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Increased micronucleus frequencies in surrogate and target cells from workers exposed to crystalline silica-containing dust

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Mining, crushing, grinding, sandblasting and construction are high-risk activities with regard to crystalline silica exposure, especially in developing countries. Respirable crystalline silica (quartz and cristobalite) inhaled from occupational sources has been reclassified as a human carcinogen in 1997 by the International Agency for Research on Cancer. However, the biological activity of crystalline silica has been found to be variable among different industries, and this has formed the basis for further *in vivo*/*in vitro* mechanistic research and epidemiologic studies. This study was conducted for genotoxicity evaluation in a population of workers (e.g. glass industry workers, sandblasters, and stone grinders) mainly exposed to crystalline silica in four different workplaces in Turkey. The micronucleus (MN) assay was applied both in peripheral blood lymphocytes (PBL) as a surrogate tissue and in nasal epithelial cells (NEC) as a target tissue of the respiratory tract. Our study revealed significantly higher MN frequencies in the workers ($n = 50$) versus the control group ($n = 29$) ($P < 0.001$) and indicated a significant effect of occupational exposure on MN induction in both of the tissues. For the NEC target tissue, the difference in MN frequencies between the workers and control group was 3-fold, whereas in peripheral tissue, it was 2-fold. Respirable dust and crystalline silica levels exceeding limit values and mineralogical/elemental dust composition of the dust of at least 70% SiO_2 were used as markers of crystalline silica exposure in each of the workplaces. Moreover, 24% of the current workers were found to have early radiographical changes (profusion category of 1). In conclusion, although the PBL are not primary target cells for respiratory particulate toxicants, an evident increase in MN frequencies in this surrogate tissue was observed, alongside with a significant increase in NEC and may be an indicator of the accumulated genetic damage associated with crystalline silica exposure.

Introduction

Exposure to crystalline silica occurs worldwide in a large variety of industries and occupations and can be explained by its extensive natural occurrence in the earth's crust as well as the wide use of materials in which it is contained. When the International Agency for Research on Cancer (IARC) reclassified crystalline silica as human carcinogen in 1997 (1), it became the subject of further epidemiologic and mechanistic studies. The target of many of these investigations was to ascertain the substantial conclusion by IARC that 'the carcinogenicity is not found in all industrial circumstances' (1). Epidemiological research mainly involved pooling strategies of former study data for quantitative risk assessments in order to evaluate the relationships among diseases and occupational exposures to crystalline silica (2–4). Mechanistic investigations using cell culture or animal models were performed to explain for the variability of the toxic potency of crystalline silica (5–10). Nowadays, it is well accepted that the biological responses of crystalline silica can be influenced by inherent characteristics of silica dust such as size, crystallinity, form and micromorphology as well as by the modification of its physicochemical properties, e.g. due to grinding, thermal treatment and etching (11,12).

The numerous health risks and diverse sources of exposure are features that make crystalline silica an important and challenging focus of research as evidenced from numerous reviews (13–22). To evaluate health risks by crystalline silica and silica-containing dusts, apart from traditional epidemiological studies and experimental studies, also molecular epidemiological studies have been conducted for the development and/or validation of biomarkers of exposure, effects or disease susceptibility (13). Examples relating to crystalline silica-containing dust exposure are studies in coal miners (23–25), foundry and pottery workers (26), granite foundry workers (27), cement plant workers (28), sandblasters (29) and stone crushers (30). With regard to crystalline silica exposure at workplaces, such molecular epidemiologic studies have proven to be important in gaining relevant human data under real exposure conditions, thereby bridging mechanistic experimental studies with the human situation. A major role of effect biomarkers is to obtain the early signs of upcoming disease and put forward strategies to prevent the development of irreversible outcomes such as occupational carcinogenic risk by the use of genotoxicity assays. In relation to exposure to crystalline silica-containing dusts, so far, such investigations have been performed using peripheral blood lymphocytes (PBL)/leukocytes, measuring chromosome aberrations and sister chromatid exchanges (30), micronuclei (25), DNA strand breaks (comet assay) (26) or the oxidative DNA adduct 8-hydroxydeoxyguanosine (27,31) in these cells. Although peripheral blood cells are generally accepted as an appropriate

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surrogate tissue in biomarker studies, one can debate whether they are also representative for epithelial cells of the respiratory tract, which form the principle target cells of inhaled particles.

