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**15-F_{2t} Isoprostane as biomarker of oxidative stress induced by tobacco smoke and occupational exposition to formaldehyde in workers of plastic laminates**

Valeria Romanazzi\textsuperscript{a*}, Valentina Pirro\textsuperscript{b}, Valeria Bellisario\textsuperscript{a}, Giulio Mengozzi\textsuperscript{c}, Marco Peluso\textsuperscript{d}, Marco Pazzi\textsuperscript{b}, Massimiliano Bugiani\textsuperscript{a}, Giuseppe Verlato\textsuperscript{e}, and Roberto Bono\textsuperscript{a}.

\textsuperscript{a} Department of Public Health and Pediatric Sciences, University of Torino; Via Santena 5 bis, 10126 Torino; ITALY
\textsuperscript{b} Department of Chemistry, University of Torino; Via Pietro Giuria 5, 10126 Torino; ITALY
\textsuperscript{c} Clinical Chemistry Laboratory, San Giovanni Battista Hospital; C.so Bramante 88, 10126 Torino; ITALY
\textsuperscript{d} Cancer Risk Factor Branch, ISPO-Cancer Prevention and Research Institute; Via Cosimo il Vecchio 2, 50139 Firenze; ITALY
\textsuperscript{e} Unit of Epidemiology and Medical Statistics, University of Verona; Strada Le Grazie 8, 37134 Verona; ITALY

*CORRESPONDENCE TO:
Dr. Valeria Romanazzi, Ph.D.
University of Torino
Department of Environmental Health and Microbiology
Via Santena 5 bis,
10126 Torino - ITALY
Phone: (+39) 011-670-5818
Fax: (+39) 011-236-5818
e-mail: valeria.romanazzi@unito.it
ABSTRACT

**Background:** Formaldehyde (FA) is a suspected human carcinogen capable of inducing oxidative stress through different metabolic ways. FA may origin from tobacco smoke, several environmental sources, as well as occupational sources, like furnishing industries specialized in the production of pressed-wood and laminate products.

**Object:** Our aim was to investigate the role of tobacco smoke and occupational exposure to air-FA in the induction of oxidative stress status by comparing FA-exposed with non-exposed subjects who smoked or did not.

**Methods:** Enrollment of 105 subjects was made in an industry of plastic laminates, including both workers directly exposed to FA and non-exposed office personnel, as control group. 15-F_{2t} Isoprostane (15-F_{2t} IsoP), detected by ELISA technique and urinary cotinine, detected by GC-MS, were used for evaluating oxidative stress and tobacco smoke exposure, respectively. Air-FA levels were detected by GC-MS.

**Results:** FA concentrations were significantly higher in subjects occupationally exposed than those in controls. Smoking habits and air-FA exposures independently induce the formation of 15-F_{2t} IsoP and increase the oxidative stress level.

**Conclusions:** Our findings show, for the first time, that 15-F_{2t} IsoP presents a dependency from both the smoking habit and air-FA exposures, and consequently, that these breathable pollutants could be considered as two important independent risk factors in increasing the oxidative stress in human beings.
KEYWORDS

15-F₂₈ Isoprostane, oxidative stress, formaldehyde, tobacco smoke

ABBREVIATION

FA (formaldehyde), air-FA (professional exposure to formaldehyde), ELISA (enzyme-linked immuno-sorbent assay), GC-MS (gas chromatography–mass spectrometry), IsoP (15-F₂₈ Isoprostane), crea (creatinine).

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1. INTRODUCTION

Oxidants, anti-oxidants and free radicals often play a useful role in cellular signalling, control of vascular tones, cell generation, and defence against microorganisms. Their formation is the result of an evolutive process. The oxidative balance can be disturbed by several adverse environmental and/or occupational conditions, causing an uncompensated increase of pro-oxidants (Basu, 2010). As a consequence, oxidative modification of cellular macromolecules, induction of cell death by apoptosis or necrosis, damage of structural tissue may occur (Lykkesfeldt, 2007). Cells living under aerobic conditions are continuously exposed to a large number of oxidizing compounds from several endogenous and exogenous sources. Urban air is a typical exogenous mixture of chemical compounds that can induce carcinogenic activity, oxidative stress and toxicity. For example, several pollutants (PM, PAHs, benzene, etc.), emitted from cars (Rusconi et al., 2011) may: (i) inhibit cell-mediated immunity toward infectious agents, (ii) exacerbate respiratory allergy, (iii) cause DNA damage and (iv) induce lung cancer, after a long-term exposure (Rossner et al., 2008a, b). Many epidemiologists related the presence of an oxidative stress status in human beings with traffic emissions and air pollution (Rossner et al., 2011).

