controls: 13 *versus* 79 ($p < 0.023$) and 28 *versus* 79 ($p < 0.0001$). Finally, t-test confirms a general higher level of N-methylenvaline in exposed (n. 70) than controls (n. 103) ($p < 0.001$).

To evaluate possible relationships between the three biomarkers, Pearson's correlation tests were carried out after the log-transformation of data. Analyzing the whole population, the air-FA appears to be significantly correlated with N-methylenvaline ($r = 0.678$; $p < 0.0001$) and weakly correlated also to cotinine ($r = 0.291$; $p = <0.0001$). The same correlations were carried out restricting the analysis to the 120 non-smokers and the findings were the same comparing air-FA and N-methylenvaline ($r = 0.658$, $p < 0.0001$). Finally, no correlation was recorded considering FA and cotinine.

In order to assess the response of the N-methylenvaline to the increase of exposure to air-FA, independently from the profession of the subjects, one-way ANOVA test was carried out considering the four averages of N-methylenvaline sub-grouped according to quartiles

of the 173 values of air-FA (**figure 2**). The resulting high significance of the test ($p < 0.0001$) shows a direct responsibility of air-FA exposure in the N-methylenvaline formation. In particular, *post-hoc* Tukey test underlines the linearity of the response of this biomarker as function of FA exposure, particularly at lower levels. In effect, the N-methylenvaline proves significant differences comparing fourth and third quartile ($p = 0.0001$), third and second ($p = 0.0001$), fourth and second ($p = 0.0001$), but not second and first (p = N.S.).

4. DISCUSSION AND CONCLUSIONS

The pathologists and the workers in plastic laminates are two professional figures that, although very different, both use formaldehyde. In the present work we confirm these two as two risky work activities due to the exposure to this chemical. Taking into consideration its toxicological and carcinogenic properties, the widespread use of FA in many working (and life) contexts represents a potential risk factor for human health when this pollutant is breathed. As previously mentioned, the actual ceiling limit value (TLV-C) suggested by ACGIH is 0.3 ppm (0.370 mg/m^3).

Our air-FA measurements show that during the sampling day, 1 pathologist and 2 workers in the plastic laminate industry were exposed to air-FA in higher concentration than the limit recommended by ACGIH, eventuality just described by some other authors (Costa et al., 2008; Orsière et al., 2006; Pala et al., 2008). At this concern, we can assume that these high values may be episodic and due to improper use of personal protective equipment, or due to poor functionality of the aspirators. In general, the pathologists show also an exposure level dependent by some specific tasks and by the small and not perfectly ventilated indoor environments in which workers act. In particular, pathologists show higher exposure to FA when operate under hood in direct contact with FA used for the reduction of the tissues. On the whole, air-FA exposure is preventable in both the two occupational environments through better aspirators of exhausts and environmental

ventilations, even if in a pathological wards seems to be easier, at least reducing quantities of FA adopted for reduction and getting the anatomical tissue under vacuum (Di Novi et al., 2010).

The differences of N-methylenvaline between exposed and controls and between smokers and non-smokers was considered true after verifying that N-metilvaline background levels, analyzed by one-way ANOVA for all four groups above mentioned, shown any statistical differences. Therefore, N-methylenvaline was evaluated attributing the observed differences solely to determinants studied and not to different background levels of N-methylvaline. Since formaldehyde represents an alkylating pollutant present in both occupational and environmental context, the formation of N-Methylenvaline as a early biological effect resulting from air-FA exposure, was evaluated comparing two groups of voluntary subjects different for their professional exposure to FA and a control group. Due to their FA exposure, pathologists and workers of plastic laminate are able to increase their levels of N-methylenvaline two times (**table 3**, the 120 non-smokers) or more times (**table 2**, the whole population including the smokers). Conversely, the two occupational exposures to FA show, practically, concentrations of the adduct of the same order of magnitude and not statistically different.

