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3-(2-deoxy- β -D-erythro-pentafuranosyl)pyrimido[1,2- α]purin-10(3H)-one deoxyguanosine adducts of workers exposed to asbestos fibers

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Abstract

Asbestos is the commercial name for a group of silicate minerals naturally occurring in the environment and widely used in the industry. Asbestos exposure has been associated with pulmonary fibrosis, mesothelioma, and malignancies, which may appear after a period of latency of 20–40 years. Mechanisms involved in the carcinogenic effects of asbestos are still not fully elucidated, although the oxidative stress theory suggests that phagocytic cells produce large amounts of reactive oxygen species, due to their inability to digest asbestos fiber. We have conducted a mechanistic study to evaluate the association between 3-(2-deoxy- β -D-erythro-pentafuranosyl)pyrimido[1,2- α]purin-10(3H)-one deoxyguanosine (M₁dG) adducts, a biomarker of oxidative stress and lipid peroxidation, and asbestos exposure in the peripheral blood of 327 subjects living in Tuscany and Liguria, Italy, stratified by occupational exposure to asbestos.

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Conflict of interest

The authors declare that they have no conflict of interest.

Adduct frequency was significantly greater into exposed subjects with respect to the controls. M₁dG per 10⁸ normal nucleotides were 4.0 ± 0.5 (SE) in 156 asbestos workers, employed in mechanic, naval, petrochemical, building industries, and in pottery and ceramic plants, versus a value of 2.3 ± 0.1 (SE) in 171 controls ($p < 0.001$). After stratification for occupational history, the effects persisted in 54 current asbestos workers, mainly employed in building renovation industry (2.9 ± 0.3 (SE)), and in 102 former asbestos workers (4.5 ± 0.7 (SE)), with p-values of 0.033, and <0.001 , respectively. A significant effect of smoking on heavy smokers was found ($p = 0.005$). Our study gives additional support to the oxidative stress theory, where M₁dG may reflect an additional potential mechanism of asbestos-induced toxicity.

Keywords

Asbestos; Tobacco smoking; Oxidative stress and lipid peroxidation; M₁dG adducts

1. Introduction

Asbestos is the commercial designation for a group of silicate mineral fibers that occur naturally in the environment. These fibers have high resistance to heat and to most chemicals and do not conduct electricity. Because of these properties, asbestos has been used widely in industry. Studies have shown that exposure to asbestos may increase the risk for lung cancer and mesothelioma, in addition to the risk for asbestosis and other benign lung and pleural diseases, such as pleural plaques (Case et al., 2011; Mossman et al., 2011). In 2012, the International Agency for Research on Cancer has classified all types of asbestos fibers as a known human carcinogens (Group 1) (IARC, 2012), based on the sufficient evidence for different types of cancers (Straif et al., 2009). In addition to lung cancer and mesothelioma, it has been suggested an association between asbestos exposure and gastrointestinal and colorectal cancers as well as an elevated risk for cancers of the larynx, pharynx, ovary, kidney, and esophagus (IARC, 2012).

Various investigations have identified underlying mechanisms (Toyokuni, 2009), yet the precise pathogenesis of asbestos-related diseases is not completely understood (IARC, 2012). Mechanisms involved in induced toxicity by asbestos are the production of reactive oxygen species (ROS), and increased oxidative stress levels. In particular, the “oxidative stress theory” (Toyokuni, 2009) expresses the hypothesis that phagocytic cells that adsorb asbestos fibers generate high levels of ROS due to their inability to digest minerals, and various studies showed that iron-containing asbestos fibers seem to be more carcinogenic. Malondialdehyde (MDA) is a reactive by-product of oxidative stress and peroxidation of lipids, which interact with DNA to form 3-(2-deoxy- β -D-erythro-pentafulanosyl)pyrimido[1,2- α]purin-10(3H)-one deoxyguano-sine (M₁dG) adducts (Jeong and Swenberg, 2005; Plastaras et al., 2000). M₁dG adducts, if not repaired, may block cell replication and cause base pair and frameshift mutations in repeated sequences (Marnett, 2002; Zhou et al., 2005). The generation of exocyclic M₁dG adducts have been associated with the loss of DNA methylation in the Long Interspersed Nuclear Element-1 repeated sequences, and in the promoter region of the inflammatory cytokine *interleukin-6* gene (Peluso et al., 2014). DNA adducts as well as altered patterns of DNA methylation are

important hallmarks of cancer risk, and clinical studies indeed showed that enhanced levels of exocyclic adducts may be linked to cancer development and tumor progression (Lauratti et al., 2002; Munni et al., 2004; Peluso et al., 2011; Wang et al., 1996). The generation of exocyclic M₁dG adducts reflects also the extent of exposure to environmental carcinogens (Bono et al., 2016, 2010; Peluso et al., 2013, 2010, 2015a, 2015b).

In the present study, we have examined the mechanisms of asbestos-induced toxicity in asbestos workers, employed in mechanic, naval, petrochemical, and building industries as well as in pottery and ceramic plants. We conducted a cross-sectional study aimed to compare the frequency of M₁dG adducts, a biomarker of oxidative stress and lipid peroxidation (Sorensen et al., 2003), in the peripheral blood of male workers exposed to asbestos versus a group of male controls, taking into consideration the occupational history. The levels of M₁dG adducts have been examined in 327 subjects using ³²P-postlabeling techniques (Vanhees et al., 2013).

2. Material and method

2.1. Study population

The underlying basic population consisted of asbestos workers that were enrolled from the Occupational Medicine Unit, within a follow up program of health surveillance of people previously exposed to asbestos in Tuscany (Cristaudo et al., 2010). The program consists of a complete medical examination including chest X-ray, spirometry and Diffusion Lung CO, and an accurate collection of working history, using a specific questionnaire. Asbestos workers were employed in various occupational fields, mostly in mechanic, naval and petrochemical ones, but also in building industries, pottery and ceramic plants. Control subjects were selected among volunteers, without history of previous occupation in industries entailing exposure to asbestos or other known or suspected carcinogens, by the local health service in Liguria region. Asbestos exposure occurring in occupational settings has been assessed according to job title and narrative report included in the questionnaires. The database included 327 male subjects, 55.2 ± 8.0 (SD) years of age. There were 156 asbestos male workers, 55.6 ± 9.9 (SD) years of age, 21% current smokers, and 171 male controls 54.9 ± 5.7 (SD) years of age, 19% current smokers. There was no statistically significant difference between the two groups with respect to age, gender and smoking habit. After occupational history stratification, there were 54 current asbestos, employed mainly in building renovation industry, 46.2 ± 8.3 (SD) years of age, and 102 former asbestos workers, 60.9 ± 7.0 (SD) years of age, with a mean of 11.5 ± 8.1 (SD) years of asbestos exposure. Study protocol was reviewed and approved by the General Hospital Institutional Committee. Trained personnel administered a standardized questionnaire to the subjects, in order to collect detailed information on demographic variables, life-style and occupational history. A signed informed consent to participate to the study was obtained from all the subjects before interview and blood collection.