The purpose of our study was to investigate the genotoxic potential of crystalline silica-rich dust exposure among workers involved in sandblasting, grinding and bagging activities. Therefore, the MN assay was applied in PBL as well as in nasal epithelial cells (NEC), both from the crystalline silica-exposed workers and from non-exposed officers as control group. These measurements were also related to workplace dust measurements and chest X-ray films. The cytokinesis-blocked MN (CBMN) assay in PBL has recently been proved to be predictor of cancer risk (32). It provides a convenient and reliable index of both chromosome breakage and chromosome loss (33) and thereby has become as an ideal and valuable biomarker in molecular epidemiology settings. Since NEC can be obtained easily by relatively non-invasive methods and are capable to indicate toxicity in actual target tissue by the MN assay, their usage in case–control studies is increasing (34–37).

Materials and methods

Subject selection

The study group consisted of male workers ($n = 50$) from different workplaces exposed to crystalline silica-containing dust. They were mainly involved in dusty jobs such as grinding, mixing, bagging and sandblasting. Workplace I was a mill with 8 workers doing grinding and bagging. Workplace II involved 17 outdoor sandblasters whilst, workplace III had 4 indoor sandblasters. Workplace IV consisted of 11 current and 10 former (i.e. workers who have worked at these dusty departments previously and shifted to other departments in the same factory) workers in a glass manufacturing plant, mainly involved in grinding and mixing departments. The working schedule in each workplace was 40 h/week. A control group from male office workers ($n = 29$) without dust exposure was matched for age, gender and smoking status to the workers. Each participant completed a detailed questionnaire which included questions on working conditions, possible confounding factors, smoking, alcohol consumption and nutritional habits. The sampling was carried out in the summer of 2004. Written informed consent was obtained from the donors.

Biological sampling and MN analysis

MN analysis. Chemicals. Roswell Park Memorial Institute (RPMI) medium, fetal calf serum (FCS), phytohaemagglutinine and L-glutamine were purchased from Biological Industries (Kibbutz Beit Haemek, Israel). KCl and cytochalasin B were from Sigma (St Louis, MO), dimethyl sulfoxide (DMSO), Giemsa–May Grünwald, acetic acid, methanol, ethanol, HCl, sodiumbisulphite, pararosaniline, fast green and xylene were purchased from Merck (Darmstadt, Germany). CBMN assay in PBL. From each subject, 10 ml of blood was obtained by venipuncture. Within the same day, the blood samples were transferred to the laboratory and incubated at 37°C for 72 h. Briefly, whole blood (0.5 ml) from the donors was added to 4.5 ml culture medium composed of RPMI supplemented with 20% FCS, 2% phytohaemagglutinine and 0.4% L-glutamine. Cell culture samples were incubated at 37°C for 72 h. Binucleated cells were accumulated by adding cytochalasin B in a final concentration of 6 μg/ml (cytochalasin B in DMSO) at 44 h. At the end of 72 h, samples centrifuged and re-suspended in 0.075 M KCl at 4°C for 3 min for hypotonic treatment. Cells were fixed by methanol–acetic acid (3:1; v/v) three to four times, dropped onto cold slides, air-dried and stained with Giemsa–May Grünwald. The induction of MN was evaluated by scoring a total of 1000 binucleated cells per subject at ×400 magnification (Zeiss Axioscope Microscope, Goettingen, Germany). In CBMN method, micronuclei are scored in binucleated cells according to the scoring and identification criteria of Fenech (38). The scoring of MN was performed on the coded slides. MN frequencies were expressed as MN per thousand (‰) binucleated PBL.

MN assay in NEC. NEC were obtained by scraping from the inner turbinate using a cytological brush by an otorhinolaryngologist. The collected cells were smeared directly onto wet slides and then left to dry. Within the same day, the slides from nasal brushes were transferred to the laboratory and fixed in 80% methanol and air-dried. Subsequently, the slides were stained according to Sarto *et al.* (39). Slides were treated in 1 N HCl for 2 min at room temperature, 10 min at 60°C and 2 min at room temperature, respectively. After washing in distilled water, the slides were left to dry. Feulgen staining (1 g pararosaniline in 100 ml distilled water) was then carried on at room temperature in the dark for 90 min and followed by 5 min washing in distilled water. The slides were then stained in fast green solution (0.5 g fast green in 95% ethanol) for 10 sec, kept in xylene for 10 min and finally air-dried. Evaluation was done by light microscopy at ×1000 magnification (Zeiss Axioscope 2 Microscope). For each subject, 3000 NEC were counted for the presence of MN. Scoring and identification criteria were according to Tolbert *et al.* (40). The scoring of MN was performed on the coded slides. MN frequencies were expressed as MN per thousand (‰) NEC.

