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Genotoxic effects of occupational exposure to glass fibres - A human biomonitoring study

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ABSTRACT

As part of a large human biomonitoring study, we conducted occupational monitoring in a glass fibre factory in Slovakia. Shopfloor workers (n = 80), with a matched group of administrators in the same factory (n = 36), were monitored for exposure to glass fibres and to polycyclic aromatic hydrocarbons (PAHs). The impact of occupational exposure on chromosomal aberrations, DNA damage and DNA repair, immunomodulatory markers, and the role of nutritional and lifestyle factors, as well as the effect of polymorphisms in metabolic and DNA repair genes on genetic stability, were investigated.

The (enzyme-modified) comet assay was employed to measure DNA strand breaks (SBs) and apurinic sites, oxidised and alkylated bases. Antioxidant status was estimated by resistance to H_2O_2-induced DNA damage. Base excision repair capacity was measured with an *in vitro* assay (based on the comet assay).

Exposure of workers to fibres was low, but still was associated with higher levels of SBs, and SBs plus oxidised bases, and higher sensitivity to H_2O_2. Multivariate analysis showed that exposure increased the risk of high levels of SBs by 20%. DNA damage was influenced by antioxidant enzymes catalase and glutathione S-transferase (measured in blood). DNA repair capacity was inversely correlated with DNA damage and positively with antioxidant status. An inverse correlation was found between DNA base oxidation and the percentage of

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eosinophils (involved in the inflammatory response) in peripheral blood of both exposed and reference groups. Genotypes of *XRCC1* variants rs3213245 and rs25487 significantly decreased the risk of high levels of base oxidation, to 0.50 ($p = 0.001$) and 0.59 ($p = 0.001$), respectively.

Increases in DNA damage owing to glass fibre exposure were significant but modest, and no increases were seen in chromosome aberrations or micronuclei. However, it is of concern that even low levels of exposure to these fibres can cause significant genetic damage.

1. Introduction

The recent development at the European Commission towards a carbon neutral economy, called the European Green Deal, set an ambitious goal to move towards a toxic-free environment. A key commitment of the European Green Deal has resulted in the development of a new Chemicals Strategy for Sustainability (CSS) adopted in 2020 [1] as part of the zero pollution ambition. The overall aim of CSS is to better protect citizens and the environment from harmful chemicals, and to promote use of safer and more sustainable chemicals.

Human biomonitoring was recognized by the Commission's CSS as a crucial tool for assessing aggregated chemical exposure of humans and its effects, that could help to improve chemical policies. It can provide a better estimate of internal exposure at the target organ. A main advantage of human biomonitoring is that exposure data can be complemented with specific endpoints representing biomarkers of exposure, effect and individual susceptibility and can thus predict the adverse health effects resulting from exposure. The data generated from human biomonitoring may reveal the biological links between exposure and effect by using biomarkers that can address key events in adverse outcome pathways (AOP). Thus data obtained from human biomonitoring can serve in future to support human risk assessment for the general population as well as for occupationally exposed workers.

In human biomonitoring studies circulating blood cells are considered as surrogate target cells for measuring various biomarkers of internal exposure, effect, and individual susceptibility. Human biomonitoring studies have already been performed to investigate, in human blood and body tissues, the effect of exposure to different hazardous chemicals including pesticides, biocides, pharmaceuticals, heavy metals, plasticisers, flame retardants [1,2] and fibres [3–5].

The focus of this paper is the genotoxicity of manufactured glass fibres. Fibres are defined by their aspect ratio, i.e. the ratio of the large dimension (the length or longest dimension) to a small dimension (width or diameter). The High Aspect Ratio hypothesis builds on the well-characterized and robust structure-activity relationship governing asbestos carcinogenicity, known as the fibre pathogenicity paradigm. This identifies width, length, rigidity and biopersistence of fibres as the critical determinants of mesothelioma development following inhalation [6,7]. In addition, fibres are defined as respirable (< 3 μm in diameter) or non-respirable (>3 μm in diameter). Some fibres fit the definition of nanofibres, having one dimension in nanosize. High aspect ratio nanomaterials (HARN) are defined as nanofibres with two similar external dimensions and a significantly larger third dimension (aspect ratio of 3:1 or greater) and substantially parallel sides [8].

It is well established that, following inhalation, certain forms of asbestos fibres, especially those with length > 5 μm, can elicit serious adverse health effects, including lung tumours, lung fibrosis (asbestosis) and mesothelioma, a tumour that arises from the mesothelial cells lining the chest wall and lungs. In view of the morphological similarity between asbestos fibres and HARN, there is clearly a chance that some HARN will pose a similar hazard in terms of pulmonary disease [9].

The mechanism accounting for the length-dependent pathogenicity of fibres such as asbestos or HARN is known as 'frustrated phagocytosis', referring to the inability of macrophages to completely engulf and clear long fibres. This leads to activation of pro-inflammation signalling, generation of reactive oxygen species (ROS), lysosomal disruption, cellular membrane damage and release of cell contents, causing local tissue damage and the development of a chronic inflammatory response [10,11]. A continuous inflammatory response is associated, via oxidative stress, with DNA damage and mutations and increased cell proliferation [9]; and it is known that chronic inflammation (triggered by interleukin 1 beta (IL-1β) signalling) plays a key role in the aetiology of fibre-related diseases [12,13].

In 1988 the International Agency for Research on Cancer (IARC) classified fibreglass, rock wool, slag wool, and ceramic fibres as Group 2B, i.e. possibly carcinogenic to humans. In 2002 it reassigned fibreglass, rock and slag wool, and continuous glass filaments to Group 3, not classifiable as carcinogenic to humans.

Glass fibres were investigated in several *in vitro* studies. Ong et al. [14] investigated the ability of glass fibres to induce micronucleated and multinucleated cells in cultures of V79 cells. The induced micronuclei were further analyzed to determine the mechanism of micronucleus formation by staining the kinetochore with anti-kinetochore and fluoresceinated goat anti-human immunoglobulin G (IgG) antibodies. Of three types of glass fibres, two resulted in concentration-related increases in multinucleated and micronucleated cells, indicating that the two microfibres are capable of inhibiting cytokinesis and are principally aneuploidogens. In contrast, the larger fibres neither induced micronuclei nor inhibited cytokinesis in V79 cells. Thus, the genotoxic potential of glass fibres in V79 cells may be related to their size. Cavallo et al. [15] assessed cytotoxic and oxidative effects in the human mesothelial cell line (MeT-5A) exposed to glass wool, rock wool and refractory ceramic fibres in comparison with crocidolite asbestos, using the formamidopyrimidine DNA glycosylase- (Fpg-) modified comet assay to detect direct and oxidation damage in DNA. Cells exposed to glass wool showed a dose-dependent increase in DNA damage only at the highest dose, suggesting a slight genotoxic and cytotoxic effect. Proietti et al. [16] assessed cytotoxic and genotoxic effects induced in the human alveolar cell line A549 by exposure to glass wool fibres, finding a decrease in cell viability and increase in DNA damage in cells exposed to glass wool fibres. Similarly, Rapisarda et al. [17] showed in A549 cells that glass fibres with a predominant diameter < 3 μm (97%) and length > 5 μm (93%) reduced cell viability and increased DNA damage and oxidative stress in a concentration-dependent manner, demonstrating that glass fibres produce cytotoxic and genotoxic effects related to increased oxidative stress in A549 cells.

Here we present a human biomonitoring study conducted in a factory where workers were exposed to glass fibres. The study was part of a larger occupational exposure monitoring program where we investigated the effect of asbestos [4], stone wool [3] and glass fibres using biomarkers of exposure, effect and susceptibility.

2. Materials and methods

2.1. Study design

The study groups recruited for this biomonitoring study consisted of 116 healthy adult volunteers, average age 44 years, ranging from 23 to 55 years. There were 80 workers with at least 5 years of occupational exposure to glass fibres, and 36 reference subjects from the administrative staff of the plant (Table 1). Informed consent was obtained from all subjects. The study was authorised by the Ethical Committee of the Institute of Preventive and Clinical Medicine, Bratislava (now Slovak Medical University). Interviews and sampling were carried out in

Table 1
Basic anthropometric characteristics; numbers in each group, numbers of men and women, number of smokers, mean age (years) and mean BMI (kg/m^2) with 95% confidence interval in brackets.