2.2. Preparation of the reference adduct standard

A reference adduct standard was prepared: calf-thymus-DNA was treated with 10 mM MDA (ICN Biomedicals, Irvine, CA, USA), as previously reported (Bono et al., 2010). MDA

treated calf-thymus-DNA was diluted with untreated DNA to obtain decreasing levels of the reference adduct standard to generate a calibration curve.

2.3. Blood sampling and DNA extraction and purification

Peripheral blood (8 ml) was collected by routine venipuncture with heparinized vacutainer tubes, coded, and immediately stored at -80°C until use. DNA was extracted and purified using a method that requires digestion with ribonuclease A, ribonuclease T₁ and proteinase K treatment and extraction with saturated phenol, phenol/chloroform/isoamyl alcohol (25:24:1), chloroform/isoamyl alcohol (24:1) and ethanol precipitation (Munnia et al., 2007). DNA concentration and purity were determined using a spectrophotometer. Coded DNA samples were subsequently stored at -80°C until laboratory analyses.

2.4. Mass spectrometry analysis

DNA adducts in MDA treated calf-thymus-DNA sample were analyzed by mass spectrometry (Voyager DE STR from Applied Biosystems, Framingham, MA), as reported elsewhere (Wang et al., 2012).

2.5. DNA hydrolysis and nuclease P1 treatment

Blood DNA (2–4 μg) was hydrolyzed by incubation with micrococcal nuclease (21.45 mU/ μl) and spleen phosphodiesterase (6.0 mU/ μl) in 5.0 mM Na succinate, 2.5 mM calcium chloride, pH 6.0 at 37°C for 4.5 h. Hydrolyzed samples were treated with nuclease P1 (0.1 U/ μl) in 46.6 mM sodium acetate, pH 5.0, and 0.24 mM ZnCl_2 at 37°C for 30 min. After nuclease P1 treatment, 1.8 μl of 0.16 mM Tris base was added to the sample. The nuclease P1-resistant nucleotides were incubated with 7–25 μCi of carrier-free $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (3000 Ci/mM) and polynucleotide kinase T₄ (0.75 U/ μl) to generate ^{32}P -labeled DNA adducts in bicine buffer, 20 mM bicine, 10 mM MgCl_2 , 10 mM 1,4 dithiothreitol, 0.5 mM spermidine, pH 9.0, at 37°C for 30 min.

2.6. M₁dG adduct analysis

The generation of M₁dG adducts in the blood samples of asbestos workers and controls were measured using a modified version of the ^{32}P -postlabeling assay, that was developed in our laboratory for the specific detection of M₁dG adducts in the genomic DNA (van Helden et al., 2009). This ^{32}P -postlabeling version is highly sensitive for the analysis of oxidative DNA adducts caused from various environmental carcinogens, including ROS-generating chemicals. Briefly, ^{32}P -labeled samples were applied on polyethyleneimine cellulose thin-layer chromatography plates (Macherey-Nagel, Postfach, Germany). The chromatographic analysis of M₁dG adducts was performed using a low-urea solvent system known to be effective for the detection of low molecular weight and highly polar DNA adducts: 0.35 M MgCl_2 for the preparatory chromatography; and 2.1 M lithium formate, 3.75 M urea pH 3.75 and 0.24 M sodium phosphate, 2.4 M urea pH 6.4 for the two dimensional chromatography. Detection and quantification of M₁dG adducts and normal nucleotides, i.e. diluted samples that were not treated with nuclease P1 was performed by storage phosphor imaging techniques employing intensifying screens from Molecular Dynamics (Sunnyvale, CA, USA). The intensifying screens were scanned using a Typhoon 9210 (Amersham).

Software used to process the data was ImageQuant (version 5.0) from Molecular Dynamics. After background subtraction, the levels of M₁dG adducts were expressed as relative adduct labeling (RAL) = pixel in adducted nucleotides/pixel in normal nucleotides. The levels of M₁dG adducts were corrected across experiments based on the recovery of the MDA treated calf thymus-DNA adduct standard. Higher specificity of the ³²P-labelling technique is obtained using appropriated reference standards (Peluso et al., 1990), therefore, co-chromatography was utilized to confirm the identity of adduct spots detected in the study population, as previously described (Bono et al., 2010). Co-chromatography of blood DNA samples together with the MDA treated calf-thymus-DNA adduct standard was performed to identify the M₁dG adducts detected in the chromatograms of the asbestos workers using 2.1 M lithium formate, 3.75 M urea, pH 3.75 and 0.24 M sodium phosphate, 2.4 M urea, pH 6.4 or 0.24 M sodium phosphate, 2.7 M urea, pH 6.4.

2.7. Reference adduct standard by ³²P-postlabeling and mass spectrometry

There were 5.0 M₁dG adducts $\pm$ 0.6 per 10⁶ normal nucleotides in MDA-treated calf-thymus-DNA using the ³²P-postlabeling assay. The presence of the M₁dG adduct in this sample was confirmed by matrix-assisted laser desorption/ionization time-of-flight-mass-spectrometry (MALDI-TOF-MS). A calibration curve was set up by diluting this sample with untreated calf-thymus-DNA and measuring the decreasing amount of M₁dG, r-squared = 0.99.