Radiological examination

Chest X-ray films of the subjects were obtained at the same week of biological sampling for silicosis diagnosis. The films were given to the two independent B film readers for evaluation according to the International Labour Office classifications. The consensus of these results has been used for further evaluations.

Exposure measurements

Collection and measurement of the dust samples were performed by the National Occupational Safety and Health Department of Ministry of Work of Turkey. The dust samples were collected from the level of respiration on the daily shift (8 h) of workers by dust collecting equipment (Casella AFC 123) at the same day when the biological sampling was carried out. The pore size of the filters (cellulose nitrate filter, Sartorius AG, Goettingen, Germany) was 5 μm. Respirable total dust concentrations (milligrams per cubic metre) were measured by the gravimetric analyses, and the percentage of silica of all samples was measured by Infrared Spectrophotometer according to the National Institute for Occupational Safety and Health method 7602. Additionally, the mineralogical analysis (Na_2O, MgO, Al_2O_3, SiO_2, P_2O_5, K_2O, CaO, TiO_2, MnO and Fe_2O_3) of the sandblasting dust samples from workplaces has been carried out by General Directorate of Mineral Research and Exploration (MTA) of Turkey.

Statistical analysis

Statistical analysis was performed by SPSS 11.5 (SPSS Inc., Chicago, IL). Normal distribution of the variables was tested by Shapiro–Wilk test. Data expressed as mean ± standard deviation for continuous data and nominal variables have been given as observation numbers and/or as percentage (%). Student's *t*-test and one-way analysis of variance (ANOVA) were used for the comparison of the two independent groups and more than two groups, respectively. In case the one-way ANOVA analysis was significant, it was followed by post hoc Tukey test. Nominal data were evaluated by χ^2 test. The magnitude of the linear relationship was calculated by the Pearson correlation '*r*' value. In order to define the risk factors of outcome variables (MN frequencies in PBL and NEC), multiple linear regression analysis was used. Confidence intervals were calculated for each independent variable. $P < 0.05$ results were accepted as statistically significant.

Results

General characterization of the study subjects and workplaces

The general characteristics of the study subjects and the workplaces are shown in Table I. Gender was not a confounding factor for our study since study population consisted of men only. Age and smoking habits (yes/no) were similar between subjects (see Table I). The safety precautions asked to be taken by the workers included the use of masks, gloves, special clothes and respiratory machines. Of the study group, 86% wore masks, 74% wore gloves, 4% had special clothes and 24% had special respiratory equipment.

The mineralogical/elemental analysis of sandblasting dust samples from workplace II (Na_2O = 1.9%, MgO = 1.3%, Al_2O_3 = 8.8%, SiO_2 = 70.5%, P_2O_5 = 0.1%, K_2O = 2.2%, CaO = 7.0%, TiO_2 = 0.4%, MnO = 0.1%, Fe_2O_3 = 2.2%)and workplace III (Na_2O = 2.1%, MgO = 0.7%, Al_2O_3 = 9.1%, SiO_2 = 70.1%, P_2O_5 = 0.1%, K_2O = 2.1%, CaO = 7.5%, TiO_2 = 0.3%, MnO = 0.1%, Fe_2O_3 = 2.0%) revealed percentages of SiO_2 which exceeded 70%. Al_2O_3 and CaO were identified as the second and the third most abundant constituent, respectively. A piece of rock obtained from workplace I was analysed as being 100% quartz by MTA. For quality assurance, the crystalline silica dust used in glass industry should have at least 95% quartz (crystalline silica) (Working Group Report of Raw Materials in Industry of

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Turkey, 2001). As such, the dust in workplace IV can be assumed as containing this percentage of crystalline silica. Overall, it can be assumed that all the workplaces in our study were dusty ones, with crystalline silica percentages in the dusts of at least 70%.