Formaldehyde (FA) is a breathable pollutant present in both living and working environments and is considered the prevalent carbonyl specie in urban atmosphere. FA is emitted by several primary sources (Bono et al., 2010a), it is formed in the troposphere by photochemical hydrocarbon-oxidation processes (Correa et al., 2003; Flyvholm and Andersen, 1993; Salthammer et al., 2010), and is also a component of tobacco smoke (Godish, 1989; Uchiyama et al., 2010). Production and use of FA in the manufacture of resins, paints, disinfectants, preservatives, and a variety of other chemicals or industrial products make this chemical compound potentially breathable in several (indoor) working and living environments (Kelly et al., 1999; Zhang et al., 2010b). Thus, FA is
nowadays a relevant topic to be studied in the environmental and occupational health studies (Nielsen and Wolkoff, 2010; Zhang et al., 2010b).

FA can induce local irritations, acute and chronic toxicity, and genotoxic and carcinogenic activity (IARC, 2006; Schmid and Speit, 2007; Speit et al., 2007), as confirmed by an increased incidence of nasopharyngeal cancer in some types of FA-exposed workers (Duhayon et al., 2008; Hauptmann et al., 2004), the relationship reported between FA and leukemia (Zhang et al., 2010a; Zhang et al., 2009), and a significant positive association between FA exposure and childhood asthma (McGwin et al., 2010).

Rager et al. have recently suggested that FA alters the expression of 89 microRNA (miRNA) profiles targeting mRNAs linked to numerous biological pathways, including those involved in the inflammatory response (Rager et al., 2011). In some molecular epidemiology studies, oxidative properties of FA have been reported in rats and humans (Bono et al., 2010b). Different metabolic pathways, i.e., the production of FA detoxifying enzymes after FA exposure, appeared to be involved. (Im et al., 2006; Kum et al., 2007). F₂-isoprostanes (F₂-IsoPs) are prostaglandin-like bioactive compounds formed in vivo from the free radical-catalyzed peroxidation of essential fatty acids. F₂-IsoPs are stable, robust molecules and are detectable in all human tissues and biological fluids analyzed, including plasma, urine, broncho-alveolar lavage fluid, cerebrospinal fluid, and bile. Based on their mechanism of formation, four F₂-IsoP regioisomers are generated. Compounds are denoted as 5-, 12-, 8-, or 15-series regioisomers depending on the carbon atom to which the side chain hydroxyl is attached; thus the compound under investigation belongs to the last regioisomer. Moreover, “²t” is for the – trans position of the oriented side chain to the prostane ring in the 15-

F₂t-IsoP (Roberts and Milne, 2009).

The metabolic fate of 15-F₂t-IsoP in humans has been assessed in previous studies (Mitsumoto et al., 2008; Morrow et al., 1999; Roberts and Morrow, 2000). Findings of these studies show the usefulness of 15-F₂t-IsoP to assess oxidant stress in vivo both in animal models and in humans.
In fact, they appear to be related to quite a number of human diseases, although a clear correlation between those pathological conditions and an oxidative stress status is far from being proven (Giustarini et al., 2009). Moreover, F2-IsoPs seem to play a role in acute and sub-clinical chronic inflammations (Basu et al., 2009). Since F2-IsoPs can be detected in urine samples, which sampling is possible with a non-invasive procedure, they have been proposed as a suitable biomarker for airways inflammation (Basu, 2008) and asthma diagnosis (Wedes et al., 2009), with the scientific community agreement.

Considering that FA and tobacco smoke have toxic and cancerogenic activities in different biological districts and may play a role in the onset of oxidative stress (Bono et al., 2010b; Campos et al., 2011), the potentiality of urinary 15-\( \text{F}_2 \text{t}	ext{OISO} \) as indicator of oxidative stress induced by FA and tobacco smoke was investigated in this study, for the first time.