	All	Men	Women
Exposed (n)	80	39	41
Smokers (n)	33	18	15
Mean age (years)	43.0$^{\#}$ (40.9–45.1)	42.1* (38.7–45.6)	43.8 (41.2–46.4)
BMI (kg/m^2)	25.7 (24.8–26.6)	25.9 (24.8–27.0)	25.5 (24.1–27.0)
Reference (n)	36	18	18
Smokers (n)	14	8	6
Mean age (years)	46.7$^{\#}$ (43.6–49.8)	48.9* (44.4–53.4)	44.5 (40.1–48.9)
BMI (kg/m^2)	25.7 (24.4–27.1)	27.4$^{£}$ (25.7–29.2)	24.1$^{£}$ (22.3–25.9)

$^{\#}p = 0.037$. $*p = 0.023$. $^{£}p = 0.008$

September 2001. Exposure to polycyclic aromatic hydrocarbons (PAHs) and fibres was monitored during four seasons, including the time of collection of the blood samples.

2.2. Exposure measurement in workplace

Exposure assessment was based on personal and environmental monitoring. For environmental exposure, air sampling was conducted during a shift (8 h) using stationary pumps at several sites in the factories and the administrative areas, and on 4 different occasions in winter, spring, summer and autumn (January 25, April 25, June 25, September 26, 2001) in order to obtain representative data. In these samples also fibre number, type and size distribution were determined.

The seasonal sampling of indoor air (production halls and office buildings) for PAHs was carried out by high-volume samplers. Air particulate samples were also collected in the working area during January, April, June and also in September at the time of collection of the blood samples. Samples were taken using stationary and personal samplers for PAHs (GPS-1, Graseby Andersen, USA) as well as fibre measurements. Hexane/ether extraction of GFFs and PUF plugs was followed by cleaning-up on a silica column. Total PAH levels (sum of fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(a)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, indeno(123cd)pyrene, dibenzo(ah)anthracene, benzo(ghi)perylene) were measured. Samples were analysed by HRGC/LRMS-SIM according to the USEPA TO-13 method. Along with the high-volume air sampling for the PAH analysis, air fibre sampling was carried out.

2.3. Personal PAH exposure monitoring

Personal exposure to PAH was measured using portable personal pumps powered by batteries to be carried by each study participant during an 8-hour shift prior to collection of biological samples.

To assess human exposure to PAH by breathing, a low air volume sampling method was developed, applicable for collecting the air by a personal sampler SKC 224-PCXR3, ALPHA, SKC AIRCHEK. A precleaned polyurethane foam plug (PUF) 1.5 cm in diameter and 2 cm in height was used for quantitative capturing of the PAH congeners of concern at a flow-rate of 4 L/min during 8-hr sampling in portable personal samplers powered by batteries. Samples were Soxhlet extracted and analysed by GC/MS for the presence of thirteen PAH congeners.

2.4. Fibres (stationary and personal sampling)

Fibre exposure assessment was based on personal and environmental monitoring. For environmental exposure, air sampling was conducted during a shift (8 h) using stationary pumps at several sites in the factory and the administrative areas, and on 3 different occasions in winter, spring and summer together with PAH sampling. Autumn sampling was conducted with personal samplers in the same week as blood sampling. Air particulate samples collected on membrane filters by stationary and personal samplers were analysed with an optical microscope in phase contrast. All samples were counted for artificial mineral fibres (basalt glass). The concentration of fibres in the air was calculated from their number in a measured area, an active area of the filter and a volume of sampled air. Samples were evaluated using a microscope with phase contrast (Nikon, Japan) according to the Reference Method for the Determination of Airborne Asbestos Fibre Concentration at Workplaces by Light Microscopy (Membrane Filter Method), AIA 1979, London, UK and then sorted to respirable and non-respirable. The fibre identification, morphology and quantification of the collected samples were performed by electron scanning microscopy (JEOL 5500, Japan) equipped with analyser EDS.

2.5. Questionnaires

All participants were interviewed by trained personnel and answered detailed questionnaires relating to duration of exposure, smoking habit, alcohol consumption, lifestyle and diet. The food frequency questionnaire covered the previous 3 months of food consumption. Response options were: daily intake, several times per week, weekly, monthly, never. Nutritional status was assessed using the Aberdeen food frequency questionnaire adapted to a Slovak population. Information from questionnaires was elaborated using the Alimenta database (Food Research Institute, Bratislava) supported by software developed by Mr David Grubb from the Rowett Research Institute, Aberdeen. All workers underwent an additional clinical examination including a functional spirometry test, radiological and immunological investigation.

2.6. Blood sampling

A sample of 30 ml of venous blood was collected after overnight fasting and processed immediately. Biomarkers of cellular antioxidant defence (antioxidants and antioxidant enzymes), DNA stability (DNA damage and repair, chromosomal aberrations, micronuclei) and individual susceptibility (genetic polymorphisms of important detoxification enzymes and DNA repair proteins) were measured. A urine sample (10 ml) was collected to measure cotinine, to confirm smoking status. All study participants signed an informed consent form before donating their specimens. This study was approved by the ethical committee of the Institute of Preventive and Clinical Medicine, Bratislava, and was performed according to Good Laboratory Practice. Samples were coded and analyses were performed blindly.

2.7. Haematology analysis

Blood samples were collected into EDTA-containing tubes. Haematological parameters were measured using a haematological analyzer, Sysmex K-4500 (SYSMEX TOA Medical Electronics Co. LTD, Japan). The following parameters were assessed: leukocyte count, erythrocyte count, haemoglobin, haematocrit, mean corpuscular volume, platelet count, percentage of neutrophils, neutrophil count, percentage of lymphocytes, lymphocyte count and common eosinophil and basophil percentage and count. A differential leukocyte count was carried out on blood smears stained with May-Grunwald and Giemsa–Romanowski; 200 leukocytes were examined by light microscopy.

2.8. Isolation of lymphocytes

Lymphocytes were obtained in different ways for the various assays.

For cytogenetic assays, short-term lymphocyte cultures were set up (two for each sample) by adding 0.5 ml of whole blood to 4.5 ml of RPMI medium with L-glutamine (Gibco) supplemented with 20% fetal calf serum (Gibco), and antibiotics (penicillin and streptomycin). Lymphocytes were stimulated to proliferate by adding 0.18 mg/ml of phytohaemagglutinin (PHA, Murex) and incubated at 37 °C with 5% CO_2.

For measurement of DNA damage and repair (comet assay

experiments), the blood was centrifuged for collection of plasma, and the buffy coat was recovered and mixed with RPMI 1640 medium with 10% fetal calf serum before layering over an equal volume of Lymphoprep (Nycomed, Oslo, Norway) and centrifuging at 700 x *g* for 20 min at 20 ºC. The layer above the Lymphoprep, containing lymphocytes, was removed, diluted with medium and centrifuged at 700 x *g* for 15 min at 20 ºC. For subsequent analysis of DNA damage, lymphocytes were suspended in 90% fetal calf serum, 10% DMSO, divided into aliquots and frozen slowly to −80 ºC. For the DNA repair assay, lymphocytes (5 x 10^6 in 50ßl aliquots) were snap-frozen to −80 °C in 45 mM HEPES, 0.4 M KCl, 1 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol, pH 7.8. For use, aliquots were thawed quickly and then kept on ice.

2.9. Analysis of cotinine

The concentration of cotinine in urine was measured by a radioimmunoassay method using a commercial kit from Brandeis University, Waltham, Massachusetts, USA. The method is based on competition between labelled and unlabelled cotinine for reacting with the limited amount of specific bonding sites of the antibody. To the standard diluted with buffer, ^{3}H-cotinine and anti-cotinine-CDI-thyroglobulin were added. After incubation normal rabbit serum and goat "Minnie" anti-rabbit-gamma-globulins were pipetted. The reaction mixture was incubated overnight at 4ºC. The sediment was diluted in 0.1 M NaOH. After adding the scintillation liquid the activity of the cotinine-antibody complex was measured. For every set of samples a new calibration curve was made. The concentration of cotinine is expressed as mg cotinine /ml creatinine in urine. Creatinine was measured (two-point rate test) by biochemical analyser VITROS 250.