MN frequencies in PBL and NEC, exposure measurements and radiological examinations

The MN frequencies measured in the samples of the workers were found to be significantly increased when compared to those of their controls, both in the PBL and the NEC ($P < 0.001$). Stratification according to smoking status and to being former or current workers also revealed significant MN increase of workers versus control group in both of the cell types (Table II). Furthermore, in each group (current workers, former workers and total), higher MN frequencies for the PBL and NEC were found in smoker workers than non-smoker workers as well as in smoker controls than non-smoker controls. In this grouping, the only significant finding was in smoker workers versus non-smoker workers in NEC (see Table II). According to the job title, either being sandblaster or grinder, there was no difference among workers in the MN frequencies of the PBL and NEC ($P = 0.921$; $P = 0.981$, respectively). Concurrently, there was no significant difference for the MN frequencies among the workplaces ($P > 0.05$), except the workers in workplace I versus workers in workplace IV with significant increase in NEC MN frequency ($P = 0.035$). Age was significantly correlated to MN frequencies in PBL as well as in NEC ($r = 0.36$, $P = 0.009$; $r = 0.39$, $P = 0.005$, respectively) for workers, while there was no statistically significant association within the control group ($P > 0.05$). Moreover, for the current workers, the duration of exposure was significantly correlated to MN frequencies in PBL as well as NEC ($r = 0.39$, $P = 0.013$; $r = 0.36$, $P = 0.021$, respectively). Exposure durations for all workers were also correlated with MN frequencies in the PBL ($r = 0.36$, $P = 0.01$) while it was not significant with the NEC ($P > 0.05$). The cumulative dust exposures of the workers were estimated by multiplying the duration of exposure of a worker (year) by each workplace's mean dust measurements (respirable total dust or SiO_2 percent). The cumulative SiO_2 percent exposure amount in the workplace I and workplace II was significantly higher than the workplace IV ($P = 0.018$ and $P = 0.009$, respectively). The correlation between MN frequency and cumulative SiO_2percent exposure of the workers was statistically significant for both PBL and NEC ($r = 0.37$, $P = 0.021$; $r = 0.34$, $P = 0.032$, respectively), whereas the relationship was not significant for the cumulative respirable total dust exposure ($P > 0.05$). Also, a significant correlation between the MN frequencies in PBL and NEC ($r = 0.73$, $P < 0.001$) was found for all study subjects.

Occupational dust exposure had the most significant effect on PBL and NEC MN frequencies of our study population (Table III). Age had also significant effect on the MN frequencies in both cell types, whereas smoking had significant effect only for NEC MN frequency (see Table III).

To determine the most effective parameters on MN frequencies of workers, cumulative SiO_2 percent exposure, duration of exposure, age and smoking (i.e. those found to have effect on the MN frequencies in univariate statistics) were included to the regression analysis (Table IV). Significant effects of smoking as well as of age were found on the MN frequency in the NEC (Table IV).

With regard to the radiological evaluation, agreement between the B readers was found 0.87 with a kappa value of 0.658, indicating compatibility. Among all X-ray films evaluated, 43 were found to be suitable for evaluation. Thirteen of the workers

Table I. Demographic characteristics of the study population

	Controls ($n = 29$)	Workers ($n = 50$)		
		Current	Former	Total
Age[a] mean ± SD	33.66 ± 7.15	35.10 ± 8.19	37.50 ± 8.76	35.58 ± 8.27
Age < 40 (n)[b]	20	27	6	33
Age ≥ 40 (n)	9	13	4	17
Employment (years) mean ± SD		7.79 ± 7.30	2.96 ± 1.52	6.82 ± 6.83
Smoker (n)[b]	11	18	6	24
Non-smoker	18	22	4	26

SD, standard deviation.
[a]$P < 0.05$; controls versus each worker group.
[b]$P < 0.05$; controls versus each worker group (χ^2 test).