2.8. Statistical analysis

The levels of M₁dG adducts were expressed as adducted nucleotides per 10⁸ normal nucleotides. M₁dG data were log transformed to stabilize the variance and normalize the distribution. Mean concentrations of M₁dG adducts of the study population across variable levels were compared by analysis of covariance, introducing into each model terms for age (continuous), education level (years), smoking habit (non-smokers, former smokers, and current smokers), environmental tobacco smoking (ETS), exposure status (asbestos workers and controls), as appropriate. Cumulative smoking exposure in current smokers was also determined in terms of pack-years by multiplying the number of years smoked with the average number of packs per day (Price et al., 1999). Based on pack-years of smoking, subjects were classified as non-smokers (0.0 pack-years), light smokers (0.1–20.0 pack-years), moderate smokers (20.1–40.0 pack-years), and heavy smokers (>40 pack-years). Then, the entire population was grouped according to occupational history in three categories: (a) current asbestos workers, (b) former asbestos workers, and (c) controls. Asbestos workers were also grouped according to quartiles for duration of asbestos exposure (years) before statistical analyses. All statistical tests were two-sided and $p < 0.05$ was considered to be statistically significant. The data were analyzed using SPSS 20.0 (IBM SPSS Statistics, New York, NY).

3. Results

To look for the genotoxic effects caused by environmental exposure to asbestos fibers, we have analyzed the amount of exocyclic M₁dG adducts in the peripheral blood of asbestos workers using ³²P-postlabeling techniques (Vanhees et al., 2013). A characteristic pattern of

M₁dG adduct spot was detected in the chromatographic plates of the study population. The intensity of M₁dG adduct spots was generally stronger in the chromatograms of the asbestos workers as compared to the controls. Taking into account the occupational history, more intense adducts spots were found in the chromatograms of the former asbestos workers as compared to controls, with intermediate values in those of the current asbestos workers. The presence of M₁dG adducts in the DNA extracted and purified from the blood samples of the asbestos workers was confirmed by co-chromatography.

Table 1 shows that the mean level of exocyclic M₁dG adducts was 3.1 **per** 10⁸ normal nucleotides in the study population. Values tended to be higher among older people and subjects with low education. When smoking habit was considered, the levels of M₁dG adducts tended to be higher in current and former smokers, 3.3 ± 0.6 (SE), and 3.4 ± 0.5 (SE) respectively, as compared to non-smokers, 2.7 ± 0.2 (SE), although a statistically significant difference was not observed in the study population. After considering cumulative smoking exposure, the highest levels of M₁dG adducts were detected in current smokers (>40 pack years). M₁dG adducts were 7.2 ± 2.4 (SE) in heavy smokers, versus a value of 2.7 ± 0.2 (SE) in non-smokers ($p = 0.005$). Any association with ETS was not found.

Subsequently, it has been then examined the effect of occupational asbestos exposures after stratification for both occupational asbestos exposure and occupational asbestos history. Our findings indicate that the levels of exocyclic M₁dG adducts in the peripheral blood of the study population were significantly higher, up to about 2-fold, into the group of exposed to asbestos with respect to the controls. As shown in Table 2, the levels of M₁dG adducts per 10⁸ normal nucleotides of asbestos workers and unexposed controls were 4.0 ± 0.5 (SE), and 2.3 ± 0.1 (SE), respectively, showing a significant difference between the two groups ($p < 0.001$), even after adjusting for confounding factors.

When tobacco smoking and occupational asbestos exposures were analyzed together (Table 2), significant relationships with asbestos were detected in non-smokers ($p = 0.008$), former smokers ($p = 0.022$), and current smokers ($p < 0.001$). After stratification for occupational history, the significant effects associated to asbestos exposure persisted in the current asbestos workers, 2.9 ± 0.3 , when compared to unexposed controls 2.3 ± 0.1 ($p = 0.033$), and was much stronger comparing controls to former asbestos workers, 4.5 ± 0.7 ($p < 0.001$).

Next, we have considered the relationship between tobacco smoking and asbestos history (Table 2). We found that the significant increase observed in the group of former asbestos workers, when compared to unexposed controls, persisted in the three strata of tobacco smokers, i.e., non-smokers ($p = 0.007$), ex-smokers ($p = 0.017$), and current smokers ($p = 0.016$), after correction for confounding factors. Conversely, no difference with unexposed controls was detected in the group of current asbestos workers. Additionally, although no significant evidence of interaction could be observed, the levels of exocyclic M₁dG adducts in current smokers is 67% higher when compared to non-smokers in the group of former asbestos workers (36% higher in smokers currently exposed to asbestos).

Finally, we have evaluated the relationship between M₁dG adducts and the duration of asbestos exposures to study the adduct persistence. Table 2 shows that the amounts of M₁dG adducts of asbestos workers among non-smokers tended to increase with the duration of exposure and to reach some kind of steady-state with longer period of occupational exposure. A significant trend was observed (p-value for trend = 0.004). Conversely, any significant association between the period of occupational asbestos exposure and the generation of M₁dG adducts was not observed in both former and current smokers.

4 Discussion

Asbestos-related chronic diseases are a common clinical problem and a major health concern worldwide, but the mechanisms by which exposure to asbestos fibers causes chronic diseases are not completely understood (IARC, 2012). Therefore, it is worthy examining the association between M₁dG adducts, a biomarker of oxidative stress and lipid peroxidation (Sorensen et al., 2003), and asbestos exposure in the peripheral blood of asbestos workers using ³²P-postlabeling techniques (van Helden et al., 2009). The use of biomarkers in epidemiological studies designed to examine environmental health hazard allows not only to design studies with lower number of individuals in comparison to cohort study, but also to predict the risk of cancer when appropriate biomarkers are evaluated.

The results of the current study indicated the ability of asbestos fibers to induce the production of a large amount of exocyclic M₁dG adducts in the peripheral blood of asbestos workers, employed in several industries, as demonstrated by the significantly higher amounts of M₁dG adducts of the asbestos workers with respect to the control group. A series of other reports have shown increased levels of genetic damage in asbestos workers using a biomarker approach (Afaghi et al., 2015; Govercin et al., 2014; Marczynski et al., 2000; Marini et al., 2011; Tagesson et al., 1993; Yoshida et al., 2001; Zhao et al., 2006). For example, increased amounts of 8-oxo-7,8-dihydro-2'-deoxyguanosine, a biomarker of oxidative stress and oxidative damage to DNA (Brancato et al., 2016), were found in workers who were exposed to asbestos fibers in occupational settings (Afaghi et al., 2015; Marczynski et al., 2000). An increase in micronucleus frequency was also observed in patients diagnosed with pleural plaques secondary to asbestos exposure (Govercin et al., 2014; Marini et al., 2011). Other reports have shown high frequencies of oxidative damage in the urine of asbestos workers (Tagesson et al., 1993; Yoshida et al., 2001).