2.10. Measurement of markers of oxidative stress and antioxidant protection

2.10.1. Measurement of antioxidant enzyme activity

After plasma separation, erythrocytes were washed three times with isotonic saline (0.9% sodium chloride). Centrifugation for each washing was at 1500xg for 10 min at 4 °C. Erythrocytes were then lysed with hypotonic solution (distilled water) and the hemolysate was used (at suitable dilutions) for measurement of activities of antioxidant enzymes. The activity of glutathione peroxidase (GPx) was determined by the kinetic method according to Paglia and Valentine [18], Catalase (CAT) was measured spectrophotometrically by a modified method of Cavarocchi et al. [19] and glutathione-S-transferase (GST) by a kinetic method according to Habig et al. [20]. The activity of superoxide dismutase (SOD) was estimated by a commercial test kit (Randox Lab Ltd., U.K.).

2.10.2. Measurement of ceruloplasmin oxidase

Ceruloplasmin (CPL) oxidase activity in plasma was assayed with the use of *o*-dianisidine dihydrochloride according to the method of Schosinsky et al. [21].

2.10.3. Measurement of antioxidant micronutrients

Plasma vitamin C [22], α-tocopherol, γ-tocopherol, β-carotene, retinol, xanthophyl and lycopene were measured by HPLC [23].

2.11. Measurement of markers of DNA stability

2.11.1. DNA damage by the comet assay

The comet assay (single cell alkaline gel electrophoresis) is a simple, sensitive and validated method for measuring DNA damage [24,25]. Lymphocytes were centrifuged, washed in PBS, recentrifuged, and suspended in low melting point agarose (1 % in PBS) at 37 °C. Aliquots of 85 µl of agarose containing 5 x 10^4 cells were placed on glass microscope slides (precoated with 1% normal melting point agarose and dried), and covered with glass cover slips. Slides were placed at 4 °C to allow the gel to set, and cells were then lysed for 1 h at 4 °C in 2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris-HCl, pH 10, 1% Triton X-100. Lysis removes membranes, cytoplasm and most nuclear proteins, leaving DNA as nucleoids. To measure DNA strand breaks (SBs), the slides were immersed in 0.3 M NaOH, 1 mM Na_2EDTA for 40 min at 4 °C, and then subjected to electrophoresis at 0.8 V/cm for 30 min at an ambient temperature of 4 °C. After neutralisation, gels were stained with 4',6-diamidine-2'-phenylindole dihydrochloride, and viewed by fluorescence microscopy. DNA loops in which supercoiling is relaxed by strand breakage extend under electrophoresis to form 'comet tails', and the relative intensity of DNA in the tail indicates the DNA break frequency. Tail intensity was assessed by visual scoring; 100 comets selected at random were graded according to degree of damage into 5 classes (0−4) to give an overall score for each gel of between 0 and 400 arbitrary units. (The visual score is directly correlated with % tail DNA [26].) To measure oxidised bases, the nucleoids were incubated, in the gel, with endonuclease III (EndoIII) which recognises oxidised pyrimidines or formamidopyrimidine DNA gycosylase (Fpg) to detect altered purines including formamidopyrimidines and 8-oxoguanine, in 40 mM HEPES, 0.1 M KCl, 0.5 mM Na_2EDTA, 0.2 mg/ml bovine serum albumin, pH 8.0, or with this buffer alone, for 30 min at 37 °C [4]. These enzymes remove the bases, and the resulting abasic sites (apurinic/apyrimidinic or AP sites) are converted to breaks by an associated AP endonuclease activity. The enzyme 3-methyladenine DNA glycosylase II (AlkA) was used in a similar way to analyse DNA alkylation [27]. Alkaline treatment and electrophoresis then followed. (AlkA does not have an AP endonuclease activity, but the abasic sites are alkali-labile and so appear as breaks after alkaline treatment.) Comets were stained with DAPI and evaluated as above.

Net enzyme-sensitive sites (netFpg, netEndoIII and netAlkA), calculated by subtracting the comet score after incubation with buffer alone from the score with enzyme, indicate the extent of base oxidation/alkylation. Total damage, i.e. SBs+enzyme sites (SBs+Fpg, SBs+EndoIII, SBs+AlkA), is also shown.

2.11.2. Measurement of DNA repair

The snap-frozen samples of lymphocytes were thawed, and lysis was completed by adding 12 µl of 1% Triton X-100. The lysate was centrifuged at 14,000 x g for 5 min at 4ºC. The supernatant was mixed with 4 volumes of 45 mM HEPES, 0.25 mM EDTA, 2% glycerol, 0.3 mg/ml bovine serum albumin, pH 7.8. To prepare substrate DNA with specific oxidative damage, HeLa cells (human transformed endothelial cells) were treated with the photosensitiser Ro 19–8022 (Hoffmann La Roche, Basel, Switzerland) at 0.2 µM plus visible light (4 min irradiation on ice at 330 mm from a 1000 W tungsten halogen lamp) to oxidise guanine to 8-oxoguanine. The cells were embedded in agarose and lysed as for the standard comet assay, and then incubated with 40 µl of lymphocyte extract for 0, 10 or 20 min at 37 °C. Alkaline treatment and electrophoresis followed as in the standard comet assay. The increase in DNA breaks between 0 and 10 min was taken as the measure of repair incision for statistical analysis.

2.11.3. Sensitivity to hydrogen peroxide

Lymphocyte samples were exposed to 50 µM H_2O_2 for 5 min on ice. After rinsing with PBS, the cells were embedded in agarose for measurement of strand breaks as described above. The increase in score over that of untreated lymphocytes indicates the sensitivity to H_2O_2 and is inversely correlated with antioxidant status.

2.11.4. Chromosomal aberrations

Lymphocytes were harvested at 48 h following stimulation, colchicine (Sigma, 0.75 µg/ml) being added 2 h before harvest. The cells were centrifuged and subjected to hypotonic shock in 0.075 M KCl for 20 min at 37 ∘C. The lymphocytes were fixed twice in methanol:acetic acid (3:1) and air-dried preparations were made. The slides were stained with 5% Giemsa-Romanowski solution for 10 min.

One hundred well-spread metaphases per person were examined. All

the standard chromosomal abnormalities, chromatid and chromosome gaps, breaks and exchanges were recorded. However, because of the controversy over the meaning of gaps, they were not included in the statistical analysis. Chromosomal damage was expressed as percentage of aberrant cells and number of breaks per cell [28].

2.11.5. Micronucleus analysis

Cytochalasin B (Sigma), final concentration 6 μg/ml, was added to the PHA-stimulated lymphocytes 44 h after the start of culture. At 72 h of incubation, cells were centrifuged, resuspended in 0.075 M KCl, immediately centrifuged again and treated twice with fixative (methanol:acetic acid, 3:1). The fixed cells were dropped onto slides, air dried and stained with 5% aqueous Giemsa solution for 10 min.

Cytochalasin B inhibits cytoplasmic cleavage without preventing mitosis. Thus cells that have divided are readily identified by the presence of two nuclei. Micronucleus analysis was performed on 2000 binucleated lymphocytes with preserved cytoplasm for each subject. Micronuclei were accepted only if they were morphologically identical to, but smaller than, normal nuclei, had a diameter between 1/16 and 1/3 of the main nucleus, were non-refractile, and were not linked to the main nucleus via a nucleoplasmic bridge (though they might sometimes overlap the boundary of the main nucleus) [29]. Cells from reference and exposed groups showed very similar high proliferation levels (Nuclear Division Factor – NDI between 1.7 and 1.8). Only proliferating cells were analysed, as is customary.