Aiming to consider the role of tobacco smoke in the methylation of human hemoglobin and in the consequent N-methylenvaline formation, number of cigarettes smoked *per* day was correlated to urinary cotinine value for each subject. Figure 1 demonstrated the high sensitivity and specificity of this internal dose marker through the very high “r” of correlation. In **table 2** analysis of cotinine in the three groups analyzed shows a significant higher exposure to tobacco smoke in the two categories of professional exposed to FA (Daher et al., 2010). This aspect, even if apparently justified by the presence of formaldehyde in tobacco smoke does not seem to be due to absorption of the gas by this route but to sociological reasons. however, among the pathologists, the number of

smokers so low (n=6) does not allow to attribute any role to the profession in highest concentration of cotinine than controls (Tukey *post-hoc* test not significant). Instead, a significant higher concentrations of cotinine in workers of plastic laminates (Tukey *post-hoc* test: $p < 0.001$) is probably dependent by their smoking habits, larger as number of smokers and as number of cigarettes smoked *per* day (typical of the blue collars) than in controls (all white collars).

Even if in minor magnitude, data on N-methylenvaline reported in **table 3** still show a significantly higher concentration of N-methylenvaline for pathologists non-smokers although to a lesser extent ($p < 0.023$) compared to the population including the smokers ($p < 0.0001$). Instead, the plastic laminate workers show a significant higher concentration of N-methylenvaline than controls, independently by tobacco smoke habit.

In conclusion, we can assume the positive relationship between air-FA professionally exposure and N-methylenvaline formation. The analysis of variance one-way ANOVA, performed for this purpose shows a general positivity of the model ($p < 0.0001$) and underlines the role of confounding factor for tobacco smoke. To test this last habit/exposure we used cotinine, which proved its sensitivity and specificity in showing the levels of exposure to tobacco smoke. In general, the two occupational exposures (pathologists and plastic laminate workers) show higher levels of exposure to formaldehyde when compared to controls, and similar levels when compared among themselves. Some criticisms can be summarized considering: a) the exposure variability, b) the different time described by the two measurements, namely the 7 hours of sampling for air-FA and 120 days for N-methylenvaline, the life of the erythrocytes, c) the N-methylenvaline variability (possible genetic polymorphisms).

5. ACKNOWLEDGMENTS

This study was made possible by a grant of from Piedmont Region, Italy (grants 2008). The authors kindly thank to doctors: prof. Bussolati, P. De Giuli, G. Cera, heads of the pathology wards of Torino, Alba, and Mondovì, G. Monchiero, D. Bogetti, P. Corino, M. Favilla, Mr. M. Cravero and all the workers (pathologists, plastic laminate workers, and subjects non exposed) for their voluntary and generous collaboration.

FIGURE CAPTIONS

Figure 1. Correlation between urinary cotinine and number of cigarettes/die referred to the whole population (n. 173). $p < 0.0001$, r = 0.814.

Figure 2. Box-plots of the four group of N-methylenvaline data according to the four quartile of air-FA exposure in µg/m^3: I quartile = 159.2-558.4, II = 66 One-way ANOVA test: $p = 0.0001$

REFERENCES

Bono R, Arossa W, Scursatone E, Meineri V and Gilli G. Tobacco smoke habits in a group of adolescents: responsibility of the cohabitants in the active and passive exposure. 1997;75:95-9.

Bono R, Vincenti M, Meineri V, Pignata C, Saglia U, Giachino O and Scursatone E. Formation of N-(2-hydroxyethyl)valine due to exposure to ethylene oxide via tobacco smoke: A risk factor for onset of cancer. Environ Res 1999;81:62-71.

Bono R, Vincenti M, Schiliro T, Traversi D, Pignata C, Scursatone E, Dotti G and Gilli G. Cotinine and N-(2-hydroxyethyl) valine as markers of passive exposure to tobacco smoke in children. J Expo Anal Env Epid 2005;15:66-73.

Bono R, Vincenti M, Schiliro T, Scursatone E, Pignata C and Gilli G. N-methylenvaline in a group of subjects occupationally exposed to formaldehyde. Toxicol Lett 2006;161:10-17.