Healthy subjects, working in one of the world’s leading manufacturers of decorative laminate located in the north-western part of Italy, were enrolled as volunteers. Workers exposed to FA and non-exposed office personnel, as control group, were statistically compared. To evaluate the FA exposure, air-FA was quantified, while tobacco exposure was measured by urinary cotinine, a metabolite of nicotine.

2. MATERIAL AND METHODS

2.1 Epidemiological sample.

51 healthy male workers of an industry of decorative laminates were recruited as subjects potentially exposed to FA. The decorative laminate sheeting is made of melamine and phenolic resins reacting with aldehydes during the thermosetting process. The resins are laminated onto layers of kraft paper topped with a decorative sheet. Other 54 male subjects were enrolled as controls from some offices and laboratories of the National Health System, where FA was not used.
All the subjects involved in this study live and work in Bra (town located in Piedmont region, Italy, counting 28,000 inhabitants, 250 meters a.s.l.) or in its immediate surroundings. Only males were selected for this epidemiological investigation, since gender is actually debated as confounding factor in the 15-F_{2t} IsoP formation, moreover only male workers are usually employed in plastic laminate industries. All subjects were informed about the objective of the study and gave a written informed consent, voluntarily.

All samplings were executed during summer. For each subject, air-FA samples were passively collected for an entire working shift (i.e., from 6 a.m. to 2 p.m.; about 8 h) in the middle of the working week (Wednesday). A spot of the first urine in the morning was also collected for urinary cotinine and 15-F_{2t} IsoP determinations.

2.2 Questionnaire.
At the end of the working shift, a questionnaire was administered to each subject to acquire information about individual and clinical features (age, place of residence, hobbies, and therapies), smoking habits, profession (qualifications, seniority, and job-specific work), the presence and use of environmental and personal devices to prevent air exposure and health risks. More in detail, the description of smoking habits for all subjects was established a-priori. Both subjects who never smoked and smokers who had ceased smoking from at least 1 month were classified as “non-smokers”, while subjects who smoked at least one cigarette per day were classified as “smokers”.

2.3 Air-FA sampling and analysis.
FA air sample was collected from each subject for the whole working shift (8 h) using a passive, personal air sampler working with radial symmetry (Radiello®), clipped near the breathing zone of the subject. Samplers were equipped with a specific sorbent tube containing 35–50 florisil mesh coated with 2,4-dinitrophenylhydrazine (DNPH). DNPH reacts with FA yielding 2,4-
dinitrophenylhydrazone which was subsequently quantified by a GC-MS method, within the
following 10 days. Cartridges were stored at -80°C before GC-MS analysis. Each sample was
eluted with 3 ml of toluene and shaken at room temperature for 15 min. An aliquot was transferred
into a vial and then injected in a capillary Agilent Technologies 6890 gas chromatograph, interfaced
to a 5973 MSD Inert Agilent single quadrupole mass spectrometer. A Gerstel CIS4 PTV injection
system utilized an initial temperature of 65 °C followed by heating at 5 °C/s; with a final
temperature of 320 °C, held for 10 min. The injection volume was 2 µl in splitless mode. The
capillary column used was a HP-5MS 30m×0.25mm×0.25µm film thickness. Initial column
temperature was 70°C, and increased at 20°C/min up to 220°C and at 30°C/min up to 300°C. The
carrier gas was ultrapure He (1.0 ml/min). The transfer-line temperature was set at 280°C. The mass
spectrometer operated in electron impact and Selected Ion Monitoring (SIM) mode. The monitored
m/z values for FA were 63, 79, 180 and 210, while the ones for the internal standard
(isovaleraldehyde-DNPH) were 177, 206, 223 and 166. The FA calibration curve was built by
fortifying 3 ml of toluene so as to obtain a concentration range from 0.10 µg/ml to 10 µg/ml. The
fortified toluene was analyzed as for the samples.

The limit of detection (LOD) was calculated as the concentration of the analyte that gives a signal
equal to the average background of the blank (Sblank) plus three times its standard deviation (LOD
= Sblank + 3*SD Sblank), while the limit of quantification (LOQ) was estimated as twice of the
LOD value.

LOD and LOQ were respectively 0.05 µg/ml and 0.10 µg/ml. Coefficients of variation (CV%)
calculated to test repeatability were below 5%.