2.11.6. Genotyping

Genomic DNA was isolated from frozen peripheral blood samples collected into potassium-EDTA-containing tubes (Sarstedt, Nümbrecht, Germany), using a phenol-chloroform method [5]. The DNA concentration was measured by spectrophotometry employing NanoDrop System (NanoDrop, Minneapolis, MN, USA), and DNA purity was determined by the A260/A280 ratio. Two deletion polymorphisms in *GSTM1* and *GSTT1* and 14 single nucleotide polymorphisms (SNPs), three in drug metabolism enzymes *GSTP1* rs1695, *NQO1* rs1800566 and *EPHX1* rs2234922 as well as 11 in DNA repair genes, *XPA* rs1800975, *XPC* rs2228001and rs2279017, *XPD* rs13181 and rs1799793, *XPG* rs17655 *OGG1* rs1052133, *XRCC1* rs3213245 and rs25487, *XRCC2* rs3218536 and *XRCC3* rs861539, were assessed. Genotyping was performed using 5' nuclease assay chemistry with TaqMan® MGB probes (Applied Biosystems, Warrington, UK). Genotyping reactions were carried out in 96-well formats, using SNP genotyping Assays™, TaqMan® Genotyping Master Mix and 10 ng of genomic DNA, according to supplier's recommendations. ABI PRISM® 7500 Sequence Detection Systems (Applied Biosystems) was employed for end-point detection and allele calling. 10% of samples were randomly validated by restriction fragment length polymorphism (RFLP) method. Presence of *GSTT1* and *GSTM1* deletions was assessed by multiplex PCR, using β-globin gene as an internal positive control as published previously [5].

2.12. Statistical methods

To compare the exposed group with the controls, Mann-Whitney U and Fisher exact test were used for continuous or categorical variables, respectively. The log-normal multivariate regression model was applied to investigate the relationship between each variable of interest and a set of predictors selected through a stepwise process. This statistical model allows estimation of the percentage change in mean frequency of the variable of interest for a unitary increase of the predictor on a continuous scale or with respect to a reference level for categorical predictors. While the association between exposure to glass fibres and the biomarkers of genetic damage is well substantiated by literature and mechanistic evidence, the study has a number of secondary hypotheses that were not completely explored. Although these results might be affected by multiple comparison, we decided not to adjust them, for the sake of generating new potentially innovative evidence [30].

3. Results and discussion

A relatively large molecular epidemiology study was conducted in Slovakia as part of the EU Framework 5 project FIBRETOX, in three mineral fibre factories, producing asbestos, stone wool, and glass fibres. Exposure monitoring and sampling were performed in 2000–2001 with the aim to investigate the effect of occupational exposure on markers of DNA instability [3–5]. Possible effects on immunomodulatory markers [31], as well as the role of nutritional and lifestyle factors, and the effect of polymorphisms in metabolic and DNA repair genes on genetic stability were also investigated [32–34]. Occupationally exposed workers and reference groups of non-exposed subjects, matched for age, sex and smoking habit, were clinically examined, and they completed nutritional and lifestyle questionnaires. Peripheral blood lymphocytes were sampled, to represent the overall effects of exposure in the body [35]. We measured SBs and AP sites using the standard alkaline comet assay, oxidised DNA bases with the Fpg- and EndoIII-modified comet assay and alkylation damage to DNA with AlkA. In addition, an *in vitro* assay for base excision repair capacity, also based on the comet assay, was applied [3–5]. Altogether 388 subjects were investigated. Workers were exposed to asbestos or mineral fibres for periods between 5 and 40 years and most of the data related to exposure to asbestos (n = 131) and stone wool (n = 141) in two factories have been published, but not those from the third, glass fibre factory (n = 116).

Here we present results from this third factory. We studied the impact of occupational exposure to glass fibres on chromosomal aberrations, DNA damage and DNA repair, their immunomodulatory effect, and the role of nutritional and lifestyle factors, and investigated the effect of polymorphisms in metabolic and DNA repair genes on genetic stability. Basic anthropometric characteristics and frequency of smokers did not differ between exposed and reference groups except for mean age (43.0 vs. 46.7 years, $p = 0.037$), this difference being seen only in men (42.1 vs. 48.9 years, $p = 0.023$). Reference group males also had higher BMIs than reference women (27.4 vs. 24.1 years, $p = 0.008$) (Table 1).

The exposure to fibres and PAHs was measured seasonally in spring, summer, autumn, and winter. Thirteen PAH congeners were determined in all the samples. PAH levels found in the administrative building were comparable with the other two fibre factories monitored (data not shown). However, total PAH concentrations in the production hall were lower compared to offices (Table 2). From thirteen congeners measured only fluoranthene and pyrene and in two seasons also anthracene were higher in the production hall (Table S1). This could be due to the rather clean operating conditions during glass fibre production and a very hot,

Table 2
Total and mean polycyclic aromatic hydrocarbons (PAH)* levels in production and office workplaces determined in different seasons.

	Concentration (ng/m^3)				
	Production		Office		*p*-Value
Season	Cumulative concentration	Mean ± SEM	Cumulative concentration	Mean ± SEM	
Winter	51	4.19 ± 1.96	101	8.39 ± 5.86	0.291
Spring	112	9.28 ± 5.15	194	16.15 ± 13.37	0.630
Summer	45	3.70 ± 1.74	139	11.53 ± 9.45	0.755
Autumn	49	4.05 ± 2.18	156	12.94 ± 10.69	0.443

* Sum and mean of PAHs [fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(a)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, indeno(123cd)pyrene, dibenzo(ah)anthracene, benzo(ghi)perylene] collected by personal and high volume samplers.

humid but well ventilated atmosphere in the room where the high-volume apparatus was deployed. No significant difference between seasons (winter and summer levels) was observed (as is usually reported for outdoor air) (Table 2, S1). These levels of PAH contamination can be considered as very low from the point of view of occupational exposure.

The presence of glass fibres was confirmed in the exposed group and some of them might belong to HARN. However, concentrations of glass fibres measured by both stationary as well as personal monitors were very low, below the occupational limit (0.2 fibre/cm^3) (Table S2, S3). Exposed workers had significantly higher DNA damage (SBs as well as overall DNA damage – SBs+Fpg sites, SBs+EndoIII sites; Figure 1) and their lymphocytes were more sensitive to additional exposure to H_2O_2. (Induction of SBs by H_2O_2 is an indication of sensitivity to oxidative damage – inversely reflecting antioxidant defence.) These effects were more pronounced in exposed men compared with women (SBs, overall damage SBs+Fpg sites, SBs+EndoIII sites, SBs+AlkA sites, H_2O_2 sensitivity) and they also had significantly higher specific base damage (net EndoIII sites indicative of oxidised pyrimidines) compared with men in the reference group. None of these DNA instability markers was significantly higher in exposed women compared to women in the reference group (Table 3). In all cases, increases in exposed compared to controls were modest, though they were always higher in exposed subjects, both men and women. The level of damage in reference women tended to be higher then in reference men. This could explain why the difference between exposed and reference groups of women did not reach statistical significance. Results are in line with published *in vitro* data showing induction of DNA damage in a human mesothelial cell line [15] and in A549 cells [16] after exposure to glass fibres.

As the overal exposure to PAHs was very low and was higher in the reference group (office workers) compared to the exposed group (Table 2), it is obvious that PAHs are not the primary cause of higher DNA damage in the lymphocytes of exposed workers - but fibres are potentially responsible. Recent studies have reported that components of glass fibres such as magnesium oxide and silicon dioxide can exhibit genotoxic effects in various systems [36,37]. Thus, co-exposure with these impurities might be responsible for the effects seen. In view of the very low levels of glass fibres in the workplace, it may be that there is no threshold concentration for DNA damage by this kind of DNA damaging agent, and so potentially even a low exposure to glass fibres may be harmful. We also assume that a proportion of non-respirable fibres can belong to the group of carcinogenic HARN.

The effect of occupational exposure on SB levels was confirmed by multivariate analysis, showing that exposure to glass fibres increased the risk of high SBs by 1.20 times ($p = 0.044$) (Table 4). Furthermore, SB levels were also influenced by catalase activity in blood. Catalase is an important cellular antioxidant enzyme, one of the body's natural protection mechanisms, participating in the decomposition of constantly formed H_2O_2 into the final products molecular oxygen and water [38]. A relatively high negative correlation was found between catalase activity measured in blood and both SBs ($r = -0.406$, $p < 0.001$) and SBs+EndoIII ($r = -0.386$, $p < 0.001$). The highest tertile values show a reduction of the risk of high SBs to 0.67 ($p < 0.001$). High catalase levels had a similar effect on SBs+Fpg sites (ExpB = 0.76, p = 0.010). Moreover, intakes of two nutrients, dietary fibre and total carotenoids, were associated in multivariate analysis with SB levels. While high dietary fibre intake (> 8 g per day) decreased the risk of high SBs to 0.72 ($p = 0.020$), an elevated consumption of total carotenoids increased the risk of high SBs by 1.28 times ($p = 0.029$) for the second and by 1.39 times ($p = 0.013$) for the third tertile values. However, consumption of food with high levels of carotenoids is not necessarily reflected in the blood levels of carotenoids as not all carotenoids are absorbed. Accordingly, carotenoids measured in blood did not show any association with DNA damage. The list of nutrients whose consumption varied significantly between exposed and reference groups is provided in Table S4.