Bono R, Degan R, Pazzi M, Romanazzi V and Rovere R. Benzene and formaldehyde in air of two winter Olympic venues of "Torino 2006". Environ Int 2010a;36:269-275.

Bono R, Romanazzi V, Munnia A, Piro S, Allione A, Ricceri F, Guarrera S, Pignata C, Matullo G, Wang PG, Giese RW and Peluso M. Malondialdehyde-Deoxyguanosine Adduct Formation in Workers of Pathology Wards: The Role of Air Formaldehyde Exposure. Chem Res Toxicol 2010b;23:1342-1348.

Carraro E, Gasparini S, Petrini T, Oitana P and Gilli G. Immune response prevalence to formaldehyde-human serum albumin molecular adduct in a healthy population. 1997;16:215-8.

Chaw YF, Crane LE, Lange P and Shapiro R. Isolation and identification of cross-links from formaldehyde-treated nucleic acids. 1980;19:5525-31.

Costa S, Coelho P, Costa C, Silva S, Mayan O, Santos LS, Gaspar J and Teixeira JP. Genotoxic damage in pathology anatomy laboratory workers exposed to formaldehyde. Toxicology 2008;252:40-48.

Daher N, Saleh R, Jaroudi E, Sheheitli H, Badr T, Sepetdjian E, Al Rashidi M, Saliba N and Shihadeh A. Comparison of carcinogen, carbon monoxide, and ultrafine particle emissions from narghile waterpipe and cigarette smoking: Sidestream smoke measurements and assessment of second-hand smoke emission factors. Atmos Environ 2010;44:8-14.

Di Novi C, Minniti D, Barbaro S, Zampirolo MG, Cimino A and Bussolati G. Vacuum-based preservation of surgical specimens: An environmentally-safe step towards a formalin-free hospital. Sci Total Environ 2010;408:3092-3095.

Doi S, Suzuki S, Morishita M, Yamada M, Kanda Y, Torii S and Sakamoto T. The prevalence of IgE sensitization to formaldehyde in asthmatic children. Allergy 2003;58:668-671.

Duhayon S, Hoet P, Van Maele-Fabry G and Lison D. Carcinogenic potential of formaldehyde in occupational settings: a critical assessment and possible impact on occupational exposure levels. Int Arch Occ Env Hea 2008;81:695-710.

Eller PM. Niosh Manual of Analytical Methods, 3rd Edition. 1984;188:6-Chas.

Goulding R. Formaldehyde (Environmental-Health Criteria 89) - WHO. J Roy Soc Health 1991;111:38-38.

Hauptmann M, Lubin JH, Stewart PA, Hayes RB and Blair A. Mortality from solid cancers among workers in formaldehyde industries. Am J Epidemiol 2004;159:1117-1130.

Heck HD, Casanova M and Starr TB. Formaldehyde Toxicity - New Understanding. Crit Rev Toxicol 1990;20:397-426.

IARC, International Agency for Research on Cancer - IARC monographs on the evaluation of carcinogenic risks to humans / World Health Organization, Formaldehyde, 2-butoxyethanol and 1-tert-butoxypropan-2-ol. 2006, 88, 1-478.

Kautiainen A, Tornqvist M, Svensson K and Ostermangolkar S. Adducts of Malonaldehyde and a Few Other Aldehydes to Hemoglobin. Carcinogenesis 1989;10:2123-2130.

Kelly TJ, Smith DL and Satola J. Emission rates of formaldehyde from materials and consumer products found in California homes. Environ Sci Technol 1999;33:81-88.

Kim H, Kim YD and Cho SH. Formaldehyde exposure levels and serum antibodies to formaldehyde-human serum albumin of Korean medical students. Arch Environ Health 1999;54:115-118.

Lu K, Ye WJ, Zhou L, Collins LB, Chen X, Gold A, Ball LM and Swenberg JA. Structural Characterization of Formaldehyde-Induced Cross-Links Between Amino Acids and Deoxynucleosides and Their Oligomers. J Am Chem Soc 2010;132:3388-3399.