2.4 Urine: collection and analyses.
Two aliquots of fresh urine were collected in the early morning and approximately at the same time from each volunteer, and stored at −80 °C prior to analysis, performed within 20 working days. One aliquot of urine was used for cotinine quantification, the other one for 15-F_{2t} IsoP determination.

First, urinary creatinine (crea) was determined by the kinetic Jaffé procedure (Bartels and Cikes, 1969) so as to normalize the excretion rate of urinary cotinine and 15-F_{2t} IsoP.

Urinary cotinine was measured in order to consider the possible role played by tobacco smoke for the subjects under study. 10 ml of urine was transferred into a glass tube and 4 g of NaCl, 500 μl of NaOH (5M) and 10 μl of cotinine-d3 (internal standard) were added. Subsequently, for two times, 2 ml of trichloromethane (CHCl₃) were added to the sample to perform liquid–liquid extraction which was carried out in a shaking wheel for 15 min. Sample was then centrifuged for 10 min at 1000 g and the resulting organic phase was collected in a new glass tube and evaporated to dryness in a rotary evaporator at room temperature. The dry residue was reconstitute in 200 μl of CHCl₃ and transferred into a conical vial for GC-MS determination (Bono et al., 2005). GC-MS analysis was performed using an Agilent Technologies 6890 gas chromatograph, interfaced to a 5973 MSD Inert Agilent mass spectrometer. A Gerstel CIS4 PTV injection system utilized an initial temperature of 50 °C followed by heating at 10 °C/s; with a final temperature of 300 °C, held for 10 min. The injection volume was 1 μl in the split mode. The capillary column used was a HP-5MS 30m×0.25mm×0.25 μm film thickness. The initial column temperature was 50 °C, increased at 15 °C/min up to 300 °C. The carrier gas was ultrapure Helium (1.0 ml/min). The transfer-line temperature was set at 280 °C. The mass spectrometer operated in electron impact and SIM mode.

The monitored m/z values for cotinine were: 98, 118, 176; while the ones for the internal standard were 101, 121, 179.

The cotinine calibration curve was built by fortifying a blank urine pool of non-smoking subjects, to obtain a concentration range from 0.02 µg/ml to 2 µg/ml. The fortified urine was extracted as for the samples. LOD and LOQ (calculated as previously described) were respectively 0.01 µg/ml and
0.02 µg/ml. Coefficients of variation calculated to test repeatability were below 5% for both cotinine and the internal standard.

15-F_{2t} IsoP in urine was measured by ELISA technique performed with a specific microplate kit (Oxford, MI, USA), according to manufacturer’s instructions. The declared limit of detection is 0.2 ng/ml and cross-reactivity can likely occur for some other isoprostanes: prostaglandin E_{2}, prostaglandin D_{2}, and arachidonic acid (< 0.01%), 9α,11β prostaglandin F_{2α} (4.1%), 13,14 dihydro-15-keto-PGF_{2α} (3.0%) and. Dilution 1:4 was adopted to achieve better accuracy in the competitive ELISA method.

Because of the high percentage of 15-F_{2t} IsoP excreted in human urine conjugated to glucuronic acid (over 50%), a preliminary incubation with β-glucuronidase for 2 h at 37°C was performed, in order to detect the entire quantity of 15-F_{2t} IsoP present in each urine sample.

2.5 Statistical analysis.

The analysis was performed by means of Stata 12 Statistical Package (StataCorp LP, Lakeway Drive, TX, USA). Table 1 shows some general epidemiological aspects of the resulting groups of volunteers (according to their FA exposure and smoking habits).

Where suggested by distributional diagnostic plots (symmetry plot, quantile plot) and descriptive statistic inspection (looking at variance stability among categories), appropriate linear transformation was applied on data. Untransformed 15-F_{2t} IsoP, urinary cotinine and air-FA values were compared among smoking and exposure categories by means of Nonparametric equality-of-medians test.

Multiple regression analysis with robust standard error estimation was performed to assess the relationship between urinary 15-F_{2t} IsoP values with urinary cotinine and air-FA concentrations. For each category, means and 95% confidence intervals of transformed 15-F_{2t} IsoP values were
calculated as margins of responses from predictions of the complete fitted model integrating over the covariates.

