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Assessment of genotoxic effects related to chronic low level exposure to ionizing radiation using biomarkers for DNA damage and repair

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The first objective of our study was to analyse whether biomarkers for genotoxic effects (DNA breaks and alkali-labile sites and micronucleus and non-disjunction frequencies) could be fully validated for biomonitoring workers chronically exposed to ionizing radiation (IR). Blood samples of controls and individuals chronically exposed to IR were analysed. The interindividual variation was reduced when the comet data were adjusted for inter-experimental variation, but remained statistically significant. No differences were found between groups, either for smoking or for exposure status. The second objective was to determine whether the Comet assay can be used to evaluate global repair phenotype as a susceptibility biomarker for IR-induced DNA damage in nuclear workers. A pilot study was performed and blood from workers exposed or not to radiation was submitted to a challenging dose of γ-rays. The repair kinetics of each individual donor were analysed by Comet assay at different time points and compared with the frequencies of biomarkers of genotoxic effects. There was a statistically significant interaction between biomarkers assessing the same damage (micronucleus and Comet assays). Multivariate analysis showed that micronucleus frequencies were positively influenced by age and the percentage of residual tail length was negatively influenced by the interaction between smoking and exposure status. The general conclusions from our study are: (i) a positive correlation exists between mechanistically related biomarkers; (ii) multivariate regression analysis confirmed that the interaction between smoking and exposure to IR negatively and statistically significantly influenced residual tail length; (iii) use of the Comet assay for the estimation of global repair phenotype with respect to IR is recommended because it is simple, fast and differences in *in vitro* repair capacity can be detected.

Introduction

To improve cancer prevention in workers chronically exposed to low level ionizing radiation (IR) in nuclear power plants, occupational medicine applies essentially biomonitoring of exposure and effects. In the future it may also include biomonitoring of susceptibility, relating genotype and phenotype polymorphisms to differential risk for cancer.

As far as the biomonitoring of IR exposure is concerned, it is restricted to the estimation of the duration of occupational exposure, dose and type of irradiation. Several methodologies have been developed to perform the biomonitoring of effects, but their sensitivity and specificity are often too low to detect genetic effects considering the annual occupational limit of 20 mSv. Assessment of the frequencies of dicentrics and chromosome translocations by Giemsa staining (Lloyd *et al*., 1992) or by chromosome-specific fluorescence *in situ* hybridization (FISH) painting in general is considered the most sensitive method (reviewed in IARC, 1997). However, the technique is time consuming and demands expertise. Scoring of ~1000 metaphases for translocations after chromosome painting allows the detection of doses of ~0.3 Gy (Snigiryova *et al*., 1997). According to Thierens *et al*. (1999), reaching the detection limit of 0.02 Gy after *in vitro* exposure to X-rays should require scoring of 10 000 metaphases with conventional Giemsa analysis of dicentrics; the detection of 0.25 Gy after *in vitro* exposure to γ-rays, using a cocktail of chromosome 2, 4 and 8 painting probes, should require a minimum of 1000 cells.

In the last decade the *ex vivo*/*in vitro* lymphocyte micronucleus (MN) test (Fenech and Morley, 1985) combined with pan-(peri-)centromeric FISH probes has provided information not only on the induction of acentric fragments ($MNCen^-$) but also on chromosome loss ($MNCen^+$) (Elhajouji *et al*., 1995; Van Hummelen *et al*., 1995). Scoring only the $MNCen^-$ was suggested to have higher sensitivity with a lower workload and cost (Vral *et al*., 1997). Based on the last observation, the scoring of $MNCen^-$ only improves the detection level from 0.25 to 0.1 Gy. Double FISH labelling of cytochalasin-blocked cells with chromosome-specific probes permits the assessment of chromosome non-disjunction (NDJ) (Kirsch-Volders *et al*., 1996; Elhajouji *et al*., 1997). Chromosome loss and NDJ were shown not to have significant consequences in human fibroblasts after *in vitro* exposure to X-rays (Kirsch-Volders *et al*., 1996) and in mouse splenocytes after *in vivo* exposure to the same mutagen (Hande *et al*., 1997). In addition, we have demonstrated a statistically significant increased rate of NDJ in human peripheral blood lymphocytes (PBLs) after *in vitro* exposure to 1 Gy γ-rays (Touil *et al*., 2000). Only 2000 binucleated interphase cells (BN) were required to reach this conclusion.

Another straightforward technique for the quantification of DNA damage in a cell-by-cell approach is the single cell gel electrophoresis or Comet assay (Singh *et al*., 1988). Its application under alkaline conditions as a sensitive technique in the regular health screening of workers who are occupationally exposed to genotoxic environmental agents to assess the possibility of different types of DNA damage [DNA strand breaks and alkali-labile sites (ALS)] is well documented (reviewed by Moller *et al*., 2000). For IR, the assay is relatively sensitive [the detection limit of γ-rays in peripheral blood lymphocytes (PBLs) and the number of cells needed for this analysis were, respectively, 0.6 cGy and 50 cells] (Malyapa

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Table I. Characteristics of the study population

	Controls	Exposed
Number of workers	19	28
Age (years)	42.2 ± 6.8*	50.0 ± 6.3[a]
Years of exposure	–	20 years
Adsorbed dose (mSv)		
range	–	19.5 – 242.3
mean ± SD	–	82.3 ± 44.7[a]
Smoking status		
% smokers	58%	61%
cigarettes/day	15.6 ± 8.1*	19.8 ± 6.3[a]
Alcohol (drinks/week)	12.1 ± 12.1*	9.2 ± 9.4[a]
Fruits (piece/week)	8.9 ± 10.3*	9.0 ± 7.7[a]

[a]Mean ± standard deviation.

et al., 1998). Recently, De Boeck *et al*. (2000) further validated the methodology for biomonitoring purposes with the introduction of an internal standard.