As mentioned before, the oxidative stress theory (Toyokuni, 2009) suggests that asbestos exposure may cause altered cellular redox state and increased ROS levels, resulting in a persistent inflammation and macrophages and neutrophils activation in lung parenchyma, a condition which produces ROS that may diffuse to endothelial cells of lung microvascular endothelium (Bernstein et al., 2013), and subsequently to peripheral blood. ROS may indirectly cause genetic damage in addition to the activation of intracellular signaling pathways, stimulation of cell proliferation, and production of epigenetic alterations (IARC, 2012). The mechanisms underlying ROS generation by asbestos may be in part caused by the activation of alveolar macrophages and neutrophils during phagocytosis (Yoshida et al., 2001). In support to this hypothesis, we have previously demonstrated an association between oxidative burst of activated cells and M₁dG generation (Gungor et al., 2010b). Also,

Fenton-type reactions catalyzed by existing iron on the asbestos fibers (Ghio et al., 2004), alterations of the structure and function of cellular membrane (Shah et al., 2015), as well as intracellular glutathione decreased amounts (Afaq et al., 2000) may be involved in the asbestos-related ROS generation. Autophagy in epithelial lung cells seems also to be implicated in molecular mechanisms of asbestos toxicity (Lin et al., 2014). In particular, chrysotile-induced autophagy has been associated with altered signaling via the AKT/mTOR and JNK2 pathways but independent of apoptosis. mTOR pathway may also mediate protein translational function (Xu et al., 2016). Additionally, chrysotile asbestos-induced autophagy has been partially mediated by ROS- and TP53-dependent mechanisms (Lin et al., 2014).

Chronic asbestos-related diseases may appear after a period of latency of 20–40 years following initial exposure to asbestos fibers (IARC, 2012). When we have taken into consideration the occupational history of exposed asbestos workers, significant differences of exocyclic adduct levels were observed between both the current asbestos workers and the former asbestos workers in respect to the controls, with the highest levels of M₁dG adducts in the group of formerly asbestos workers. Nevertheless, this association persisted only in the group of former asbestos workers when tobacco smoking and occupational asbestos history were analyzed together. It is worthy of notice that the production of M₁dG adducts may reflect recent exposures to asbestos-containing materials as well as to asbestos fibers that have been accumulated in lung parenchyma, where those fibers may cause chronic inflammation, leading to the development of a marked oxidative stress. Increased lung fiber burden may play a main role in aggravating the levels of M₁dG of former exposed.

A next result of the present study shows that the production of M₁dG adducts tended to increase with the duration of occupational exposure to asbestos among the group of non-smoker former asbestos workers, with a leveling off of M₁dG adducts production at longer exposure, possibly related to the saturation of metabolic and DNA repair enzymes (Peluso et al., 2001). As regards on public health, it should be noted that the large differences were observed between formerly exposed workers, workers no longer exposed but requiring close medical observation for their condition of subjects at high risk of asbestos associated diseases, and the currently exposed workers. Despite the fact that conditions at workplace are very different, currently exposed asbestos workers still show higher levels of genetic damage than the controls. A possible reason should be the uncorrected use of safety equipment, but also the need of both revision and improvement of these devices. Anyhow, it's very important to make people understand the significance of the use of safety equipment, following the recommendations of safety manager and physician.

On the other hand, the underlying basic population was employed in mechanic, naval, petrochemical, and building industries and pottery and ceramic plants, so the individuals might have experienced co-exposures to other respiratory carcinogens, such as crystalline silica dust, heavy metals, volatile organic compounds, and polycyclic aromatic hydrocarbons. An example of this interaction comes from a study measuring levels of M₁dG adducts in a population resident nearby a large petrochemical complex in Sardinia island, Italy (Peluso et al., 2015a). In that study, the children living in the residential community around the industrial estate had increased levels of genetic damage in comparison to the controls, indicating that some of the air pollutants emitted from the petrochemical complex,

such as volatile organic compounds, were involved in the production of M₁dG adducts. Recently, we have conducted a cross-sectional study to evaluate the prevalence of M₁dG adducts in silica dust exposed workers in Tuscany region, Italy. In this study, significant increases of M₁dG adducts were found in the exposed workers with respect to the controls (Peluso et al., 2015a). Chronic inflammation as well as alcohol intake may result in increased production of ROS, which may subsequently induce the generation of M₁dG adducts (Gungor et al., 2010a; Munnia et al., 2004; Sapkota et al., 2014). Indeed, investigations in RAW264.7 macro-phages have recently shown the presence of high basal levels of exocyclic adducts (Wauchope et al., 2015). Therefore, we cannot exclude that the generation of M₁dG adducts observed in the asbestos workers may be aggravated when concurrent exposures to other volatile carcinogens occur in work-places or in the presence of chronic inflammation.