Table II. PBL and NEC MN frequencies among workers and control group

	Exposed	n	Control	n	P
PBL MN (‰)					
All					
Non-smokers	12.00 ± 2.95	24	4.91 ± 3.15	11	<0.001
Smokers	12.92 ± 5.07	26	6.00 ± 2.68	18	<0.001
Total	12.48 ± 4.17	50	5.59 ± 2.86	29	<0.001
Current workers					
Non-smokers	12.11 ± 3.10	18	4.91 ± 3.15	11	<0.001
Smokers	12.95 ± 5.48	22	6.00 ± 2.68	18	<0.001
Total	12.58 ± 4.53	40	5.59 ± 2.86	29	<0.001
Former workers					
Non-smokers	11.67 ± 2.66	6	4.91 ± 3.15	11	<0.001
Smokers	12.75 ± 1.89	4	6.00 ± 2.68	18	<0.001
Total	12.10 ± 2.33	10	5.59 ± 2.86	29	<0.001
NEC MN (‰)					
All					
Non-smokers	7.28 ± 1.75	24	2.70 ± 1.41	11	<0.001
Smokers	9.25 ± 2.47*	26	2.92 ± 1.76	18	<0.001
Total	8.30 ± 2.35	50	2.84 ± 1.61	29	<0.001
Current workers					
Non-smokers	7.44 ± 1.71	18	2.70 ± 1.41	11	<0.001
Smokers	9.12 ± 2.22**	22	2.92 ± 1.76	18	<0.001
Total	8.37 ± 215	40	2.84 ± 1.61	29	<0.001
Former workers					
Non-smokers	6.78 ± 1.92	6	2.70 ± 1.41	11	<0.001
Smokers	9.92 ± 3.96	4	2.92 ± 1.76	18	0.004
Total	8.03 ± 3.14	10	2.84 ± 1.62	29	<0.001

*$P = 0.002$ versus non-smoker workers.
**$P = 0.012$ versus non-smoker workers.

Table III. Multiple linear regression analysis of PBL and NEC MN frequencies (‰) of the subjects (workers + controls, $n = 79$)

	PBL MN $R^2 = 0.505$		NEC MN $R^2 = 0.674$	
	B[a] (95% CIs)	P	B (95% CIs)	P
Age	0.17 (0.07–0.27)	**0.002**	0.08 (0.02–0.13)	**0.006**
Smoking	1.41 (−0.21 to 3.03)	0.087	1.55 (0.67–2.44)	**0.001**
Occupational exposure	6.71 (5.06–8.37)	**<0.001**	5.47 (4.56–6.37)	**<0.001**

The P values of significantly effective variables on MN frequencies are given in bold. Independent variables: age (years), smoking (yes/no) and occupational exposure (yes/no). CIs, confidence intervals.
[a]B, regression coefficient (slope).

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Table IV. Multiple linear regression analysis of PBL and NEC MN frequencies (‰) of current workers

	PBL MN $R^2 = 0.213$		NEC MN $R^2 = 0.418$	
	B^a (95% CIs)	*P*	*B* (95% CIs)	*P*
Age	0.22 (−0.01 to 0.44)	0.058	0.13 (0.04–0.22)	**0.007**
Smoking	1.35 (−1.28 to 3.98)	0.304	1.96 (0.89–3.04)	**<0.001**
Duration of exposure	1.60 (−0.19 to 3.40)	0.079	0.66 (−0.73 to 1.40)	0.076
SiO_2 percent exposure	−0.17 (−0.36 to 0.03)	0.095	−0.07 (−0.15 to 0.01)	0.085

The *P* values of significantly effective variables on MN frequencies are given in bold. Independent variables: age (years), smoking (yes/no), duration of exposure (years) and SiO_2 percent exposure (cumulative SiO_2 percent exposure). CIs, confidence intervals.
[a]B, regression coefficient (slope).

were diagnosed with radiological profusion category 1. Seven of these were smokers, three were ex-smokers and three were non-smokers. Among the former workers, 50% were diagnosed to have silicosis (profusion category 1). In current workers, the rate was 24%. Among workers, 48% suffered from respiratory symptoms such as coughing and sputum.