McGwin G, Lienert J and Kennedy JI. Formaldehyde Exposure and Asthma in Children: A Systematic Review. Environ Health Persp 2010;118:313-317.

Miraglia N, Assennato G, Clonfero E, Fustinoni S and Sannolo N. [Biologically effective dose biomarkers]. 2004;26:298-301.

Nielsen GD and Wolkoff P. Cancer effects of formaldehyde: a proposal for an indoor air guideline value. 2010;84:423-46.

Orsière T, Sari-Minodier I, Iarmarcovai G and Botta A. Genotoxic risk assessment of pathology and anatomy laboratory workers exposed to formaldehyde by use of personal air sampling and analysis of DNA damage in peripheral lymphocytes. Mutat Res-Gen Tox En 2006;605:30-41.

Pala M, Ugolini D, Ceppi M, Rizzo F, Maiorana L, Bolognesi C, Schilio T, Gilli G, Bigatti P, Bono R and Vecchio D. Occupational exposure to formaldehyde and biological monitoring of Research Institute workers. Cancer Detect Prev 2008;32:121-126.

Saito Y, Nishio K, Yoshida Y and Niki E. Cytotoxic effect of formaldehyde with free radicals via increment of cellular reactive oxygen species. Toxicology 2005;210:235-245.

Schmid O and Speit G. Genotoxic effects induced by formaldehyde in human blood and implications for the interpretation of biomonitoring studies. Mutagenesis 2007;22:69-74.

Shaham J, Bomstein Y, Meltzer A and Ribak J. DNA-protein crosslinks, a biomarker of exposure to formaldehyde - In vitro and in vivo studies - Response. Carcinogenesis 1996;17:2098-2101.

Shaham J, Bomstein Y, Gurvich R, Rashkovsky M and Kaufman Z. DNA-protein crosslinks and p53 protein expression in relation to occupational exposure to formaldehyde. Occup Environ Med 2003;60:403-409.

Speit G, Schutz P, Hogel J and Schmid O. Characterization of the genotoxic potential of formaldehyde in V79 cells. Mutagenesis 2007;22:387-394.

Thrasher JD, Broughton A and Micevich P. Antibodies and Immune Profiles of Individuals Occupationally Exposed to Formaldehyde - 6 Case-Reports. Am J Ind Med 1988;14:479-488.

Thrasher JD, Broughton A and Madison R. Immune Activation and Autoantibodies in Humans with Long-Term Inhalation Exposure to Formaldehyde. Arch Environ Health 1990;45:217-223.

Tornqvist M, Mowrer J, Jensen S and Ehrenberg L. Monitoring of environmental cancer initiators through hemoglobin adducts by a modified Edman degradation method. 1986;154:255-66.

Tornqvist M and Kautiainen A. Adducted Proteins for Identification of Endogenous Electrophiles. Environ Health Persp 1993;99:39-44.

Zhang L, Steinmaus C, Eastmond DA, Xin XK and Smith MT. Formaldehyde exposure and leukemia: a new meta-analysis and potential mechanisms. 2009;681:150-68.

Zhitkovich A and Costa M. A Simple, Sensitive Assay to Detect DNA-Protein Cross-Links in Intact-Cells and Invivo. Carcinogenesis 1992;13:1485-1489.

Table 1. General description of some individual characteristics of all the 173 volunteers.

	Exposed to formaldehyde		
	pathologists	**plastic laminate workers**	**controls**
Subjects (n.)	19	51	103
Age (mean of years) *	38	40,9	39.1
Gender (m/f)	8/11	51/0	30/73
Smoking habits (yes/no)	6/13	23/28	24/79

*ANOVA test *p* = not significant

Table

Table 2. Air-FA, N-methylenvaline, and cotinine in the three category of air-FA exposure. In brackets the log-transformation.