The biomonitoring of susceptibility is not yet applicable due to the lack of scientifically sound assessment of our capacity to quantify the effects of genetic polymorphisms on the cellular response to IR. Moreover, bioethical issues related to the implementation of genetic screening for IR susceptibility in occupational medicine need to be addressed in all their aspects before the implementation of methods can be considered. However, it is important to realize that the development of scientifically adequate methods to assess individual susceptibility to IR exposure may be helpful not only to prevent undesirable exposure, but also to allow a more accurate interpretation of the genotoxic effects observed during occupational follow-up. Therefore, genotyping and phenotyping studies of those polymorphisms which are relevant to exposure to IR are becoming essential. As far as IR is concerned, DNA repair, cell cycle and apoptosis genes are the most important. Information on genetic polymorphisms of repair genes is needed before implementing genotyping as a tool in biomonitoring. Polymorphisms of several related repair genes have been identified (e.g. OGG1, XRCC1 and XRCC5), some of which possibly play a role in clinical radiosensitivity (Price *et al*., 1997). However, development and evaluation of a global 'phenotype' repair assay to assess the global repair capacity for IR-induced DNA damage might reflect the genotype status and be helpful in identifying hypersensitive individuals (Leprat *et al*., 1998).

This work has addressed two major questions: (i) are biomarkers of effects (DNA breaks and ALS, MN and NDJ frequencies) fully validated for biomonitoring of workers chronically exposed to ionizing radiation; (ii) can the Comet assay be used to evaluate global repair phenotype as a susceptibility biomarker for IR-induced DNA damage in nuclear workers. To achieve the second objective, a pilot study was performed and blood from workers exposed or not to a chronic dose of IR was submitted to a challenging dose of γ-rays. The repair kinetics of each individual donor were analysed by alkaline Comet assay and compared with the frequencies of biomarkers of effect (DNA breaks and ALS, MN and NDJ frequencies).

Materials and methods

Study population

The characteristics of the study populations are summarized in Table I. A group of workers (28 males) occupationally exposed to low doses of IR in a nuclear power plant, with 20 years average duration of employment, and a group of controls (19 men) chosen from among administrative employees were monitored. Exposure was estimated from personnel dosimeter records, the doses ranged from 19.54 to 242 mSv. Moreover, information on age, smoking habits, intake of vitamins, use of therapeutic drugs and previous exposures to diagnostic X-rays was recorded. We were unable to perform an analysis of all the biomarkers for each individual because of lack of material.

Sampling

Blood (10 ml) drawn by venipuncture into heparinized tubes (Vacutainer; Benton Dickinson, Oxford, UK) was delivered to the laboratory by car within 4 h, protected from light. Samples from the controls and exposed subjects were handled concurrently. All assays were run on coded samples.

Biomarkers of genotoxic effect

Alkaline Comet assay

From each individual, 2.8 ml of whole blood was diluted with 3.2 ml of sterile Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS). An aliquot of 6 ml of the diluted blood was layered on top of a 3 ml Ficoll-Paque gradient (Pharmacia-Biotech, Brussels, Belgium) and centrifuged at 400 *g* for 45 min at room temperature. The interphase containing the mononuclear cells was collected with a Pasteur pipette, transferred to a separate tube and washed three times with PBS. For each electrophoresis, internal Comet slides from a human K562 erythroleukaemia cell line (Lozzio and Lozzio, 1975), treated or untreated with 2 mM ethylmethane sulfonate, were included as positive and negative standards, respectively. The procedure for the preparation of these samples has been described in a previous work (De Boeck *et al*., 2000). The Comet assay was performed according to the method described by Singh *et al*. (1988), with some modifications as reported (De Boeck *et al*, 1998). Briefly, 1000–5000 cells/ml in 0.8% low melting point agarose was layered on top of an ordinary microscope slide that had been pre-coated with 1% normal melting point agarose. The Comet slides were left in lysis solution overnight at 4°C (2.5 M NaCl, 10 mM Tris, 100 mM Na_2EDTA, pH 10) supplemented just before use with 10% DMSO and 1% Triton X-100. The coded slides from the human blood donors were transferred to an electrophoresis unit together with 200 µl of H_2O_2 (10 min).

Cytokinesis-blocked micronucleus (MN) assay

Whole blood cultures were made as follows: 1.6 ml of whole blood was placed in culture flasks at a concentration of 1×10^6 cells/ml and incubated in a humidified CO_2 incubator at 37°C in Ham's F-10 medium supplemented with 15% fetal calf serum (FCS) (Gibco, Paisley, UK). The lymphocytes were stimulated with 2% phytohaemagglutinin (PHA 16; Murex Biotech, Belgium) and treated with 6 µg/ml cytochalasin-B (Sigma Chemical Co., Belgium) at 44 h. At 72 h cells were subjected to a cold hypotonic treatment (0.075 M KCl), immediately centrifuged at 400 *g* for 8 min and fixed three times with methanol/acetic acid (3:1). The fixed cells were dropped onto humidified slides and air dried. For MN analysis the slides were stained directly with 5% Giemsa for 20 min, rinsed and air dried. The remaining slides were stored at –20°C until use in FISH analysis.

FISH with chromosome-specific centromeric probes

FISH with probes for centromeric regions of chromosomes 1 (pUCl.77) and 17 (D17Z1) was applied to slides from MN preparations. The probes were labelled by nick translation according to the instructions of the supplier (Life Technologies, BRL). FISH was performed as described by Touil *et al*. (2000). Briefly, the slides were treated with RNase (Sigma) (0.1 mg/ml in 2× SSC) for 1 h and pepsin solution for 10 min in a water bath (37°C). Thereafter, the slides were denatured at 80°C together with the probes. Following an overnight hybridization at 37°C, the slides were washed with 50% formamide in 2× SSC at 42°C. Detection of the biotinylated probe for chromosome 17 was performed with avidin–FITC (fluorescein avidin D; Vector Laboratories, Burlingame, CA) and biotinylated goat anti-avidin antibodies (Vector Laboratories), allowing signal amplification. The digoxigenin-labelled probe (chromosome 1) was detected using a mouse anti-digoxigenin antibody (Boehringer Mannheim, Germany) followed by a Texas Red-conjugated sheep anti-mouse antibody (Amersham, Little Chalfont, UK). After dehydrating in an ethanol series (50, 75 and 98%), the cells were counterstained with 4′,6-diamido-2 phenylindole (DAPI) (Boehringer Mannheim, Germany) in a phenylenediamine antifade solution.