3. RESULTS

As reported in Table 1, subjects were classified according to their professional exposure to FA (exposed and non-exposed), and their smoking habits (smokers and non-smokers). As reported above, gender was not considered for describing the epidemiological sample population. Among the non-exposed subjects, 14 out of 54 are smokers (26%), while the percentage of smokers into the FA-exposed population grows up to 47%. The difference between the number of smokers into the FA-exposed and non-exposed categories appears to be statistically significant.

Means and standard deviations for age, urinary cotinine, urinary 15-F_{2t} IsoP and air-FA levels are reported in Table 2. Both the overall and single category values are reported.

From Table 2 outcomes, it is worth noting that the mean age of studied groups were comparable, and any statistical differences were recorded excluding “age of the subjects” as possible confounding factor. Considering the air-FA concentrations (ranged from 49 to 444 µg/m³ for FA-exposed subjects, and from 16 to 110 µg/m³ for the control group), three FA-exposed workers presented air-FA concentrations greater than 370 µg/m³, namely the absolute exposure limit that should not be exceeded at any time (TLV-ceiling), suggested by the American Conference Governmental Industrial Hygienists (ACGIH). Moreover, the air-FA concentrations for the FA-exposed workers appear to be significantly higher at 5% probability than those for the non-exposed subjects (Fisher exact p <0.0001). Conversely, urinary cotinine concentrations appear to be significantly higher for smokers than those for non-smokers (Fisher exact p <0.0001). Finally, considered the four kind of exposures, 15-F_{2t} IsoP levels appear to be higher for FA-exposed workers and smokers than those for non-exposed subjects and non-smokers, respectively; instead,
15-F_{2t} IsoP in the two groups having only one exposure shows intermediate mean levels (Fisher exact $p < 0.0001$).

Due to non-normal distribution and heteroskedasticity (the variance increases linearly with the mean), for further analysis appropriate log-transformation was applied to 15-F_{2t} IsoP levels, air-FA and cotinine concentrations, in order to stabilize the variance and normalize the distributions.

Table 3 shows the outcomes for the linear regression analyses performed considering the transformed 15-F_{2t} IsoP levels versus transformed air-FA and urinary cotinine concentrations, first singly (Mod.1 and Mod.2, respectively), and then in association between them (Mod.3).

Despite the higher air-FA values for smokers than those for non-smokers, there are no significant linear relationship between urinary cotinine and air-FA concentrations ($p=0.24$; $R=0.04$; $B=0.018$; C.I. $95\% = -0.054, 0.09$). The standardized regression coefficient (Beta) show the smoking contribution to IsoP biosynthesis as equal in subjects exposed and not exposed to FA; in particular, each increase of one S.D. unit both for Log-FA and Log-cotinine induces an Log-IsoP increase of approximately 35%.

15-F_{2t} IsoP levels increase significantly with both air-FA and urinary cotinine concentrations. The complete model confirms the multivariate relationship without substantial confounding effects. No age main effect significant at 5% level and no significant at 5% level interactions were detected.

Table 4 reports the means and 95% confidence intervals of 15-F_{2t} IsoP levels for all the studied categories, as estimated by the multiple regression analysis. The smoking effect is not significantly different from the FA exposure effect. In addition, the two effects seem to be less than additive in logarithmic scale (2.3 versus 3.3 expected, $p <0.05$).

Figure 1 shows the added-variable plots - or partial regression plot – in which the effect of adding one variable (FA or tobacco smoke exposure) to the model containing the other one can be evaluated. Although both regression lines are significantly different from 0, the residuals are very scattered around them, suggesting (as confirmed by the low R$^2$ value) that the model is able to
predict only part (20%) of the large 15-F_{2t} IsoP variability. Evident outliers (influential position or evident lack of residual normality distribution) from the regression lines are not evident.

4. DISCUSSION

In this study we investigated the relationship occurring between human exposure to FA and oxidative stress - measured by urinary 15-F_{2t} IsoP - in a population of male subjects. These subjects were recruited in a laminate industry in Piedmont region (Italy) and information about FA and tobacco smoke exposures was collected. They were classified as FA-exposed and non-exposed (control group) subjects and as smokers or non-smokers too.