The role of smoking habit has been also investigated in the study, but despite the fact that current smokers had greater amounts of DNA damage in their blood, no significant influence of tobacco smoking on M₁dG adducts has been found in the study population. Nevertheless, when current smokers were classified based on cumulative smoking exposure (Price et al., 1999), significantly higher levels of M₁dG were observed in the group of heavy smokers as compared to non-smokers. Our results indicate that cumulative smoking exposure, expressed as pack-years, should be taken into account to properly evaluate the association between cigarette smoking and genetic damage. Furthermore, when tobacco smoking and occupational asbestos history were considered together, our data seem to suggest a possible synergistic effect of asbestos exposure on heavy smoking, at least among former asbestos workers. Indeed, although no significant evidence of interaction could be observed, the levels of exocyclic M₁dG adducts in current smokers were 67% higher when compared to non-smokers in the group of former asbestos workers (36% higher in smokers currently exposed to asbestos). These findings are somehow expected, since the association of M₁dG adducts frequency with tobacco smoking seems obvious because heavy smokers inhale a broad range of chemical carcinogens derived from tobacco and pyrolysis products, including ROS involved in oxidative stress and lipid peroxidation. Earlier investigations have reported higher M₁dG levels in smokers (Bono et al., 2010; Peluso et al., 2010; Zhang et al., 2002), but their results were not confirmed by other studies, in which not further significant differences associated to smoking status were reported (Bono et al., 2016; Everett et al., 2001; Leuratti et al., 2002; Peluso et al., 2010). Whereas it is possible that the effects of smoking were not completely discerned in the study population due to the low prevalence of heavy smokers, we have to point out that we have only analyzed a single DNA adduct, whereas cigarette smoking may induce many different types of DNA damage. Present findings may be influenced by the use of peripheral blood, which contains a mixture of different cellular types, most of the with a relatively short lifespan, from hours to weeks (Savela and Hemminki, 1991). Stronger associations with smoking habit were found from previous investigations which have been conducted using the nasal epithelium (Bono et al., 2016, 2010; Peluso et al., 1997; Zhao et al., 1997), which constitutes one of the portal of entry for the air carcinogens (Zhao et al., 1997). For instance, the levels of M₁dG in the nasal epithelium of office personnel were significantly correlated with the concentrations of urinary cotinine, a major proximate metabolite of nicotine.

Among the strengths of our study is the use of the ^{32}P -postlabeling assay, a sensitive technique for the detection of different kind of DNA damage, including M₁dG adducts (Phillips and Venitt, 2013), and the assay reproducibility (Peluso et al., 2005). Nevertheless, this method is unable to determine the precise DNA adduct structure, unless if the technique is combined with appropriate internal standards (Peluso et al., 1990), or coupled with MALDI-TOF-MS techniques (Bono et al., 2010; Peluso et al., 2011; Vanhees et al., 2013). Furthermore, M₁dG adducts are a form of genetic damage that is quite persistent, with a relatively long half-life of 12.5 days (Marnett, 1999). If unrepaired, M₁dG adducts may result in base pair substitutions, e.g. M₁dG $\rightarrow$ A $\sim$ T $>$ C, and frameshift mutations (VanderVeen et al., 2003), and inhibition of DNA transcription at adduct sites (Cline et al., 2010). Conversely, a limitation of the present study is the lack of dose-response analysis. However, retrospectively assessing asbestos exposures in workers with no longer asbestos exposures may be quite misleading, especially because of the recall bias. On the other hand, also exposure assessment in the current exposed asbestos workers may have been unreliable after the ban of exposure to asbestos that is considered illegal in Italy.

The observation that exocyclic M₁dG adducts were produced in the blood of the asbestos workers reflects an additional mechanism of asbestos-induced toxicity. Persistent genetic damage may ultimately contribute to the general decline of the physiological mechanisms designed to maintain cellular homeostasis, including apoptosis, senescence, uncontrolled proliferation and genome instability. Therefore, this cross-sectional study provides support to the hypothesis that asbestos fibers damage cells through an oxidative mechanism. These results highlight the need of interventions and policy initiatives in both current and former asbestos groups to further reduce the level of exposure to asbestos fibers and to prevent potential health hazards.

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References

- Afaghi A, Oryan S, Rahzani K, Abdollahi M, 2015 Study on genotoxicity, oxidative stress biomarkers and clinical symptoms in workers of an asbestos cement factory. *EXCLI J* 14, 1067–1077. [PubMed: 27004050]
- Afaq F, Abidi P, Rahman Q, 2000 N-acetyl L-cysteine attenuates oxidant-mediated toxicity induced by chrysotile fibers. *Toxicol. Lett* 117, 53–60. [PubMed: 11033233]
- Bernstein D, Dunnigan J, Hesterberg T, Brown R, Velasco JA, Barrera R, Hoskins J, Gibbs A, 2013 Health risk of chrysotile revisited. *Crit. Rev. Toxicol* 43, 154–183. [PubMed: 23346982]
- Bono R, Romanazzi V, Munnia A, Piro S, Allione A, Ricceri F, Guarrera S, Pignata C, Matullo G, Wang P, Giese RW, Peluso M, 2010 Malondialdehyde-deoxyguanosine adduct formation in workers of pathology wards: the role of air formaldehyde exposure. *Chem. Res. Toxicol* 23, 1342–1348. [PubMed: 20707408]
- Bono R, Munnia A, Romanazzi V, Bellisario V, Cellai F, Peluso MEM, 2016 Formaldehyde-induced toxicity in the nasal epithelia of workers of a plastic laminate plant. *Toxicol. Res* 5, 752–760.
- Brancato B, Munnia A, Cellai F, Ceni E, Mello T, Bianchi S, Catarzi S, Risso GG, Galli A, Peluso ME, 2016 8-Oxo-7,8-dihydro-2'-deoxyguanosine and other lesions along the coding strand of the exon 5