Discussion

Molecular epidemiology as bridging between epidemiologic and mechanistic studies has a crucial role in predicting severe diseases in occupational settings. In relation to crystalline silica dust exposure, there is a need for biomarkers to predict the likelihood of silicosis and lung cancer development. Advantages of using biomarkers of early biological effects in disease aetiology studies are that fewer persons may be needed than in a cohort study that evaluates the disease outcome. Such studies can be performed quickly, as they are generally cross-sectional or short-term longitudinal investigations. Also, because recent exposure often has the greatest impact on early biological effect biomarkers, highly accurate exposure assessments can be achieved (41). At that point, our workplace study can be settled in the proper place. Our objective was to conduct an occupational molecular epidemiologic study in workers exposed to crystalline silica-rich dust, specifically by applying the MN assay in PBL and NEC together with workplace dust measurements and chest X-ray films.

In our study, sandblasting, grinding and bagging were the main jobs of the workers which were directly related to crystalline silica exposure. According to the elemental composition of the bulk samples collected from the workplaces, each contained >70% crystalline silica. Moreover, in the glass industry, the ground dust was 100% crystalline silica. As such, the effects evaluated in our present study could be attributed mainly to crystalline silica.

In studies that examine the relationship between occupational exposure to airborne particulates and pulmonary diseases, early radiographical categories (including 0/1 and 1/0) are considered as early stages of pneumoconiosis (42). In our study, 24% of the current workers and 50% of the former workers were found to have silicosis in profusion category 1 (1/0, 1/1 and 1/2), whereas the controls all had normal chest radiographs. The ranking of the workplaces according to the silicosis diagnosis were workplace I (43%), workplace IV (33%) and workplace II (20%), respectively. Consistent with these observations, the cumulative SiO_2 percent exposure in workplace I was found to be the highest among these workplaces. Furthermore, in all workplaces, the respirable crystalline silica limit value of Turkey (0.25 mg/m^3) has been exceeded and the crystalline silica content of the respirable dust was in a range of 8.9–12.5%. These exposure measurement outcomes revealed a marked crystalline silica exposure of our study population.

Silicosis and other occupational lung diseases are still important, even in the most developed countries. In Turkey, 1000 silicosis and silicotuberculosis cases out of 1208 occupational diseases have been recorded in 2007 according to the Statistical Yearbook of Ministry of Labour and Security of Turkey. These records comprised only the workers covered by the social security system. Workers adjacent to the sandblasting operations are at increased risk for developing acute silicosis, and hence, this has been banned in many industrialized countries. However, sandblasting remains an important process in cleaning and preparation of surfaces for painting and other industrial uses. This has resulted in the use of substitutes for silica sand, those containing abrasive property and low crystalline silica content (43). Sandblasting with silica-containing dusts is widely used in small-scale workplaces in Turkey, since crystalline silica can be provided easily and cheaply. Moreover, those sandblasters do not have social security, since their occupational diseases are not included in official statistics (29). Recently, denim sandblasting has been shown to be another crystalline silica exposure activity leading to severe acute silicosis cases and deaths among workers without social security in Turkey (44,45). Taken together, the incidence of silicosis could be higher than the recorded values for Turkey; hence, the health consequences should be strictly evaluated for immediate prevention actions. In our workplace study, sandblasters comprised nearly the half of the study workers. Sandblasters in workplace II ($n = 17$) were processing outdoor sandblasting, whereas indoor sandblasting was carried out in workplace III ($n = 4$). In the study of Sevinc *et al.* (29) which has been carried on indoor sandblasters in Turkey, the respirable crystalline silica concentrations were found to exceed the Turkish limit value, as it was in our sandblasting workplaces. In the study of Sevinc *et al.* (29), the silicosis ratio was found to be 36%, whereas in our study, the ratio was 20% for outdoor sandblasters. Since the chest radiograph films were not available from the indoor sandblasters, we do not have information on the prevalence of silicosis among them. There has not been any other study evaluating dust measurements parallel to chest X-ray investigations for Turkey. Basaran *et al.* (26) measured respirable crystalline silica concentrations in foundries and found that it exceeded the limit value of Turkey; however, they did not have the evaluations for silicosis risk.