	Exposed to formaldehyde		
	pathologists	**plastic laminate workers**	**Controls***
Number	19	51	103
FA µg/m^3 (log-transformed data)			
Min	14.9 (1.172)	49.1 (1.691)	5.3 (0.725)
Max	558.4 (2.747)	444.5 (2.648)	134.6 (2.129)
Median	189.6 (2.278)	195.1 (2.290)	29.8 (1.474)
Mean	188.6 (2.110)	210.1 (2.265)	41.4 (1.507)
SD	144.2 (0.442)	104.5 (0.235)	29.4 (0.321)
p ANOVA	<0.0001		
p (19 *vs* 51) tukey	N.S.		
p (19 *vs* 103) tukey	<0.0001		
p (51 *vs* 103) tukey	<0.0001		
p (70 *vs* 103) t-test	<0.0001		
N-methylenvaline nmol/gr of globin (log-transformed data)			
Min	22.13 (1.345)	30.80 (1.489)	0.7 (-0.156)
Max	1606 (3.206)	1242 (3.094)	894.3 (2.951)
Median	369.3 (2.567)	278.4 (2.445)	65.8 (1.818)
Mean	377.9 (2.365)	342.8 (2.420)	144.8 (1.739)
SD	362.7 (0.501)	259.2 (0.338)	204.8 (0.689)
p ANOVA	< 0.0001		
p (19 *vs* 51) tukey	N.S.		
p (19 *vs* 103) tukey	<0.0001		
p (51 *vs* 103) tukey	<0.0001		
p (70 *vs* 103) t-test	<0.0001		
cotinine ng/ml (log-transformed data)			
Min	3.1 (0.491)	1.0 (0)	0.8 (-0.097)
Max	1963 (3.293)	3306 (3.519)	1644 (3.216)
Median	8.0 (0.903)	45.0 (1.653)	10.0 (1)
Mean	409.1 (1.521)	704.3 (1.794)	129.6 (1.164)
SD	706.0 (1.076)	954.2 (1.278)	307.4 (0.867)
p (ANOVA)	0.002		
p (19 *vs* 51) tukey	N.S.		
p (19 *vs* 103) tukey	N.S.		
p (51 *vs* 103) tukey	0.001		
p (70 *vs* 103) t-test	0.001		