- of the tumour suppressor gene P53 in a breast cancer case-control study. *DNA Res.: Int. J. Rapid Publ. Rep. Genes Genomes* 23, 395–402.
- Case BW, Abraham JL, Meeker G, Pooley FD, Pinkerton KE, 2011 Applying definitions of asbestos to environmental and low-dose exposure levels and health effects particularly malignant mesothelioma. *J. Toxicol. Environ. Health B Crit. Rev* 14, 3–39. [PubMed: 21534084]
- Cline SD, Lodeiro MF, Marnett LJ, Cameron CE, Arnold JJ, 2010 Arrest of human mitochondrial RNA polymerase transcription by the biological aldehyde adduct of DNA, M1dG. *Nucleic Acids Res* 38, 7546–7557. [PubMed: 20671026]
- Cristaudo A, Foddìs R, Guglielmi G, 2010 Methodology and results of an experience of medical surveillance of people previously exposed to asbestos in Tuscany. *Giornale italiano di medicina del lavoro ed ergonomia* 32, 385–388. [PubMed: 21438305]
- Everett SM, Singh R, Leuratti C, White KL, Neville P, Greenwood D, Marnett LJ, Schorah CJ, Forman D, Shuker D, Axon AT, 2001 Levels of malondialdehyde-deoxyguanosine in the gastric mucosa: relationship with lipid peroxidation, ascorbic acid, and *Helicobacter pylori*. *Cancer Epidemiol. Biomark. Prev* 10, 369–376.
- Ghio AJ, Churg A, Roggli VL, 2004 Ferruginous bodies: implications in the mechanism of fiber and particle toxicity. *Toxicol. Pathol* 32, 643–649. [PubMed: 15513907]
- Govercin M, Tomatir AG, Evyapan F, Acikbas I, Coskun G, Akdag B, 2014 Elevated micronucleus frequencies in patients with pleural plaque secondary to environmental exposure to asbestos. *Genet. Mol. Res* 13, 598–604. [PubMed: 24615025]
- Gungor N, Knaapen AM, Munnia A, Peluso M, Haenen GR, Chiu RK, Godschalk RW, van Schooten FJ, 2010a Genotoxic effects of neutrophils and hypochlorous acid. *Mutagenesis* 25, 149–154. [PubMed: 19892774]
- Gungor N, Pennings JL, Knaapen AM, Chiu RK, Peluso M, Godschalk RW, Van Schooten FJ, 2010b Transcriptional profiling of the acute pulmonary inflammatory response induced by LPS: role of neutrophils. *Respir. Res* 11, 24. [PubMed: 20184723]
- IARC, 2012 Biological agents. volume 100 B. a review of human carcinogens. IARC Monogr. Eval. Carcinog. Risks Hum 100, 1–441.
- Jeong YC, Swenberg JA, 2005 Formation of M1G-dR from endogenous and exogenous ROS-inducing chemicals. *Free Radic. Biol. Med* 39, 1021–1029. [PubMed: 16198229]
- Leuratti C, Watson MA, Deag EJ, Welch A, Singh R, Gottschalg E, Marnett LJ, Atkin W, Day NE, Shuker DE, Bingham SA, 2002 Detection of malondialdehyde DNA adducts in human colorectal mucosa: relationship with diet and the presence of adenomas. *Cancer Epidemiol. Biomark. Prev* 11, 267–273.
- Lin Z, Liu T, Kamp DW, Wang Y, He H, Zhou X, Li D, Yang L, Zhao B, Liu G, 2014 AKT/mTOR and c-Jun N-terminal kinase signaling pathways are required for chrysotile asbestos-induced autophagy. *Free Radic. Biol. Med* 72, 296–307. [PubMed: 24735948]
- Marczynski B, Rozynek P, Kraus T, Schlosser S, Raithel HJ, Baur X, 2000 Levels of 8-hydroxy-2'-deoxyguanosine in DNA of white blood cells from workers highly exposed to asbestos in Germany. *Mutat. Res* 468, 195–202. [PubMed: 10882896]
- Marini V, Michelazzi L, Cioe A, Fucile C, Spigno F, Robbiano L, 2011 Exposure to asbestos: correlation between blood levels of mesothelin and frequency of micronuclei in peripheral blood lymphocytes. *Mutat. Res* 721, 114–117. [PubMed: 21238604]
- Marnett LJ, 1999 Lipid peroxidation-DNA damage by malondialdehyde. *Mutat. Res* 424, 83–95. [PubMed: 10064852]
- Marnett LJ, 2002 Oxy radicals: lipid peroxidation and DNA damage. *Toxicology* 181–182, 219–222.
- Mossman BT, Lippmann M, Hesterberg TW, Kelsey KT, Barchowsky A, Bonner JC, 2011 Pulmonary endpoints (lung carcinomas and asbestosis) following inhalation exposure to asbestos. *J. Toxicol. Environ. Health B Crit. Rev* 14, 76–121. [PubMed: 21534086]
- Munnia A, Amasio ME, Peluso M, 2004 Exocyclic malondialdehyde and aromatic DNA adducts in larynx tissues. *Free Radic. Biol. Med* 37, 850–858. [PubMed: 15304257]
- Munnia A, Saletta F, Allione A, Piro S, Confortini M, Matullo G, Peluso M, 2007 32P-Post-labelling method improvements for aromatic compound related molecular epidemiology studies. *Mutagenesis* 22, 381–385. [PubMed: 17716983]