Global repair phenotype Comet assay

For the study of repair kinetics of control and exposed workers, 2.8 ml of blood per donor was stored at 4°C in the dark until the following day. Previous experiments have shown that whole blood can be stored under these conditions for at least 24 h without affecting the level of DNA damage in lymphocytes (Vaghef *et al*., 1997).

Blood samples from 10 nuclear workers and 10 control individuals were used for the repair study. For each individual, two whole blood cultures of 35 ml were prepared. The blood was placed in culture flasks at a concentration of 1×10^6 cells/ml and incubated in a humidified CO_2 incubator at 37°C in Ham's F-10 medium supplemented with 15% FCS. The lymphocytes were stimulated with 2% PHA. After 24 h, cells were exposed to ^{60}Co γ-rays in the G_1 phase of the cell cycle. The blood cultures were irradiated *in vitro* with 2 Gy at 0.1 Gy/min in a water bath at 0°C to prevent any repair. Irradiation was performed on whole blood to better mimic the *in vivo* situation. Dosimetry was performed at the position of the samples with a NE2571 cylindrical ionization chamber and NE2570 dosimeter (Nuclear Enterprises, Reading, UK), applying the IAEA 1987 Code of Practice. Aliquots (2×5 ml) were removed from the cultures before and immediately after exposure and the Comet slides were stored in lysis solution at 4°C with internal standards prepared on the same day. The remaining blood cultures (25 ml), having been kept on ice, were incubated at 37°C to allow acclimatization for 20 min. This time was established in preliminary experiments. Thereafter, repair assessments were performed after *in vitro* irradiation (0 min) at times 5, 10, 30, 60 and 120 min, aliquots of 5 ml of blood cultures being centrifuged at 400 *g* for 10 min. The comet slides were prepared as described above (see Alkaline Comet assay). The slides prepared at different sampling times, all originating from the same individual, were kept overnight at 4°C in lysis solution with those obtained before and immediately after irradiation and internal standards prepared on the same day. They were processed together in the same electrophoresis session the following day.

Reproducibility of the assay

Whole blood collected from one volunteer was subjected to *in vitro* challenge with 2 Gy γ-rays followed by repair, as described above for the global repair phenotype. Four electrophoreses were performed to assess the reproducibility of the assay.

Analysis

Comet assay

For the Comet assay analysis 100 cells per donor from two cultures were randomly captured on coded slides using a Leitz fluorescence microscope (×25 objective) coupled to a CCD camera and image analysis system (Komet 3.0; Kinetic Imaging, Liverpool, UK). The length of the comet tail in μm (TL), the percentage of DNA damage in the tail (TDNA) and the product of TL and TDNA in μm (TM) were recorded.

MN assay

For the MN analysis micronuclei (MNi) were scored in both BN and in non-divided lymphocytes (mononucleated cells, i.e. Monos) on a same slide prepared 72 h after mitogen stimulation. The scoring criteria followed those proposed by Thierens *et al*. (1999). 2000 BN per individual (1000 BN per culture) were examined for the presence of one, two or more MNi and data are presented as the frequency of micronucleated binucleate cells per thousand binucleates (‰MNBN). The mononucleated lymphocytes with or without MNi were examined and data are presented as the frequency of micronucleated mononucleate cells (‰MNMonos).

FISH with chromosome-specific centromeric probes

For chromosomal NDJ, to restrict the scoring to the first mitosis and exclude artefacts, only BN having the diploid number of hybridization signals were analysed. The distribution of signals for both probes in BN was scored as 2/2, 1/3 and 0/4 for, respectively, two spots in each of the two nuclei, one spot in one nucleus and three spots in the second, no spots in one nucleus and four spots in the second. Both the 1/3 and 0/4 combinations were scored as a single NDJ cell. ‰NDJ represents the number of NDJ events observed per 1000 BN. The events involving chromosome 1 were scored independently of those involving chromosome 17 but were recorded in parallel in the same cells. The preparations were examined with a Zeiss Axioscop microscope (Carl Zeiss, Oberkochen, Germany) equipped with a filter (filter block 9; Zeiss) to visualize the fluorescein/Texas red-labelled probe and DAPI counterstaining. To estimate chromosomal NDJ frequencies (%NDJ) for the total genome, assuming that NDJ occurs randomly over the chromosomes, the initial frequencies of NDJ for both chromosomes were multiplied by 23/2.

Repair kinetics

For analysis of repair kinetics, the initial DNA damage and residual DNA damage were calculated as follows (Leprat *et al*., 1998):
initial DNA damage = DNA damage immediately after *in vitro* irradiation – DNA damage in control cells before *in vitro* irradiation;
per cent residual DNA damage at time *t* after irradiation (%RD) = 100×(DNA damage at time *t* after irradiation – DNA damage in control cells before irradiation) ÷ (DNA damage immediately after irradiation – DNA damage in control cells before irradiation).

Three parameters of RD were recorded: per cent residual tail length (%RDTL), per cent residual tail DNA (%RDTDNA) and per cent residual tail moment (%RDTM).

Statistical evaluation of data

For all genotoxicity tests, the interindividual variation was analysed by a Student's *t*-test.

TL, TDNA, TM, ‰MNMonos, ‰MNBN and %NDJ showed significant departures from a normal distribution. Therefore, all data underwent log transformation and a two-way ANOVA design was performed to evaluate the possible influences of smoking and exposure on the biomarkers. A simple regression analysis was performed to verify the correlation between all genotoxicity parameters and age for each group (exposed workers and controls separately) and for all workers together.

In addition, a multiple regression analysis (stepwise multivariate analysis) was performed to determine the relative influence of the different independent variables (cumulative dose, smoking and age in the exposed group and exposure, smoking and age in all workers) on genotoxicity parameters. Statistical analyses were performed using the SPSS and SAS packages.

Results

Biomarkers of effect

The comet, MN and NDJ data are summarized in Table II. The results compare the control and exposed groups. In addition, the control and exposed groups were subdivided into control non-smokers (CNS), control smokers (CS), exposed non-smokers (ENS) and exposed smokers (ES).