In the present study, we determined micronuclei in lymphocyte as well as in nasal epithelial samples (NEC) from the workers. The NEC were specifically included in the present study, since peripheral blood cells do not form a direct target after inhalation of poorly soluble particles such as silica. While the MN formation in the NEC is considered to reflect the direct interaction with the silica particles upon nasal deposition, the observed MN frequencies in the PBL will be caused by effect mediators released from the respiratory tract following silica inhalation. These mediators include stable diffusible reactive oxygen species (ROS) that can be generated in aqueous environment from surface-associated free radicals or oxidative groups (e.g. $SiO\bullet$ and $SiO_2\bullet$) and/or upon silica-induced pulmonary

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inflammation by activated alveolar macrophages and recruited neutrophils (46). Apart from the ROS, other mediators released from the lung during inflammation may also be involved in clastogenic actions (e.g. cytokines or growth factors). The formation of ROS during silica-induced inflammation is considered to be a crucial mechanism responsible for mutagenesis and carcinogenesis in silica-exposed lungs (5,46). In support of this mechanism, increased MN frequencies have been observed in subjects with chronic obstructive pulmonary disease (47), demonstrating that pulmonary stress and ROS can cause genotoxicity in peripheral cells. The MN assay has been considered to be an effective biomarker of disease and processes associated with induction of DNA damage (48).

Cytogenetic monitoring has been traditionally used for the surveillance of populations exposed to genotoxic agents by several biomarkers (49). While the significance of many of these biomarkers in terms of predicting cancer is still largely unknown (50), the CBMN assay in PBL has recently been validated as a predictor of cancer [reviewed by Bonassi *et al.* (32)]. Thus, the data gained by our study can be considered as valuable.

The MN assay has been adopted by numerous laboratories for the measurement of MN in PBL and, to a lesser extent, in epithelial cells, erythrocytes and fibroblasts, with the overall aim to determine the impact of environmental, genetic and/or lifestyle factors on genomic stability in human populations (48). To the best of our knowledge, our study is the first to determine one and the same genotoxicity marker—MN—in both PBL and NEC of workers exposed to crystalline silica-containing dust. As such, this allowed us to evaluate the effects of exposure to this classified human carcinogen in surrogate cells as well as in target cells of the respiratory tract. The usage of NEC MN assay in human biomonitoring studies as target cells has revealed increased genotoxicity for anatomy and pathology laboratory and dental laboratory workers (36,37), for styrene-exposed workers (34) and for mortuary science students exposed to embalming fluid-containing formaldehyde (35). With regard to exposure to particles or fibres in occupational settings, we did not come across any study in which the MN assay was applied to NEC. The CBMN assay with PBL, however, has been used in previous studies in asbestos workers (51), coal workers (25) and metal dust-exposed workers (52,53).

In relation to silica exposure, only few studies exist in which genotoxicity has been determined. In stone crushers, the sister chromatid exchange assay and the chromosomal aberration assay were used (30), oxidative DNA adducts (8-hydroxydeoxyguanosine) were evaluated in granite workers (27) and in healthy and silicotic coal workers (31), the comet assay was used in a study among foundry and pottery workers (26) and finally CBMN assay and sister chromatid exchange assay were measured in coal workers (25). It is important to keep in mind that the various genotoxicity tests used in the aforementioned studies likely reflect different mechanisms of genotoxic action: for instance, 8-hydroxydeoxyguanosine adducts are known to be caused by oxidative attack of the DNA and this oxidative lesion has therefore also been forwarded as a biomarker of oxidative stress (54). The comet assay represents a sensitive measure of global DNA damage measured in individual cells. The chromosomal aberration test and the sister chromatid exchange assay are both indicators of structural damage to the chromosomes in the metaphases of the mitosis. MN, measured in the present study, may originate from acentric fragments (chromosome fragments lacking a centromere) and/or whole chromosomes. Pancentromeric and centromeric probes or kinetochore antibodies allow for the discrimination between chromosome loss and chromosome breakage (33).