We also found an inverse correlation between GST levels measured in peripheral blood and SBs+EndoIII sites ($r = -0.330$, $p < 0.001$) values. The levels of GST in the second tertile reduced the risk of high SBs+EndoIII sites to 0.79 ($p = 0.039$), while for the highest tertile, it was reduced to 0.66 ($p = 0.001$). An inverse association between GST and SBs and EndoIII sensitive sites was reported already in an analysis of combined data from all three biomonitoring studies [5]. Interestingly, an opposite effect was found for GPx values, which positively correlated with SBs+EndoIII sites ($r = 0.217$, $p = 0.023$). In multivariate analysis (Table 4) the highest GPx tertile values increased the risk of high SBs+EndoIII by 1.38 times (p = 0.008).

DNA repair capacity at 8-oxoguanine shows an inverse correlation with DNA damage markers (SBs, SBs+Fpg sites, SBs+EndoIII sites) consistent with the role of DNA repair in removing DNA oxidation damage. As expected strong correlations are seen between SBs and total DNA damage which includes SBs as well as enzyme-sensitive sites. DNA repair capacity also has an inverse relationship with sensitivity to H_2O_2; low sensitivity to SB-induction by H_2O_2 is a measure of antioxidant resistance, and so it seems that high capacity for repair of base oxidation is associated with cellular antioxidant status (Table 6, Table S5).

We also measured the influence of several polymorphisms in metabolic and DNA repair genes on DNA damage values (Table S6, Table 4, Table 5). Their frequencies did not differ between exposed and reference groups except for *XRCC3* ($p = 0.019$). However, the *XRCC1* rs25487 minor T allele was associated with lower SBs values in dominant model (65 ± 4 for CT+TT vs. 82 ± 6 for CC genotypes, $p = 0.031$). Accordingly, in a multivariate model CT+TT genotypes reduced the risk of high SBs to 0.74 ($p = 0.001$). Minor G rs3213245 allele was associated with lower netFpg values in all and exposed subjects while minor T allele for rs25487 polymorphism was protective with respect to SBs+EndoIII in both all and exposed subjects, and SBs+AlkA in exposed subjects. These results were confirmed for netFpg sites also in multivariate analysis, where genotypes of both *XRCC1* variants rs3213245 and rs25487 significantly decreased the risk of high netFpg sites to 0.50 ($p = 0.001$) and 0.59 ($p = 0.001$), respectively. We previously published a report on

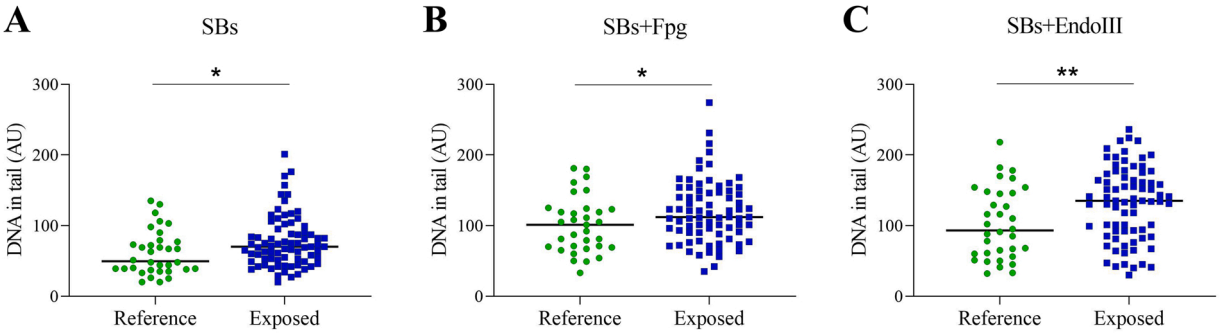

Fig. 1. DNA damage – SBs (A), SBs+Fpg (B), and SBs+EndoIII (C) (measured in lymphocytes by the comet assay in arbitrary units). The horizontal line represents the median. * $p < 0.05$, * * $p < 0.01$; SBs – strand breaks; Fpg – formamidopyrimidine DNA glycosylase sensitive sites, EndoIII – endonuclease III sensitive sites.

Table 3
DNA damage and repair measured in lymphocytes with the comet assay; *p*-values shown in the table relate to exposed/control comparisons.

Variables	**Exposed** (mean ± SEM)	N	**Reference** (mean ± SEM)	N	*p*-Value
Repair (8-oxoG)					
All	122 ± 6	74	128 ± 9	31	0.679
Men	128 ± 8	38	126 ± 14	15	0.903
Women	115 ± 9	36	130 ± 10	16	0.634
Smokers	123 ± 10	31	112 ± 14	13	0.563
Non-smokers	121 ± 8	43	140 ± 10	18	0.198
SBs					
All	77 ± 4	76	61 ± 5	34	**0.017**
Men	75 ± 6	38	57 ± 8	17	**0.036**
Women	79 ± 6	38	65 ± 7	17	0.181
Smokers	81 ± 5	33	69 ± 8	12	0.237
Non-smokers	74 ± 6	43	56 ± 7	22	**0.032**
SBs+Fpg					
All	119 ± 5	76	101 ± 7	33	**0.044**
Men	116 ± 7	39	102 ± 12	16	0.354
Women	123 ± 8	37	100 ± 8	17	0.061
Smokers	123 ± 7	33	105 ± 10	13	0.178
Non-smokers	116 ± 7	43	98 ± 9	20	0.123
netFpg					
All	43 ± 3	75	38 ± 4	31	0.622
Men	41 ± 5	38	42 ± 5	15	0.906
Women	46 ± 5	37	35 ± 5	16	0.191
Smokers	42 ± 5	33	36 ± 7	11	0.218
Non-smokers	44 ± 4	42	40 ± 4	20	0.519
SBs+EndoIII					
All	132 ± 6	76	102 ± 9	34	**0.007**
Men	136 ± 7	38	96 ± 15	16	**0.009**
Women	127 ± 9	38	108 ± 10	18	0.215
Smokers	139 ± 9	33	107 ± 12	12	0.059
Non-smokers	126 ± 8	43	99 ± 12	22	0.063
netEndoIII					
All	56 ± 4	75	43 ± 6	33	0.079
Men	61 ± 6	38	38 ± 8	16	**0.034**
Women	51 ± 6	37	47 ± 8	17	0.653
Smokers	59 ± 7	33	40 ± 9	11	0.163
Non-smokers	54 ± 5	42	44 ± 7	22	0.258
SBs+AlkA					
All	166 ± 11	73	153 ± 18	35	0.206
Men	185 ± 16	36	112 ± 20*	17	**0.003**
Women	147 ± 13	37	191 ± 28*	18	0.274
Smokers	181 ± 17	32	132 ± 19	14	0.066
Non-smokers	153 ± 14	41	167 ± 28	21	0.818
netAlkA					
All	83 ± 9	78	92 ± 17	36	0.954
Men	100 ± 15	39	54 ± 14	18	0.087
Women	65 ± 10	39	130 ± 29	18	0.087
Smokers	96 ± 16	33	73 ± 18	14	0.553
Non-smokers	73 ± 11	45	104 ± 25	22	0.626
H_2O_2 sensitivity					
All	274 ± 6	76	255 ± 7	36	**0.027**
Men	282 ± 7	39	244 ± 12	18	**0.013**
Women	265 ± 9	37	266 ± 7	18	0.900
Smokers	271 ± 8	33	256 ± 11	14	0.333
Non-smokers	276 ± 8	43	255 ± 9	22	**0.049**

Difference between men and women * $p = 0.020$. SBs – strand breaks; Fpg - formamidopyrimidine DNA glycosylase sensitive sites; EndoIII – endonuclease III sensitive sites; AlkA - 3-methyladenine DNA glycosylase II sensitive sites.

Table 4
Multivariate analysis for DNA damage markers.