DNA damage data

The results for TL, TDNA and TM were corrected by the calibration model described in a previous work (De Boeck *et al*., 2000). TL, TDNA and TM showed similar patterns (Table II). No statistically significant effect of smoking or of exposure at the 95% confidence level was found within each group and between groups.

Micronucleus assay

A highly and statistically significant interindividual variability was found ($P < 0.001$) for MN frequencies in both MNMonos and MNBN using Student's *t*-test.

The frequency of MNMonos was not statistically different between controls and exposed workers (Table II). The frequency of MNMonos showed an increased level in CS as compared with CNS. However, this increase was not statistically significant. In ES there was a tendency for an increase in MNMonos but the difference between ENS and ES was not statistically significant.

No statistically significant increase in MNBN frequency was found in workers exposed to IR as compared with controls. In the control group the mean frequency of MNBN was higher in CS than in CNS, but the difference was not statistically significant. In the exposed group no clear effect of smoking was observed.

Chromosome NDJ

Chromosome-specific malsegregation was studied using FISH with specific centromeric probes for chromosomes 1 and 17. No statistically significant differences in NDJ frequencies between the two chromosomes were found. No significant differences in NDJ for chromosome 1 and for chromosome 17 frequencies between individuals in the control and exposed groups were detected. Frequencies of BN where NDJ was observed in the same cell for both chromosomes were extremely low. The data are shown for each group (Table II). No statistically significant differences between groups were found either for smoking or for exposure.

Global repair phenotype assay

Reliability of the assay

Reliability was determined using the intraclass correlation

Table II. Mean values ± standard deviations (SD) of biomarkers of effects

		Control	CNS	CS	Exposed	ENS	ES
TL (μm)	N	18	7	11	27	12	15
	Mean ± SD	1.00 ± 0.50	1.00 ± 0.41	1.00 ± 0.56	1.00 ± 0.60	1.20 ± 0.71	0.80 ± 0.47
	Range	0.51 − 2.40	0.64 − 1.70	0.51 − 2.40	0.37 − 3.00	0.37 − 3.00	0.37 − 1.90
TDNA (%)	N	18	7	11	27	12	15
	Mean ± SD	1.10 ± 0.75	1.10 ± 0.74	1.10 ± 0.79	0.9 ± 0.43	1.00 ± 0.58	0.80 ± 0.25
	Range	0.28 − 3.00	0.51 − 2.70	0.28 − 3.00	0.27 − 3.00	0.27 − 2.00	0.51 − 1.60
Tn (μm)	N	18	7	11	27	12	15
	Mean ± SD	1.03 ± 1.10	1.26 ± 1.55	0.89 ± 0.75	1.06 ± 1.29	1.55 ± 1.80	0.70 ± 0.45
	Range	4.71 − 0.14	4.71 − 0.25	0.14− 0.78	0.09 − 6.25	0.09− 6.25	0.25 − 1.68
MNMonos (‰)	N	17	8	9	26	11	15
	Mean ± SD	5.40 ± 3.70	4.90 ± 4.20	5.90 ± 3.50	4.50 ± 2.40	3.90 ± 2.50	5.00 ± 2.30
	Range	0.69 − 13.00.40	0.69 − 13.00	0.69 − 12.60	0.42 − 9.60	0.42 − 8.60	1.00 − 9.60
MNBN (‰)	N	17	8	9	26	11	15
	Mean ± SD	12.70 ± 7.90	10.20 ± 5.80	14.80 ± 9.20	13.7 ± 6.50	14.80 ± 5.70	12.90 ± 7.10
	Range	3.50 − 29.50	3.50 − 21.00	5.50 − 29.50	6.00 − 33.00	6.00 − 24.00	6.00 − 33.00
NDJ (%)	N	17	7	10	20	9	11
	Mean ± SD	15.00 ± 5.70	16.60 ± 8.30	14.00 ± 2.90	15.00 ± 5.20	14.50 ± 6.20	15.40 ± 4.50
	Range	7.50 − 33.30	7.50 − 33.30	10.40 − 19.50	6.80 − 24.10	6.80 − 24.10	9.30 − 23.20

TL (length of the tail in the comet in μm), TDNA (% DNA damage in the tail) and TM (TL * TDNA in μm) are parameters used to assess DNA damage; MNMonos: ‰ micronucleated mononucleates (Monos); MNBN: ‰ micronucleated binucleates (BN); NDJ: non-disjunction frequency. The assessment of these biomarkers was performed in control non-smokers (CNS), control smokers (CS), exposed non-smokers (ENS) and exposed smokers (ES).

Table III. Base line damage in both control and exposed individuals in whole blood cultures before the *in vitro* challenging assay with Gy γ-rays

		Controls	Exposed workers	
TL	N	10	10	
	Mean ± SD	28.2 ± 7.4	28.9 ± 13.2	$P < 0.49$
	Range	16.0 − 39.0	15.0 − 58.0	
TDNA	N	10	10	
	Mean ± SD	14.4 ± 4.7	$P < 0.95$	
	Range	6.0 − 21.0	7.3 − 33.0	
TM	N	10	10	
	Mean ± SD	11.3 ± 5.4	10.8 ± 6.7	$P < 0.85$
	Range	5.9 ± 22.0	4.0 ± 26.00	

N, the number of donors per group; SD, standard deviation; TL, tail length; TDNA, tail DNA; and TM, tail moment.

coefficient (ICC). There was significant ICC between the electrophoreses for the DNA repair parameters %RDTDNA and %RDTM ($P = 0.0003$). This is an indication that the *in vitro* global repair phenotype assay as performed in our laboratory is reproducible given that %RDTDNA is the most stable repair parameter.

To assess the global phenotype for repair of DNA breakage and ALS induced by IR, PHA-stimulated whole blood cultures were challenged *in vitro* with an acute exposure to 2 Gy ^{60}Co γ-rays applied to cells from 10 nuclear workers and 10 controls. For the controls only four individuals were non-smokers, while in contrast seven individuals were non-smokers in the exposed group.

The repair capacity was estimated at different time points (5, 10, 30, 60 and 120 min) after irradiation by the alkaline Comet assay. This allows the quantification of initial damage, the assessment of repair phenotype and the calculation of residual DNA damage at each time point.