- Peluso M, Castegnaro M, Malaveille C, Talaska G, Vineis P, Kadlubar F, Bartsch H, 1990 32P-postlabelling analysis of DNA adducted with urinary mutagens from smokers of black tobacco. *Carcinogenesis* 11, 1307–1311. [PubMed: 2387016]
- Peluso M, Amasio E, Bonassi S, Munnia A, Altrupa F, Parodi S, 1997 Detection of DNA adducts in human nasal mucosa tissue by 32P-postlabeling analysis. *Carcinogenesis* 18, 339–344. [PubMed: 9054626]
- Peluso M, Ceppi M, Munnia A, Puntoni R, Parodi S, 2001 Analysis of 13 (32)P-DNA postlabeling studies on occupational cohorts exposed to air pollution. *Am. J. Epidemiol* 153, 546–558. [PubMed: 11257062]
- Peluso M, Hainaut P, Airoidi L, Autrup H, Dunning A, Garte S, Gormally E, Malaveille C, Matullo G, Munnia A, Riboli E, Vineis P, investigators E, 2005 Methodology of laboratory measurements in prospective studies on gene-environment interactions: the experience of GenAir. *Mutat. Res* 574, 92–104. [PubMed: 15991349]
- Peluso M, Srivatanakul P, Munnia A, Jedpiyawongse A, Ceppi M, Sangrajrang S, Piro S, Boffetta P, 2010 Malondialdehyde-deoxyguanosine adducts among workers of a Thai industrial estate and nearby residents. *Environ. Health Perspect* 118, 55–59. [PubMed: 20056580]
- Peluso M, Munnia A, Risso GG, Catarzi S, Piro S, Ceppi M, Giese RW, Brancato B, 2011 Breast fine-needle aspiration malondialdehyde deoxyguanosine adduct in breast cancer. *Free Radic. Res* 45, 477–482. [PubMed: 21250785]
- Peluso M, Munnia A, Ceppi M, Giese RW, Catelan D, Rusconi F, Godschalk RW, Biggeri A, 2013 Malondialdehyde-deoxyguanosine and bulky DNA adducts in schoolchildren resident in the proximity of the Sarroch industrial estate on Sardinia Island, Italy. *Mutagenesis* 28, 315–321. [PubMed: 23446175]
- Peluso ME, Munnia A, Bollati V, Srivatanakul P, Jedpiyawongse A, Sangrajrang S, Ceppi M, Giese RW, Boffetta P, Baccarelli AA, 2014 Aberrant methylation of hypermethylated-in-cancer-1 and exocyclic DNA adducts in tobacco smokers. *Toxicol. Sci* 137, 47–54. [PubMed: 24154486]
- Peluso ME, Munnia A, Giese RW, Chellini E, Ceppi M, Capacci F, 2015a Oxidatively damaged DNA in the nasal epithelium of workers occupationally exposed to silica dust in Tuscany region, Italy. *Mutagenesis* 30, 519–525. [PubMed: 25771384]
- Peluso MEM, Munnia A, Giese RW, Catelan D, Rocca S, Farigu S, Leoni A, Bruzzzone M, Ceppi M, Biggeri A, 2015b Exocyclic DNA adducts in sheep with skeletal fluorosis resident in the proximity of the Portoscuso-Portovesme industrial estate on Sardinia Island, Italy. *Toxicol. Res* 4, 986–993.
- Phillips DH, Venitt S, 2013 DNA and protein adducts in human tissues resulting from exposure to tobacco smoke. *Int. J. Cancer* 131, 2733–2753.
- Plastaras JP, Riggins JN, Otteneider M, Marnett LJ, 2000 Reactivity and mutagenicity of endogenous DNA oxopropenylating agents: base propenals, malondialdehyde, and N(epsilon)-oxopropenyllysine. *Chem. Res. Toxicol* 13, 1235–1242. [PubMed: 11123964]
- Price JF, Mowbray PI, Lee AJ, Rumley A, Lowe GD, Fowkes FG, 1999 Relationship between smoking and cardiovascular risk factors in the development of peripheral arterial disease and coronary artery disease: Edinburgh Artery Study. *Eur. Heart J* 20, 344–353. [PubMed: 10206381]
- Sapkota M, Hottor TK, DeVasure JM, Wyatt TA, McCaskill ML, 2014 Protective role of CYP2E1 inhibitor diallyl disulfide (DADS) on alcohol-induced malondialdehyde-deoxyguanosine (M1dG) adduct formation. *Alcohol. Clin. Exp. Res* 38, 1550–1558. [PubMed: 24891074]
- Savelle K, Hemminki K, 1991 DNA adducts in lymphocytes and granulocytes of smokers and nonsmokers detected by the 32P-postlabelling assay. *Carcinogenesis* 12, 503–508. [PubMed: 2009595]
- Shah AA, Khand F, Khand TU, 2015 Effect of smoking on serum xanthine oxidase, malondialdehyde, ascorbic acid and alpha-tocopherol levels in healthy male subjects. *Pak. J. Med. Sci* 31, 146–149. [PubMed: 25878632]
- Sorensen M, Autrup H, Moller P, Hertel O, Jensen SS, Vinzents P, Knudsen LE, Loft S, 2003 Linking exposure to environmental pollutants with biological effects. *Mutat. Res* 544, 255–271. [PubMed: 14644327]
- Straif K, Benbrahim-Tallaa L, Baan R, Grosse Y, Secretan B, El Ghissassi F, Bouvard V, Guha N, Freeman C, Galichet L, Coglianov V, W.H.O.I.A.f.R.o.C.M. W. Group, 2009 A review of human

- carcinogens—part C: metals, arsenic, dusts, and fibres. *Lancet Oncol* 10, 453–454. [PubMed: 19418618]
- Tagesson C, Chabiuk D, Axelson O, Baranski B, Palus J, Wyszynska K, 1993 Increased urinary excretion of the oxidative DNA adduct, 8-hydroxydeoxyguanosine, as a possible early indicator of occupational cancer hazards in the asbestos, rubber, and azo-dye industries. *Pol. J. Occup. Med. Environ. Health* 6, 357–368. [PubMed: 8019198]
- Toyokuni S, 2009 Mechanisms of asbestos-induced carcinogenesis. *Nagoya J. Med. Sci* 71, 1–10. [PubMed: 19358470]
- VanderVeen LA, Hashim MF, Shyr Y, Marnett LJ, 2003 Induction of frameshift and base pair substitution mutations by the major DNA adduct of the endogenous carcinogen malondialdehyde. *Proc. Natl. Acad. Sci. U. S. A* 100, 14247–14252. [PubMed: 14603032]
- Vanhees K, van Schooten FJ, van Waalwijk van Doorn-Khosrovani SB, van Helden S, Munnia A, Peluso M, Briede JJ, Haenen GR, Godschalk RW, 2013 Intrauterine exposure to flavonoids modifies antioxidant status at adulthood and decreases oxidative stress-induced DNA damage. *Free Radic. Biol. Med* 57, 154–161. [PubMed: 23295412]
- Wang M, Dhingra K, Hittelman WN, Liehr JG, de Andrade M, Li D, 1996 Lipid peroxidation-induced putative malondialdehyde-DNA adducts in human breast tissues. *Cancer Epidemiol. Biomark. Prev* 5, 705–710.
- Wang P, Gao J, Li G, Shimelis O, Giese RW, 2012 Nontargeted analysis of DNA adducts by mass-tag MS: reaction of p-benzoquinone with DNA. *Chem. Res. Toxicol* 25, 2737–2743. [PubMed: 23113579]
- Wauchope OR, Beavers WN, Galligan JJ, Mitchener MM, Kingsley PJ, Marnett LJ, 2015 Nuclear oxidation of a major peroxidation DNA adduct, M1dG, in the genome. *Chem. Res. Toxicol* 28, 2334–2342. [PubMed: 26469224]
- Xu B, Gogol M, Gaudenz K, Gerton JL, 2016 Improved transcription and translation with L-leucine stimulation of mTORC1 in Roberts syndrome. *BMC Genom* 17, 25.
- Yoshida R, Ogawa Y, Shioji I, Yu X, Shibata E, Mori I, Kubota H, Kishida A, Hisanaga N, 2001 Urinary 8-oxo-7, 8-dihydro-2'-deoxyguanosine and biopyrrins levels among construction workers with asbestos exposure history. *Ind. Health* 39, 186–188. [PubMed: 11341550]
- Zhang Y, Chen SY, Hsu T, Santella RM, 2002 Immunohistochemical detection of malondialdehyde-DNA adducts in human oral mucosa cells. *Carcinogenesis* 23, 207–211. [PubMed: 11756243]
- Zhao C, Georgellis A, Flato S, Palmberg L, Thunberg E, Hemminki K, 1997 DNA adducts in human nasal mucosa and white blood cells from smokers and non-smokers. *Carcinogenesis* 18, 2205–2208. [PubMed: 9395222]
- Zhao XH, Jia G, Liu YQ, Liu SW, Yan L, Jin Y, Liu N, 2006 Association between polymorphisms of DNA repair gene XRCC1 and DNA damage in asbestos-exposed workers. *Biomed. Environ. Sci* 19, 232–238. [PubMed: 16944782]
- Zhou X, Taghizadeh K, Dedon PC, 2005 Chemical and biological evidence for base propenals as the major source of the endogenous M1dG adduct in cellular DNA. *J. Biol. Chem* 280, 25377–25382. [PubMed: 15878883]
- van Helden YG, Keijer J, Heil SG, Pico C, Palou A, Oliver P, Munnia A, Briede JJ, Peluso M, Franssen-van Hal NL, van Schooten FJ, Godschalk RW, 2009 Beta-carotene affects oxidative stress-related DNA damage in lung epithelial cells and in ferret lung. *Carcinogenesis* 30, 2070–2076. [PubMed: 19638427]