Variable	Covariate	Categories	Exp (β) / MR	95% CI	*p*-Value
SBs	Exposure	Reference	1.00	–	–
		Exposed	1.20	1.00–1.44	**0.044**
	Catalase (kU/gHb)	<15.95	1.00	–	–
		15.95–25.44	0.96	0.77–1.19	0.685
		>25.44	0.67	0.54–0.83	**<0.001**
	Dietary fibre (g)	<5.78	1.00	–	–
		5.78–8.08	0.95	0.77–1.17	0.629
		>8.08	0.72	0.55–0.95	**0.020**
	Total carotenoids (mg)	<1.48	1.00	–	–
		1.48–3.18	1.28	1.03–1.60	**0.029**
		>3.18	1.39	1.08–1.80	**0.013**
	XRCC1 rs25487	CC	1.00	–	–
		CT+TT	0.74	0.62–0.88	**0.001**
SBs+Fpg	Catalase (kU/gHb)	<15.95	1.00	–	–
		15.95–25.44	1.00	0.83–1.21	0.995
		>25.44	0.76	0.62–0.94	**0.010**
netFpg	*XRCC1* rs3213245	AA	1.00	–	–
		AG	0.58	0.43–0.80	**0.001**
		GG	0.50	0.32–0.78	**0.003**
	XRCC1 rs25487	CC	1.00	–	–
		CT	0.76	0.55–1.07	0.115
		TT	0.59	0.35–0.99	**0.045**
	Eosinophils (%)		0.85	0.76–0.96	**0.008**
SBs+EndoIII	GST (U/gHb)	<80.01	1.00	–	–
		80.01–118.49	0.79	0.63–0.99	**0.039**
		>118.49	0.66	1.09–1.75	**0.001**
	GPx (U/gHb)	<16.07	1.00	–	–
		16.07–24.86	1.23	0.98–1.54	0.071
		>24.86	1.38	1.09–1.75	**0.008**

MR: percentage change in the mean frequency of the variable of interest for a unitary increase of the predictor on a continuous scale or with respect to a reference level for categorical predictors; GST - Glutathione-S-transferase; GPx - Glutathione peroxidase; SBs – strand breaks; Fpg – formamidopyrimidine DNA glycosylase sensitive sites; EndoIII – endonuclease III sensitive sites.

an association of metabolic genes with markers of antioxidant protection and DNA stability [5] as well as an association of a polymorphism in nucleotide excision repair gene XPA with DNA repair capacity on 8-oxo-guanine [39]. In the present study, we show a significant association between *XRCC1* and SBs as well as netFpg sites. The XRCC1 protein interacts with DNA ligase III, polymerase beta and poly (ADP-ribose) polymerase to participate in the base excision repair pathway. The XRCC1 protein does not have enzymatic activity, but acts as a scaffolding protein that interacts with multiple repair enzymes. The scaffolding allows these repair enzymes to then carry out their enzymatic steps in repairing DNA. XRCC1 has a larger function and additionally to base excision repair is also involved in single-strand break repair, and nucleotide excision repair [40].

Haematological parameters are described in Table S7. An inverse correlation found between DNA damage biomarkers and percentage of eosinophils in peripheral blood of both exposed (SBs $r = -0.344$, $p = 0.002$; SBs+Fpg $r = -0.334$, $p = 0.003$; SBs+EndoIII $r = -0.302$, $p = 0.008$; AlkA $r = -0.253$, $p = 0.031$) and reference (SBs $r = -0.409$, $p = 0.016$; SBs+Fpg $r = -0.396$, $p = 0.023$; AlkA $r = -0.458$, $p = 0.006$; Net AlkA $r = -0.400$, $p = 0.016$, Repair (8-oxoG) $r = 0.404$, $p = 0.024$) groups was confirmed by multivariate analysis for netFpg sites (Exp$\beta = 0.85$, $p = 0.008$). Eosinophils are multifunctional leukocytes involved in inflammatory responses, as well as in tissue homeostasis. In agreement with our findings they were shown to have the ability to repair both double and single strand DNA breaks [41].

EndoIII seems a more sensitive and reliable enzyme than Fpg, detecting DNA oxidation in lymphocytes of exposed subjects (Table 3). This was also seen in the asbestos biomonitoring study where we found significantly higher numbers of EndoIII sites in asbestos-exposed men compared to men in the reference group [4]. There is a correlation between Fpg and EndoIII sites, consistent with the similar nature and origin of their substrates – oxidised bases (Table 6).

Events subsequent to the initial occurrence of DNA damage influence

Table 5
DNA damage values in the whole group and exposed subjects stratified by *XRCC1* rs3213245 (5'UTR) and rs25487 (Gln399Arg) genotypes.

Additive model							
XRCC1 rs3213245			AA	AG	GG	*p*-Value[a]	*p*-Value[b]
	SBs	all	62 ± 6[&]	69 ± 5	87 ± 8[&]	0.045	**0.042**
	netFpg	all	55 ± 5*	37 ± 3*	38 ± 6	0.027	**0.036**
		exposed	62 ± 6*[&]	38 ± 4*	37 ± 9[&]	0.006	**0.007*/0.038[&]**
XRCC1 rs25487			CC	CT	TT		
	SBs+EndoIII	all	137 ± 8[&]	118 ± 7	94 ± 15[&]	0.023	**0.031**
		exposed	154 ± 9*[&]	124 ± 8*	93 ± 21[&]	0.003	**0.039*/0.007[&]**
	SBs+AlkA	exposed	202 ± 17[&]	156 ± 15	95 ± 24[&]	0.004	**0.005**
Dominant model							
XRCC1 rs3213245			AA		AG+GG		*p*-ValuecMann Whitney U test
	netFPG	all	55 ± 5		38 ± 3		**0.007**
XRCC1 rs25487			CC		CT+TT		
	SBs	all	82 ± 6		65 ± 4		**0.031**
	SBs+Fpg	all	126 ± 8		105 ± 5		**0.015**
	SBs+EndoIII	all	137 ± 8		113 ± 6		**0.019**
Recessive model							
XRCC1 rs3213245			AA+AG		GG		
	SBs	all	67 ± 4		87 ± 8		**0.022**
XRCC1 rs25487			CC+CT		TT		
	SBs+EndoIII	all	126 ± 5		94 ± 15		**0.042**
	SBs+AlkA	all	169 ± 10		106 ± 16		**0.035**
	netAlkA	all	93 ± 9		38 ± 9		**0.038**

Only variables differing significantly between genotypes are presented; *, significant difference between AA and Aa; [&], significant difference between AA and aa; p-Value[a], one-way ANOVA or Kruskal-Wallis test depending on normality, p-Value[b], multiple comparisons; *p*-Value[c], t-test or Mann-Whitney U test depending on normality; SBs – strand breaks; Fpg - formamidopyrimidine DNA glycosylase sensitive sites; EndoIII – endonuclease III sensitive sites; AlkA - 3-methyladenine DNA glycosylase II sensitive sites.

Table 6
Correlations between analyzed variables in the whole group.

		SBs	SBs+Fpg	netFpg	SBs+EndoIII	netEndoIII	SBs+AlkA	netAlkA	H_2O_2 sens
Repair (8-oxoG)	r (*p*-Value	**-0.45 (<0.001)**	**-0.32 (0.001)**	-0.12 (0.256)	**-0.29 (0.003)**	-0.09 (0.366)	**-0.21 (0.040)**	-0.08 (0.429)	**-0.20 (0.047)**
SBs	r (*p*-Value)		**0.75 (<0.001)**	0.01 (0.909)	**0.68 (<0.001)**	0.13 (0.186)	**0.47 (<0.001)**	0.16 (0.098)	**0.32 (0.001)**
SBs+Fpg	r (*p*-Value)			**0.62 (<0.001**	**0.78 (<0.001)**	**0.40 (<0.001)**	**0.45 (<0.001)**	0.18 (0.062)	**0.23 (0.017)**
NetFpg	r (*p*-Value)				**0.32 (0.001)**	**0.42 (<0.001)**	0.11 (0.294)	0.05 0.579	0.00 (0.988)
SBs+EndoIII	r (*p*-Value)					**0.75 (<0.001)**	**0.70 (<0.001)**	**0.46 (<0.001**	**0.35 (<0.001**
NetEndoIII	r (*p*-Value)						**0.52 (<0.001)**	**0.56 (<0.001)**	**0.24 (0.012)**
SBs+AlkA	r (*p*-Value)							**0.86 (<0.001)**	**0.26 (0.006)**
NetAlkA	r (*p*-Value)								0.13 (0.179)