If compared with controls, FA-exposed workers reported significantly higher concentrations of air-FA, although the mean concentration was lower than the ACGIH limit (370 µg/m^{3}) (Table 2). Good environmental work conditions and health status suggest that these high values of air-FA could essentially depend on improper work behaviour by those workers, rather than improper working conditions. On the other hand, non-exposed subjects presented an average air-FA concentration equal to 40 µg/m^{3} (Table 2) and 10% of these subjects even exceed 70 µg/m^{3} concentration, suggesting that other non-controlled domestic or environmental FA exposures may influence the results.

As previously mentioned, urinary 15-F_{2t} IsoP were investigated as indicators of an oxidative stress status due to FA and tobacco smoke exposure. The presence of aldehydes by lipid peroxydation (LPO) processes is one of the factors related to the “physiological” oxidative stress status (Qujeq et al., 2004; Sohal et al., 2002). FA exposure can increase the oxidative stress status with further release of non-specific cellular membrane inflammation mediators, especially at the upper respiratory tract (Bartsch and Nair, 2006). In the last decade, many experimental evidences suggested that cellular damage, mediated by reactive species, may play a crucial role in the
pathogenesis of respiratory disorders (Wedes et al., 2009; Yang and Omaye, 2009) - that cover a wide range of conditions: from simple acute effects due to tobacco smoke exposure in healthy subjects to chronic damages characterized by interstitial lung destruction (emphysema) or irreversible thickening.

The outcomes of this study globally show the effectiveness of urinary 15-F_2t IsoP in describing the \textit{in vivo} exposure to air-FA (Table 3 and Figure 1a). Age usually does not linearly correlate with 15-F_2t IsoP levels (Basu et al., 2009) and, within the studied population, this tendency appeared to be confirmed. The influence of tobacco smoke habits on 15-F_2t IsoP formation was also reported - as previously described by Campos (Campos et al., 2011) – although it appeared to be independent from the FA exposure effect (Table 3 and Figure 1b). Indeed, no statistically significant interaction was detected between FA and tobacco smoke exposures, at least in the logarithmic scale. In addition to FA, cigarette smoke contains quite a number of different carcinogenic compounds and reactive oxygen species (ROS) - due to tobacco pyrolisis - that potentially can lead to an oxidative stress status (Bhalla et al., 2009; Faux et al., 2009). Furthermore, FA and tobacco smoke exposures seem not to have an additive effect in logarithmic scale (multiplicative in natural scale) on the 15-F_2t IsoP formation and not to play a reciprocal confounding effect.

It is important to remark that urinary 15-F_2t IsoP is a non-specific oxidative stress indicator, and therefore, other exogenous stimuli, other than FA and tobacco smoke, may influence their urinary levels, as the wide dispersion of the residuals around the regression line (Figure 1a and b) and by the relatively low values of determination coefficient (<0.20 in the complete model) seem to suggest.

The means of 15-F_2t IsoP, as predicted by the multiple regression model (as shown in Table 4), are similar for FA-exposed/non-smokers and non-exposed/smokers, but the means of 15-F_2t IsoP for subjects exposed both to FA and tobacco smoke (FA-exposed/smokers) are less than expected for the additive model.
5. CONCLUSION

In this study, urinary 15-F$_2$IsoP levels were measured as indicator of oxidative stress status induced by FA and tobacco smoke exposures. Several endogenous/biological and exogenous/environmental or occupational factors, such as FA and tobacco smoke exposures - here investigated - affect the onset of oxidative stress and probably act synergistically in different ways. In this study a relationship between urinary 15-F$_2$ IsoP levels with FA and urinary cotinine concentrations has been reported, suggesting that primary prevention towards professional (and environmental) exposure to FA and tobacco smoke plays an important role for health care, as well as primary prevention towards several pathogenic conditions.

Finally, an enzyme-linked immunoassay procedure was adopted and proved to be appropriate for the purpose of this study. Comparison of the ELISA procedure with a GC-NCI-MS protocol for 15-F$_2$ IsoP determination can be considered as a perspective goal.
6. REFERENCES


Caption Figure 1: Partial regression plot: regression of Log (15-F2t IsoP) as dependent vs. (a) Log (Air-Fa) and (b) Log (Cotinine) as predictive variables.