Initial DNA damage induced by in vitro radiation in control and exposed worker groups

No significant effect of smoking and occupational exposure to IR on the level of initial DNA damage was found. The background level of DNA damage determined from TL, TDNA and TM was similar in both groups (Table III).

For all individuals a statistically significant increase in comet parameters (TL, TDNA and TM) in PHA-stimulated lymphocytes was detected immediately after *in vitro* exposure to 2 Gy γ-rays as compared with the DNA damage before irradiation ($P < 0.001$). The initial DNA damage for the three comet parameters was the same in both groups.

Residual DNA damage as measured by the time-dependent reduction in comet parameters in control and exposed worker groups

The *in vitro* γ-ray-induced DNA damage was progressively repaired in PHA-stimulated lymphocytes from both control and exposed workers as measured as a time-dependent reduction in the comet parameters. The interindividual variation was high, the standard deviation (SD) being elevated in both groups. Table IV shows the repair kinetics of DNA damage in both control and exposed individuals expressed as mean values of %RDTL, %RDTDNA and %RDTM. A period of faster repair (50% of DNA damage remains after 5 min) when considering %RDTM as a measure of repair capacity was observed in occupationally exposed individuals and controls. This faster repair was more pronounced in exposed than in control workers when considering %RDTDNA (48.35 ± 26.64 and 58.20 ± 29.48, respectively). At 30 min repair time the mean %RDTDNA in exposed individuals was, however, higher than in control individuals. %RDTL followed the same pattern as that for %RDTM, remaining higher at longer repair time points in the exposed group. Despite these differences in repair between groups, there were no statistically significant differences between groups (ANOVA analysis; see the *P* values in Table IV). For controls (Table V) the only statistically significant effect of smoking on repair capacity was seen for %RDTL after 30 min; this residual damage was found to be higher in CS. %RDTDNA remained higher in CS than CNS at 5, 10 and 30 min repair, but reached similar values at 60 min and were lower at 120 min, however, the *P* values were close to one at the last repair time point (i.e. 120 min). In the exposed group (Table VI) %RDTL followed an opposite trend in ES and was much lower than in ENS.

Table IV. Mean values and standard deviations (SD) of residual DNA damage in both controls and exposed individuals after *in vitro* exposure to 2 Gy γ-rays at different repair times (5, 10, 30, 60 and 120 min)

Comet parameters	Mean percentage of residual DNA damage ± SD (*P* values)				
	Repair time (min)				
	5	10	30	60	120
TL					
Control (n = 10)	64.22 ± 23.5	46.95 ± 32.5	39.80 ± 31.4	27.32 ± 29.2	11.41 ± 34.1
	(0.37)	(0.46)	(0.81)	(0.89)	(0.86)
Exposed (n = 10)	79.97 ± 31.5	57.48 ± 28.9	33.85 ± 69.0	23.05 ± 87.6	15.24 ± 57.4
TDNA					
Control (n = 10)	58.20 ± 29.4	42.79 ± 33.8	17.85 ± 20.2	14.69 ± 24.3	−3.29 ± 26.4
	(0.35)	(0.51)	(0.46)	(0.98)	(0.62)
Exposed (n = 10)	48.35 ± 28.0	31.01 ± 35.0	26.21 ± 37.9	10.01 ± 37.9	−0.77 ± 29.0
TM					
Control (n = 10)	48.78 ± 30.5	35.47 ± 28.47	15.46 ± 21.9	19.43 ± 23.6	0.53 ± 18.2
	(0.62)	(0.82)	(0.10)	(0.61)	(0.14)
Exposed (n = 10)	54.29 ± 21.2	38.16 ± 23.0	35.26 ± 27.3	24.97 ± 22.5	13.73 ± 18.0

ANOVA test was performed on non-tranformed data. %RDTL: % residual tail length, %RDTDNA: % residual tail DNA, %RDTM: % residual tail moment. The values in parentheses indicate the *P* values determined using ANOVA analyses.

Table V. Mean values and standard deviations (SD) of residual DNA damage in both control smokers and non-smoker after *in vitro* exposure to 2 Gy γ-rays at different repair times (5, 10, 30, 60 and 120 min)

Comet parameters	Mean percentage of residual DNA damage ± SD (*P* values)				
	Repair time (min)				
	5	10	30	60	120
TL					
CNS (n = 4)	46.67 ± 28.0	41.85 ± 38.7	14.23 ± 22.4	21.06 ± 34.9	−0.31 ± 24.1
	(0.06)	(0.72)	(0.03)	(0.65)	(0.43)
CS (n = 6)	75.93 ± 13.5	50.36 ± 27.0	56.85 ± 24.2	31.13 ± 23.8	19.24 ± 37.3
TDNA					
CNS (n = 4)	42.82 ± 21.5	29.62 ± 23.3	12.94 ± 21.5	19.17 ± 31.3	20.59 ± 20.5
	(0.65)	(0.58)	(0.98)	(0.52)	(0.93)
CS (n = 6)	62.15 ± 36.3	48.30 ± 38.2	23.96 ± 16.30	18.51 ± 22.0	−2.65 ± 11.5
TM					
CNS (n = 4)	33.22 ± 11.3	22.28 ± 19.9	−0.08 ± 17.3	11.60 ± 24.5	1.02 ± 23.3
	(0.11)	(0.28)	(0.07)	(0.44)	(0.95)
CS (n = 6)	60.50 ± 33.6	44.26 ± 30.3	25.82 ± 18.1	24.66 ± 21.4	0.20 ± 13.7

ANOVA test was performed on non-tranformed data. %RDTL: % residual tail length, %RDTDNA: % residual tail DNA, %RDTM: % residual tail moment. The values in parentheses indicate the *P* values determined using ANOVA analyses.

Global repair phenotype at the individual level

The global repair phenotype at the individual level aims at detecting individuals who have an elevated or diminished repair capacity in lymphocytes compared with an average level of repair. In addition, characterization of this capacity by defined parameters allows comparison between individuals. Therefore, the data for each individual were plotted separately for each time point on graphs giving ± 2 SD from the mean value calculated for the control non-smokers (CNS).