SBs – strand breaks; Fpg – formamidopyrimidine DNA glycosylase sensitive sites; EndoIII – endonuclease III sensitive sites; AlkA - 3-methyladenine DNA glycosylase II sensitive sites.

whether effects are seen at the gene or chromosome level. DNA damage is regarded as a marker of exposure, while chromosome changes are a downstream marker of effect. DNA damage can be repaired or fixed as mutations. Elevated SBs or oxidised bases are therefore likely to be indicative of higher rates of gene mutation (whether or not chromosomal damage is seen) and can be regarded as an early warning of possible risk of disease. Therefore, it is not surprising that in spite of significant increases in DNA damage, glass fibre exposure did not affect the level of chromosome aberrations or micronuclei in subjects working in the glass fibre factory (Table S8). However, smokers in the reference group had slightly but not significantly elevated levels of aberrations ($p = 0.06$) over non-smokers, consistent with higher PAH exposure in office workplaces compared with the production hall. Regarding micronucleus induction, there was a significant difference between men and women (2.56 ± 0.3 vs. 6.98 ± 0.8, $p < 0.001$) as has been previously found in many studies, including our studies of asbestos and stone wool exposure [3,4].

In our two previous related studies we showed that asbestos exposure (but not exposure to stone wool) was associated with a significant increase in chromosome aberrations, a predictive marker of cancer risk [3,4]. The effect was greater in smokers than in non-smokers. Micronucleus frequency was not influenced by exposure, but increased with age and was higher in women than in men (as has been previously reported) [3,4,42,43]. SBs in lymphocytes of stone wool-exposed workers were higher compared with the reference group and oxidised pyrimidines were significantly higher in asbestos-exposed men compared with the respective reference group [3,4]. In asbestos-exposed subjects there was also a positive association between SBs and age, and between alkylation damage to DNA and age. Moreover, oxidised pyrimidines and alkylated bases strongly correlated with duration of occupational exposure to asbestos. Repair capacity (8-oxoguanine DNA glycosylase activity) overall did not show any effect of exposure to stone wool, but was negatively correlated with micronucleus frequency, implying that unrepaired 8-oxoguanine contributes to micronucleus formation.

A recent retrospective study pooling together several cohorts with large numbers of subjects has shown that subjects with higher SB levels have a higher risk of mortality [44]. Thus, our finding that workers exposed to glass fibres had elevated DNA damage in their lymphocytes

suggests that they might be at risk of premature mortality. It is well established that biomarkers of effect (chromosomal aberrations, micronuclei) are predictive markers of cancer risk [45]. We found no increase in chromosomal damage, and so there is no evidence of an increase in cancer risk. However, our data indicate that even very low exposure to these fibres can cause significant genetic damage.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mrgentox.2022.503572.

References

[1] ⟨https://ec.europa.eu/environment/pdf/chemicals/2020/10/Strategy.pdf⟩.
[2] H. Louro, M. Heinälä, J. Bessems, J. Buekers, T. Vermeire, M. Woutersen, J. Van Engelen, T. Borges, C. Rousselle, E. Ougier, Human biomonitoring in health risk assessment in Europe: current practices and recommendations for the future, Int. J. Hyg. Environ. Health 222 (2019) 727–737.
[3] M. Dušinská, M. Barančoková, A. Kažimírová, V. Harrington, Kn Volkovová, M. Staruchová, A. Horská, L. Wsólová, A. Collins, Does occupational exposure to mineral fibres cause DNA or chromosome damage? Mutat. Res. /Fundam. Mol. Mech. Mutagen. 553 (2004) 103–110.
[4] M. Dušinská, A. Collins, A. Kažimírová, M. Barančoková, V. Harrington, Kn Volkovová, M. Staruchová, A. Horská, L. Wsólová, A. Kočan, Genotoxic effects of asbestos in humans, Mutat. Res. /Fundam. Mol. Mech. Mutagen. 553 (2004) 91–102.
[5] M. Dusinska, M. Staruchova, A. Horska, B. Smolkova, A. Collins, S. Bonassi, K. Volkovova, Are glutathione S transferases involved in DNA damage signalling? Interactions with DNA damage and repair revealed from molecular epidemiology studies, Mutat. Res 736 (2012) 130–137.
[6] C. Tran, S. Hankin, B. Ross, R. Aitken, A. Jones, K. Donaldson, V. Stone, R. Trantra, An outline scoping study to determine whether high aspect ratio nanoparticles (HARN) should raise the same concerns as do asbestos fibres, Report on project CB0406, (2008).
[7] K. Donaldson, F.A. Murphy, R. Duffin, C.A. Poland, Asbestos, carbon nanotubes and the pleural mesothelium: a review of the hypothesis regarding the role of long fibre retention in the parietal pleura, inflammation and mesothelioma, Part. Fibre Toxicol. 7 (2010) 1–17.
[8] European Chemical Agency, Appendix for nanoforms applicable to the Guidance on Registration and Substance Identification Version 2.0 January 2022; https://echa.europa.eu/documents/10162/13655/how_to_register_nano_en.pdf/f8c046ec-f60b-4349–492b-e915fd9e3ca0.
[9] F. Murphy, S. Dekkers, H. Braakhuis, L. Ma-Hock, H. Johnston, G. Janer, L. di Cristo, S. Sabella, N.R. Jacobsen, A.G. Oomen, A. Haase, T. Fernandes, V. Stone, An integrated approach to testing and assessment of high aspect ratio nanomaterials and its application for grouping based on a common mesothelioma hazard, NanoImpact 22 (2021), 100314.
[10] B.T. Mossman, A. Churg, Mechanisms in the pathogenesis of asbestosis and silicosis, Am. J. Respir. Crit. care Med. 157 (1998) 1666–1680.
[11] M. Sayan, B.T. Mossman, The NLRP3 inflammasome in pathogenic particle and fibre-associated lung inflammation and diseases, Part. Fibre Toxicol. 13 (2015) 1–15.
[12] T. Chernova, F.A. Murphy, S. Galavotti, X.-M. Sun, I.R. Powley, S. Grosso, A. Schinwald, J. Zacarias-Cabeza, K.M. Dudek, D. Dinsdale, Long-fiber carbon nanotubes replicate asbestos-induced mesothelioma with disruption of the tumor suppressor gene Cdkn2a (Ink4a/Arf), Curr. Biol. 27 (2017) 3302–3314, e3306.
[13] X. Wang, B. Sun, S. Liu, T. Xia, Structure activity relationships of engineered nanomaterials in inducing NLRP3 inflammasome activation and chronic lung fibrosis, NanoImpact 6 (2017) 99–108.
[14] T. Ong, Y. Liu, B.-Z. Zhong, W. Jones, W.-Z. Whong, Induction of micronucleated and multinucleated cells by man-made fibers in vitro in mammalian cells, J. Toxicol. Environ. Health Part A 50 (1997) 409–414.
[15] D. Cavallo, A. Campopiano, G. Cardinali, S. Casciardi, P. De Simone, D. Kovacs, B. Perniconi, G. Spagnoli, C.L. Ursini, C. Fanizza, Cytotoxic and oxidative effects induced by man-made vitreous fibers (MMVFs) in a human mesothelial cell line, Toxicology 201 (2004) 219–229.
[16] L. Proietti, A. Giallongo, A.M. Zakrzewska, I. Ammoscato, L. Lombardo, G. Frasca, V. Cardile, [Fibers glass induced cytotoxicity and genotoxicity], G Ital. Med Lav. Erg. 29 (2007) 274–276.
[17] V. Rapisarda, C. Loreto, C. Ledda, G. Musumeci, M. Bracci, L. Santarelli, M. Renis, M. Ferrante, V. Cardile, Cytotoxicity, oxidative stress and genotoxicity induced by glass fibers on human alveolar epithelial cell line A549, Toxicol. Vitr. 29 (2015) 551–557.
[18] D.E. Paglia, W.N. Valentine, Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase, J. Lab. Clin. Med. 70 (1967) 158–169.
[19] N.C. Cavarocchi, M.D. England, J.F. O'Brien, E. Solis, P. Russo, H.V. Schaff, T. A. Orszulak, J.R. Pluth, M.P. Kaye, Superoxide generation during cardiopulmonary bypass: is there a role for vitamin E? J. Surg. Res. 40 (1986) 519–527.
[20] W.H. Habig, M.J. Pabst, W.B. Jakoby, Glutathione S-transferases: the first enzymatic step in mercapturic acid formation, J. Biol. Chem. 249 (1974) 7130–7139.
[21] K.H. Schosinsky, H.P. Lehmann, M.F. Beeler, Measurement of ceruloplasmin from its oxidase activity in serum by use of o-dianisidine dihydrochloride, Clin. Chem. 20 (1974) 1556–1563.
[22] D. Cerhata, A. Bauerova, E. Ginter, Determination of ascorbic acid in blood serum using high-performance liquid chromatography and its correlation with spectrophotometric (colorimetric) determination, Ceska a Slov. Farm.: Cas. Ceske Farm. Spol. a Slov. Farm. Spol. 43 (1994) 166–168.
[23] D. Hess, H.E. Keller, B. Oberlin, R. Bonfanti, W. Schüep, Simultaneous determination of retinol, tocopherols, carotenes and lycopene in plasma by means of high-performance liquid chromatography on reversed phase, Int. J. Vitam. Nutr. Res. Int. Z. fur Vitam. -und Ernahr. J. Int. De. Vitaminol. Et. De. Nutr. 61 (1991) 232–238.
[24] A. Collins, G. Koppen, V. Valdiglesias, M. Dusinska, M. Kruszewski, P. Møller, E. Rojas, A. Dhawan, I. Benzie, E. Coskun, The comet assay as a tool for human biomonitoring studies: the ComNet project, Mutat. Res. /Rev. Mutat. Res. 759 (2014) 27–39.
[25] P. Møller, A. Azqueta, E. Boutet-Robinet, G. Koppen, S. Bonassi, M. Milić, G. Gajski, S. Costa, J.P. Teixeira, C. Costa Pereira, Minimum Information for Reporting on the Comet Assay (MIRCA): recommendations for describing comet assay procedures and results, Nat. Protoc. 15 (2020) 3817–3826.
[26] A. Collins, M. Dusinská, M. Franklin, M. Somorovská, H. Petrovská, S. Duthie, L. Fillion, M. Panayiotidis, K. Raslová, N. Vaughan, Comet assay in human biomonitoring studies: reliability, validation, and applications, Environ. Mol. Mutagen 30 (1997) 139–146.
[27] A.R. Collins, M. Dusinska, A. Horská, Detection of alkylation damage in human lymphocyte DNA with the comet assay, Acta Biochim. Pol. 48 (2001) 611–614.
[28] J.R. Savage, Classification and relationships of induced chromosomal structual changes, J. Med Genet 13 (1976) 103–122.
[29] M. Fenech, W.P. Chang, M. Kirsch-Volders, N. Holland, S. Bonassi, E. Zeiger, HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures, Mutat. Res. /Genet. Toxicol. Environ. Mutagen. 534 (2003) 65–75.
[30] K.J. Rothman, No adjustments are needed for multiple comparisons, Epidemiology (1990) 43–46.
[31] M. Kuricova, Tulinska, J., Liskova, A., Horvathova, M., Ilavska, S., Kovacikova, Z., Tatrai, E., Hurbankova, M., Cerna, S., Jahnova, E., Neubauerova, E., Wsolova, L., Wimmerova, S., Fuortes, L., Kyrtopoulos, S.A., & Dusinska, M., Chapter Immune System and Environmental Xenobiotics-The Effect of Selected Mineral Fibers and Particles on the Immune Response, (2012).
[32] A. Horská, A. Kazimírová, M. Barancoková, L. Wsólová, J. Tulinská, M. Dusinská, Genetic predisposition and health effect of occupational exposure to asbestos, Neuro Endocrinol. Lett. 27 (Suppl 2) (2006) 100–103.
[33] A. Horska, C. Mislanova, S. Bonassi, M. Ceppi, K. Volkovova, M. Dusinska, Vitamin C levels in blood are influenced by polymorphisms in glutathione S-transferases, Eur. J. Nutr. 50 (2011) 437–446.