HIGHLIGHTS

- The genotoxicity of asbestos exposure was investigated in asbestos workers.
- The analysis was performed considering smoking as well as exposure duration.
- M₁dG generation was more evidenced in the former asbestos workers.
- Our study gives additional support to the oxidative stress theory.

Table 1

Mean levels of 3-(2-deoxy- β -D-*erythro*-pentafuranosyl) pyrimido[1,2- α]purin-10 (3H)-one deoxyguanosine (M₁dG) adducts per 10⁸ normal nucleotides in the peripheral blood according to demographic and life-style characteristics.

Peripheral blood M ₁ dG adducts			
	N ^a	Mean Levels $\pm$ SE	p-values ^b
<i>Mean exocyclic adducts levels</i>	327	3.1 $\pm$ 0.5	
<i>Age</i>			
52 years ^c	104	0.2 $\pm$ 2.4	
53–58 years	118	3.7 $\pm$ 0.6	0.118
59 years	105	3.0 $\pm$ 0.2	0.240
<i>Education (years)</i>			
5 ^c	23	1.4 $\pm$ 4.5	
6–8	125	3.4 $\pm$ 0.5	0.939
9–13	124	2.6 $\pm$ 0.2	0.440
>13	48	2.5 $\pm$ 0.2	0.627
<i>Tobacco smoking</i>			
Non-smokers ^c	136	0.2 $\pm$ 2.7	
Ex-smokers	121	3.4 $\pm$ 0.5	0.949
Current smokers	70	3.3 $\pm$ 0.6	0.327
<i>Cumulative smoking</i>			
Light smokers (0.1–20.0 pack-years)	20	3.1 $\pm$ 0.5	0.500
Moderate smokers (20.1–40.0 pack-years)	29	2.3 $\pm$ 0.3	0.869
Heavy smokers (>40 pack years)	12	7.2 $\pm$ 2.4	0.005
<i>Environmental tobacco smoke (ETS) exposure</i>			
At home			
Non-exposed to ETS ^c	145	3.1 $\pm$ 0.4	
Exposed to ETS	181	3.1 $\pm$ 0.2	0.854
At work			
Non-exposed to ETS ^c	174	3.1 $\pm$ 0.4	0.750
Exposed to ETS	152	3.1 $\pm$ 0.3	

^aSome figure do not add up to the total because of missing values.

^bP-values after adjusting for the confounding factors.

^cReference level.

Table 2

Mean levels of 3-(2-deoxy- β -D-*erythro*-pentafuranosyl)pyrimido[1,2- α]purin-10 (3H)-one deoxyguanosine (M₁dG) adducts per 10⁸ normal nucleotides in asbestos workers as compared to the controls, considering tobacco smoking, occupational asbestos exposures and history as well as duration of asbestos exposure among non-smokers.

Peripheral blood M ₁ dG adducts			
	^a N	Mean levels SE	p-values ^b
Occupational asbestos exposure			
Controls ^c	171	2.3±0.1	
Asbestos workers	156	4.0±0.5	<0.001
Tobacco smoking and asbestos exposures			
Non-smokers			
Controls ^c	74	2.1±0.2	
Asbestos workers	62	3.3±0.4	0.008
Ex-smokers			
Controls ^c	64	2.4±0.1	
Asbestos workers	57	4.0±0.5	0.022
Current smokers			
Controls ^c	33	2.3±0.1	
Asbestos workers	37	4.0±0.5	<0.001
Occupational asbestos history			
Controls ^c	171	2.3±0.1	
Former asbestos workers ^d	102	4.5±0.7	<0.001
Current asbestos workers ^e	54	2.9±0.3	0.033
Tobacco smoking and asbestos exposure history			
Non-smokers			
Controls ^c	74	2.1±0.2	
Former asbestos workers	46	3.7±0.5	0.007
Current asbestos workers	16	2.5±0.4	0.589
Ex-smokers			
Controls ^c	64	2.4±0.2	
Former asbestos workers	43	4.9±1.5	0.017
Current asbestos workers	14	2.5±0.5	0.701
Smokers			
Controls ^c	33	2.4±0.2	
Former asbestos workers	13	6.2±2.2	0.016
Current asbestos workers	24	3.4±0.3	0.120
Duration of asbestos exposure			

Peripheral blood M ₁ dG adducts			
<i>Non-smokers</i>			
Controls ^c	74	2.1±0.2	
<3 years	10	2.4±0.6	0.999
4–10 years	16	3.9±0.7	0.055
11–19 years	21	3.5±0.9	0.284
>20 years	15	3.3±0.3	0.096
<i>p-value for trend</i>			0.004

^aSome figure do not add up to the total because of missing values.

^b*P*-values after adjusting for the confounding factors.

^cReference level.

^dEmployed in mechanic, naval, petrochemical, and building industries and in pottery and ceramic plants.

^eMainly employed in building renovation.