Figures 1–3 show %RDTL, %RDTDNA and %RDTM, respectively, measured 5, 10, 60 and 120 min after 2 Gy exposure for both control and exposed individuals.

As seen in Figure 1, two donors appeared to be deficient in repair capacity at the 120 min time point, one individual being CS and the other ENS. The corresponding %RDTL was closed to 90%.

Interestingly, three individuals appeared to have a high %RDTDNA after 5 min repair incubation. Two of these were CS and also showed an abnormal repair capacity at 10 min (Figure 2). One of the ENS appeared to have a high repair capacity at all time points.

%RDTM, which is in fact the product of both %RDTL and %RDTDNA, detected more individuals with less efficient repair capacity (Figure 3). Some of the donors who were CS indeed appeared to have a higher %RD damage during the faster repair period (i.e. 5 and 10 min).

Evaluation of the biomarkers

The analysis of possible mechanistic relationships between biomarkers was performed in the total population (Table VII). Significant and positive correlations were found between the different parameters (TL, TDNA and TM) assessing DNA damage (Comet assay). MNBN and MNMonos were also significantly correlated. TL correlated significantly with MNBN and TM with NDJ. For %RDTDNA, a statistically significant and positive correlation with %RDTM was found.

Table VI. Mean values and standard deviations (SD) of residual DNA damage in both exposed smokers and non-smokers after *in vitro* exposure to 2 Gy γ-rays at different repair times (5, 10, 30, 60 and 120 min)

Comet parameters	Mean percentage of residual DNA damage ± SD (*P* values)				
	Repair time (min)				
	5	10	30	60	120
TL					
ENS (n = 7)	64.36 ± 20.4 (0.21)	59.47 ± 23.9 (0.60)	46.38 ± 29.5 (0.24)	41.06 ± 29.5 (0.34)	41.11 ± 23.2 (0.07)
ES (n = 3)	81.92 ± 14.0	69.72 ± 26.7	71.95 ± 5.4	65.36 ± 30.3	6.03 ± 22.5
TDNA					
ENS (n = 7)	54.79 ± 28.9 (0.31)	36.79 ± 36.8 (0.48)	25.45 ± 35.9 (0.92)	9.30 ± 45.1 (0.87)	0.61 ± 28.6 (0.84)
ES (n = 3)	33.39 ± 9.6	17.52 ± 21.2	28.32 ± 39.7	14.04 ± 16.8	−4.00 ± 23.8
TM					
ENS (n = 7)	55.33 ± 20.3 (0.83)	39.39 ± 25.1 (0.82)	31.31 ± 26.9 (0.54)	23.93 ± 26.6 (0.84)	17.14 ± 17.2 (0.41)
ES (n = 3)	51.86 ± 23.0	35.27 ± 16.7	44.49 ± 26.1	27.39 ± 5.5	5.76 ± 17.4

ANOVA test was performed on non-tranformed data. %RDTL: % residual tail length, %RDTDNA: % residual tail DNA, %RDTM: % residual tail moment. The values in parentheses indicate the *P* values determined using ANOVA analyses.

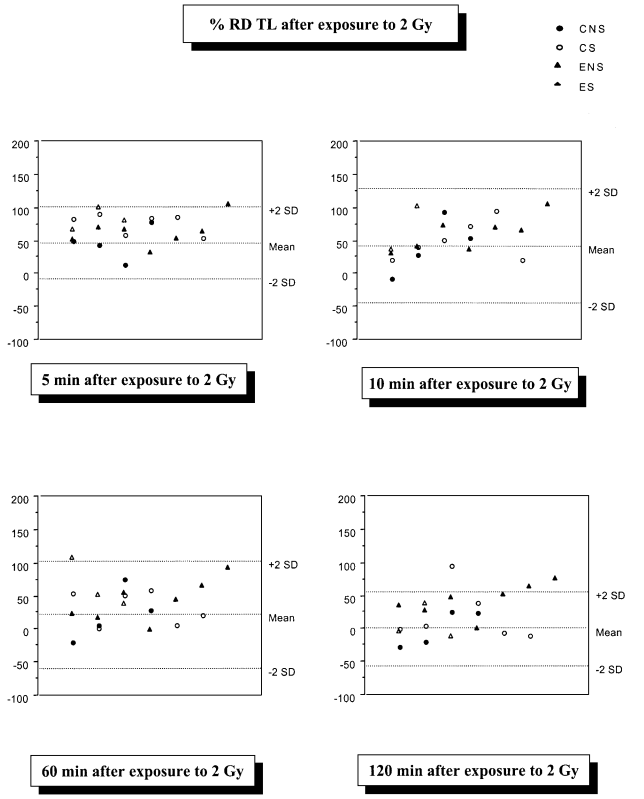

Fig. 1. %RDTL measured 5, 10, 60 and 120 min after 2 Gy exposure in control non-smoking and smoking individuals (CNS and CS, respectively) and in exposed non-smokers and smokers (ENS and ES, respectively). The area between the dotted lines defines the normal response (i.e. the mean of CNS ± 2 SD).