[34] M. Staruchova, A.R. Collins, K. Volkovova, C. Mislanová, Z. Kovacikova, J. Tulinska, A. Kocan, L. Staruch, L. Wsolova, M. Dusinska, Occupational exposure to mineral fibres. Biomarkers of oxidative damage and antioxidant defence and associations with DNA damage and repair, Mutagenesis 23 (2008) 249–260.
[35] M. Dusinska, A.R. Collins, The comet assay in human biomonitoring: gene–environment interactions, Mutagenesis 23 (2008) 191–205.
[36] A. Mahmoud, Ö. Ezgi, A. Merve, G. Özhan, In Vitro Toxicological Assessment of Magnesium Oxide Nanoparticle Exposure in Several Mammalian Cell Types, Int. J. Toxicol. 35 (2016) 429–437.
[37] B. Mangalampalli, N. Dumala, R. Perumalla Venkata, P. Grover, Genotoxicity, biochemical, and biodistribution studies of magnesium oxide nano and microparticles in albino wistar rats after 28-day repeated oral exposure, Environ. Toxicol. 33 (2018) 396–410.
[38] N. Shangari, P.J. O'Brien, Catalase activity assays, Curr. Protoc. Toxicol., Chapter 7 Unit 7.7 (2006) 1–15.
[39] M. Dusinská, Z. Dzupinková, L. Wsólová, V. Harrington, A.R. Collins, Possible involvement of XPA in repair of oxidative DNA damage deduced from analysis of damage, repair and genotype in a human population study, Mutagenesis 21 (2006) 205–211.
[40] R.E. London, The structural basis of XRCC1-mediated DNA repair, DNA Repair 30 (2015) 90–103.
[41] S. Salati, E. Bianchi, R. Zini, E. Tenedini, D. Quaglino, R. Manfredini, S. Ferrari, Eosinophils, but not neutrophils, exhibit an efficient DNA repair machinery and high nucleolar activity, Haematologica 92 (2007) 1311–1318.
[42] A. Wojda, E. Ziętkiewicz, M. Witt, Effects of age and gender on micronucleus and chromosome nondisjunction frequencies in centenarians and younger subjects, Mutagenesis 22 (2007) 195–200.
[43] H. Thierens, A. Vral, R. Morthier, B. Aousalah, L. De Ridder, Cytogenetic monitoring of hospital workers occupationally exposed to ionizing radiation using the micronucleus centromere assay, Mutagenesis 15 (2000) 245–249.
[44] S. Bonassi, M. Ceppi, P. Møller, A. Azqueta, M. Milić, M. Neri, G. Brunborg, R. Godschalk, G. Koppen, S.A.S. Langie, J.P. Teixeira, M. Bruzzone, J. Da Silva, D. Benedetti, D. Cavallo, C.L. Ursini, L. Giovannelli, S. Moretti, P. Riso, C. Del Bo, P. Russo, M. Dobrzyńska, I.A. Goroshinskaya, E.I. Surikova, M. Staruchova, M. Barančokova, K. Volkovova, A. Kažimirova, B. Smolkova, B. Laffon, V. Valdiglesias, S. Pastor, R. Marcos, A. Hernández, G. Gajski, B. Spremo-Potparević, L. Živković, E. Boutet-Robinet, H. Perdry, P. Lebailly, C.L. Perez, N. Basaran, Z. Nemeth, A. Safar, M. Dusinska, A. Collins, DNA damage in circulating leukocytes measured with the comet assay may predict the risk of death, Sci. Rep. 11 (2021) 16793.
[45] S. Bonassi, A. Znaor, M. Ceppi, C. Lando, W.P. Chang, N. Holland, M. Kirsch-Volders, E. Zeiger, S. Ban, R. Barale, M.P. Bigatti, C. Bolognesi, A. Cebulska-Wasilewska, E. Fabianova, A. Fucic, L. Hagmar, G. Joksic, A. Martelli, L. Migliore, E. Mirkova, M.R. Scarfi, A. Zijno, H. Norppa, M. Fenech, An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans, Carcinogenesis 28 (2007) 625–631.