*Reference category

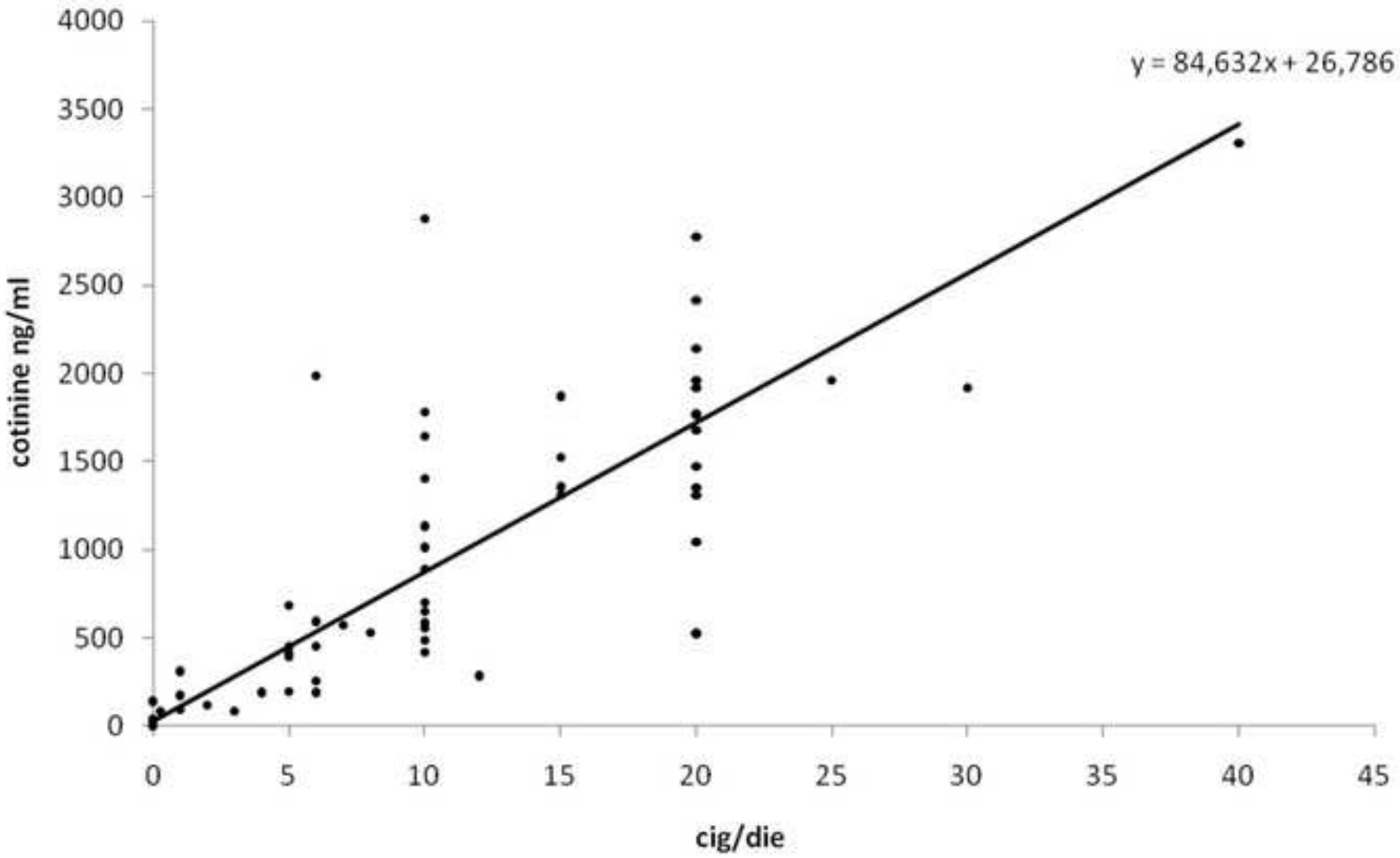
Table 3. Air-FA and N-methylenvaline in the three category of air-FA exposure of the 120 non-smokers. In brackets the log-transformation.

	Exposed to formaldehyde		
	pathologists	**Plastic laminate workers**	**Controls***
n. = 120	13	28	79
FA ug/m^3 (log-transformed data)			
Min	14.9 (1.173)	49.10 (1.691)	5.300 (0.725)
Max	335.5 (2.536)	444.5 (2.648)	134.6 (2.129)
Median	117.9 (2.072)	190.5 (2.279)	29.80 (1.474)
Mean	136.1 (1.966)	208.5 (2.248)	40.44 (1.502)
SD	106.7 (0.439)	115.9 (0.263)	28.33 (0.312)
p ANOVA	<0.0001		
p (13 *vs* 28) tukey	0.025		
p (13 vs 79) tukey	<0.0001		
p (28 vs 79) tukey	<0.0001		
p (41 *vs* 79) t-test	<0.0001		
N-methylenvaline nmol/gglobin (log-transformed data)			
Min	22.1 (1.345)	32.4 (1.511)	0.7 (-0.156)
Max	626.2 (2.797)	1214.0 (3.084)	894.3 (2.951)
Median	130.1 (2.114)	342.2 (2.254)	51.1 (1.708)
Mean	244.7 (2.182)	380.6 (2.476)	133.6 (1.694)
SD	211.7 (0.489)	258.9 (0.332)	197.5 (0.694)
P	<0.0001		
p (13 *vs* 28) Tukey	N.S.		
p (13 vs 79) Tukey	0.023		
p (28 vs 79) Tukey	<0.0001		
p (41 *vs* 79) t-test	<0.0001		

∗Reference category

Figure

Click here to download high resolution image



Figure

Click here to download high resolution image