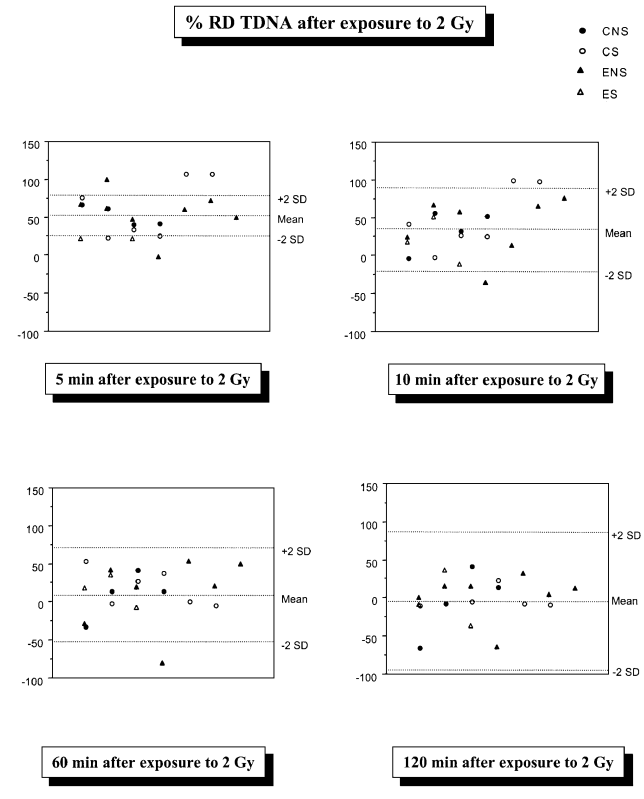

Fig. 2. %RDTDNA measured 5, 10, 60 and 120 min after 2 Gy exposure in control non-smoking and smoking individuals (CNS and CS, respectively) and in exposed non-smokers and smokers (ENS and ES, respectively). The area between the dotted lines defines the normal response (i.e. the mean of CNS ± 2 SD).

However, a negative correlation between %RDTM and %NDJ was found.

Interaction of confounders with genotoxicity markers

The influence of independent variables, namely cumulative dose and smoking habit, on genotoxicity parameters was examined by multiple regression analysis in all workers. Interactions between cumulative dose and smoking (I1), exposure and smoking (I2) and exposure and age (I3) were examined (Table VIII). MNMonos frequencies were influenced positively by age and negatively by I3 (i.e. interaction between exposure and age). MNBN frequencies were influenced positively by age and %RDTL was influenced negatively by the interaction between smoking and exposure status.

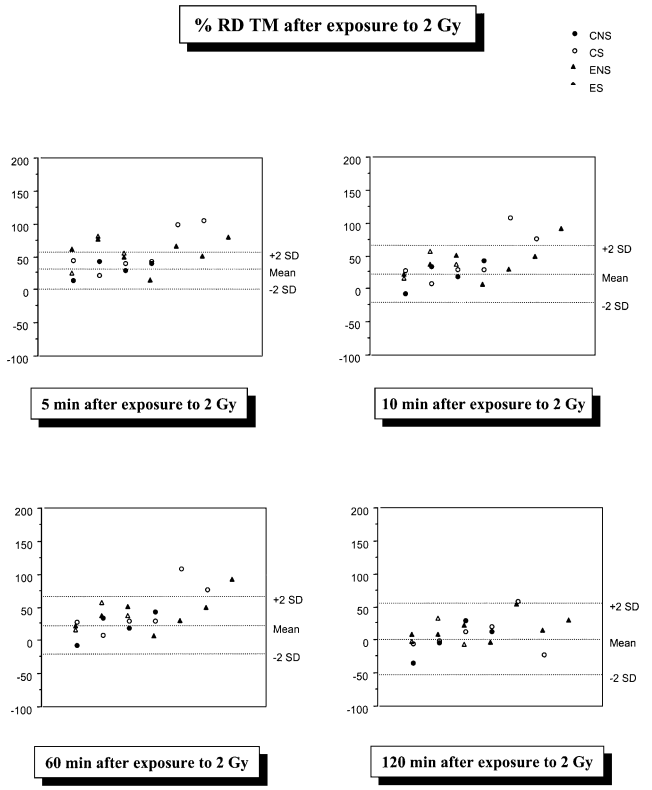

Fig. 3. %RDTM measured 5, 10, 60 and 120 min after 2 Gy exposure in control non-smoking and smoking individuals (CNS and CS, respectively) and in exposed non-smokers and smokers (ENS and ES, respectively). The area between the dotted lines defines the normal response (i.e. the mean of CNS ± 2 SD).

Discussion

This work has addressed two major questions: (i) are biomarkers of effects (DNA breaks and ALS, MN and NDJ frequencies) fully validated for biomonitoring of workers chronically exposed to ionizing radiation; (ii) can the Comet assay be used to evaluate global repair phenotype as a susceptibility biomarker for IR-induced DNA damage in nuclear workers.

The power of the present study is the use of several biomarkers assessing different types of damage after chronic low level exposure to IR. The first biomarker is the Comet assay, a biomarker of exposure and/or effect, validation of which is in progress. The other two are biomarkers of genotoxic effects. MN and non-disjunction assays were performed to analyse chromosome/genome mutations. Also, a global repair phenotype Comet assay was included in the test battery to better understand the intragroup and intergroup susceptibilities to chronic exposure to IR, taking into account smoking habits.

The controls and exposed individuals were not perfectly matched for smoking habits and age, and this could be a source of bias. Indeed, based on published data in our laboratory (Elhajouji *et al*., 1998), an increase in both MNi in Monos and in BN would be expected in the exposed group as compared with the controls since the exposed group was statistically significantly older. Therefore, multivariate analysis was applied to assess the effects of these confounders. Our study involved the assessment of genotoxicity markers in a rather limited number of subjects, especially for the global repair phenotype Comet assay. The latter should be considered as a pilot study to evaluate the method, by comparison with other biomarkers for genotoxicity, and to see whether it could be useful.

The strengths of this study are the application of recent standardized protocols for the Comet (De Boeck *et al*., 2000) and MN assays (Kirsch-Volders and Fenech, 2001).

To achieve the first objective in this work, the Comet assay, including standardization of the different comet parameters, was used. Our previous work has demonstrated that internal standards should be included with the biomonitoring samples in the electrophoreses and a calibration model should be used to minimize potential sources of bias and confounders (De Boeck *et al*., 2000). The interindividual variation was smaller but remained statistically significant when the data for the parameters TL, TDNA and TM were adjusted for both control and exposed individuals in our study. This indicates that the observed interindividual variation is not due to the experimental conditions. Moreover, these data are supported by the interindividual variation found for the biomarkers of effect (MN and NDJ). This variability between individuals may result from acquired or inherited susceptibility, personal exposure, differences in repair capabilities, nutritional status and/or the immune response.