In this study, crystalline silica-exposed workers had significantly higher MN frequencies than controls in both PBL and NEC. In line with these findings, other studies have also shown genotoxicity in relation to exposure to silica-containing dusts. Increased 8-hydroxydeoxyguanosine adducts were found in PBL of coal workers who had been chronically exposed to silica-containing dust (31). In a similar study, Pilger *et al.* (27) measured the 8-hydroxydeoxyguanosine levels in blood and urine of workers exposed to quartz and in patients with silicosis in order to examine radical-induced DNA damage and its elimination in relation to quartz exposure, silicosis and lung function. From their results, Pilger *et al.* (27) suggested that a less effective repair of 8-hydroxydeoxyguanosine was associated with a higher degree of pulmonary airway obstruction in patients with silicosis. Basaran *et al.* (26) investigated the DNA damage by comet assay among foundry and pottery workers who were exposed to a mixture of chemicals and silica and found significantly higher DNA damage in the lymphocytes of the workers than the control group. Sobti and Bhardwaj (30) found that chromosomal aberrations and sister chromatid exchanges were significantly increased against control group in PBL of stone crusher workers in India, indicating that the mutagenic risk in this specific working environment was associated with silica dust exposure. As far as we know, the CBMN assay has been used only in one study as comparable to ours; however, this was in coal workers where crystalline silica represents a constituent of the coal dust (25). Occupational exposure to the crystalline silica in our study put forward significantly increased MN frequencies in PBL and NEC and thus confirmed the increased genotoxicity with different end points for the different workplaces in the aforementioned studies. However, a major difference was that in our study, apart from surrogate cells (i.e. PBL), also target cells were evaluated for genotoxicity. Interestingly, the MN frequency rate in workers versus control was found to be higher in the target cells (NEC) than the surrogate cells (PBL) (3-fold and 2-fold increase, respectively).

Confounders such as smoking, asbestos and radon exposure at the workplaces, concomitant exposures to other possible lung carcinogens in the work environment and silica particles coated for example with clay (2) should be taken into account when addressing the issue of crystalline silica genotoxicity. For the epidemiologic and human biomarker studies for crystalline silica, it is crucial to give a critical concern to smoking as an important confounder. Therefore, in our study, care was taken that the workers and control group were matched to control for smoking, as well as other potential confounders, i.e. age, gender and exposure to other genotoxic chemicals. Indeed, Basaran *et al.* (26) found previously that the smoking caused additional risk in their study. No association of smoking and 8-hydroxydeoxyguanosine was found by the study of Pilger *et al.* (27). However, in silicosis patients who smoked, a significant correlation between 8-hydroxydeoxyguanosine in DNA and impaired lung function was observed, suggested as a superposition of smoking and silicosis-driven pulmonary airway obstruction (27). Smoking was found not to be associated with PBL 8-hydroxydeoxyguanosine in the study of Schins *et al.* (31). In all these studies, no effect of age has been seen.

In our study, the strongest effect has been attributed to the occupational exposure on MN frequencies for both PBL and NEC. Besides that, age and smoking were found as significantly affecting parameters for NEC MN, whereas for

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the PBL MN frequency, age was a significantly affecting parameter. Battershill *et al*. (55) pointed to an age-related increase of the chromosome damage in lymphocytes and emphasized the need to take into account the potential confounding effect of this variable in the design of biomonitoring studies based on chromosome damage.

In our study, the age distribution was consistent in between the workers and controls. In stratifying the groups as smokers and non-smokers, significant MN frequencies were still obvious for workers against controls. Concurrently, workers had higher MN frequencies than the non-smokers for both cell types, although this was significant only for the NEC.

In summary, crystalline silica exposure was found to be associated with genotoxicity in both target tissue (i.e. epithelial cells from the respiratory tract) and surrogate tissue (i.e. PBL) in crystalline silica-exposed workers. Importantly, we also found that the MN frequencies in PBL and NEC were significantly correlated. Therefore, the MN assay seems to be a valuable effect biomarker for dust-exposed workers, both in target and in surrogate tissues. In such a population, the MN assay has been used for the first time in present study. As a recommendation, future studies can be designed to elucidate on the potential involvement of aneugenic or clastogenic mechanisms in genotoxicity of crystalline silica by small modifications in MN assay.

In our study, all workers were employed in grinding and sandblasting jobs, in where there was direct exposure to crystalline silica. Thus, the observed genotoxicity seems to reflect mainly that exposure. In the present study, we also performed workplace dust measurements, which allowed us to compare the exposure levels to occupational standards of the world and Turkey. Finally, we evaluated the presence of silicosis in our study population, which has been discussed to be associated with silica-induced lung cancer. Our data may contribute to the hazard identification of crystalline silica.

As a conclusion, understanding the lung toxicity mechanisms of crystalline silica particles exposed via inhalation may help to prevent diseases, put forward treatment approaches, maintain the health and safety of workers and activate the legal sanctions. Specific controls, work practices, lowering the limit values could reduce exposures and prevent additional cases of silicosis.

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