In the nuclear workers TL was negatively influenced by smoking habit and TDNA by cumulative dose. Consequently, TM was influenced by the interaction of both independent variables, which is expected since TM is the product of TL and TDNA. In a recent work we clearly demonstrated that TDNA of an internal standard evaluated in 141 electrophoreses was more stable than TL (De Boeck *et al*., 2000). Therefore, we consider TDNA to be more reliable than TL for the evaluation of DNA damage. In addition, it measures the 'real' amount of breaks in the tail. The negative slope of the independent variable cumulative dose, which influences the parameter TDNA, could be seen as a protective effect of exposure to chronic low level IR for those workers, since there was no contribution of smoking habit.

As far as MNi frequencies are concerned, the frequency of MNi in both Monos and BN was determined. Since Monos are those cells that did not enter cell division after 72 h mitogen stimulation (Fenech *et al*., 1997), MNMonos frequency may indicate a cumulative effect over a long period. MNBN are those cells that may also harbour MNi present before they were stimulated to divide in culture (for a recent discussion of protocols and mechanisms see Kirsch-Volders and Fenech, 2001). In our study, although the mean frequency of MNMonos was slightly elevated as compared with exposed individuals and an opposite trend for MNBN in controls was observed, the mean frequencies of MNi in both Monos and BN did not differ significantly in the exposed group as compared with controls. A multiple regression analysis was performed to determine possible confounders which may influence the frequencies of MNi in both Monos and BN in all workers. MNi frequencies were positively influenced by age and negatively influenced by the interaction of age and cumulative dose for BN. This negative slope for the interaction factor on MNBN frequency is simply because younger people had higher cumulative doses of IR.

The level of NDJ observed in the nuclear workers was similar to that seen in the control group. It was previously demonstrated by our laboratory that after *in vitro* exposure to γ-rays NDJ correlated with increasing IR dose (Touil *et al*., 2000). NDJ events were not detectable at γ-ray doses <1 Gy. In the present study no statistically significant increase in NDJ

Table VII. Simple correlations for all workers using the SPSS package

		log TL	log TDNA	Log TM	log MNMono	log MNCB	log total NDJ	%RDTL	%RDTDNA
log TDNA	*r*	−0.6290							
	P	**−0.0001**							
log TM		−0.2690	−0.3930						
		−0.1880	**−0.0190**						
log MNMono		−0.0240	−0.0800	−0.0240					
		−0.8860	−0.6270	−0.8980					
log MNCB		−0.3470	−0.2130	−0.1970	−0.3400				
		−0.0280	−0.1870	−0.2890	**−0.0270**				
log total NDJ		−0.2750	−0.1420	−0.3810	−0.2540	−0.1060			
		−0.0990	−0.4020	**−0.0460**	−0.1470	−0.5500			
%RDTL		−0.2080	−0.3070	−0.0340	−0.3130	−0.0420	−0.2310		
		−0.3140	−0.1550	−0.9050	−0.1920	−0.8650	−0.3550		
%RDTDNA		−0.3310	−0.2620	−0.2590	−0.3900	−0.1610	−0.3330	−0.0880	
		−0.1330	−0.2390	−0.3510	−0.0990	−0.5090	−0.1910	−0.6970	
%RDTM		−0.0190	−0.3650	−0.1750	−0.1820	−0.1470	−0.5300	−0.5090	−0.0960
		−0.9310	−0.0870	−0.5340	−0.4570	−0.5490	**−0.0240**	**−0.0130**	−0.6690

TL (length of the tail in the comet in μm), TDNA (% DNA damage in the tail) and TM (TL×TDNA in μm) are parameters used to assess DNA damage. MNMonos, micronucleated mononucleates (Monos) per 1000 cells; MNBN, micronucelated binucleates (BN) per 1000 cells; NDJ, per cent non-disjunction frequency; %RDTL, per cent residual tail length; %RDTDNA, per cent residual tail DNA; %RDTM, per cent residual tail moment.

frequency with cumulative dose was observed in the exposed group. This is not surprising considering the low dose to which the workers were exposed (90.4 ± 49.3 mSv).

There are a number of factors which may prevent the detection of exposure effects by the biomarkers of effect (i.e. TDNA, MNMonos, MNBN and NDJ): (i) a low cumulative dose, which could at least explain the lack of detection of NDJ events and MNi in both Monos and BN after *in vivo* exposure in this study; (ii) death of damaged cells during cell division *in vivo* since cells, especially Monos, with chromosomal damage may be eliminated by apoptosis *in vivo*; (iii) chronic low level exposure could lead to the induction of an adaptive response (AR) and consequently damage leading to MNi in BN would be repaired in the *in vivo* situation, moreover, there was a negative influence of cumulative dose on TDNA which could also be considered as a type of AR; (iv) none of the biomarkers used actually measures a stable type of DNA damage and since the cumulative dose was relatively low, the dose received during the short period before sampling was much, much lower.

The most striking observation when using the global repair phenotype was that compared with lymphocytes from controls, lymphocytes from exposed individuals showed a higher percentage of residual TL and TM after the 120 min repair time point, suggesting better repair in the controls. No statistically significant differences in residual DNA damage between smokers and non-smoking individuals could be detected at any repair time point. The percentage of residual DNA damage for all comet parameters was found to be much lower in ES (Table VI) at later time points (120 min), while in controls %RDTL was higher in smokers at the same time points. In addition, cumulative dose and smoking habit were found to negatively influence %RDTL (Table VIII). These results suggest that exposed individuals may have shorter DNA strand breaks than controls. The active carcinogen(s) in cigarette smoke may alter the properties of DNA as a result of adduct formation or DNA crosslinks and therefore may hamper the migration of fragmented DNA during electrophoresis. The use of this biomarker as a predictor of repair capacity may be disrupted. Before drawing any conclusions, it will be necessary to reanalyse DNA damage and repair phenotype in a larger group of nuclear workers taking into consideration smoking habit.

Characterization of individual global repair capacity for acute exposure to γ-rays was attempted by the statistical approach proposed by Leprat *et al*. (1998). They analysed repair only at 60 min after irradiation, whereas our data present DNA repair for the three residual forms of damage (i.e. %RDTL, %RDTDNA and %RDTM) after several incubation times (5, 10, 30, 60 and 120 min). This aims at a better analysis of the differences in repair of single-strands breaks, which is assumed to be complete within 15 min, and repair of double-strand breaks, which generally takes >1 h.

Considering %RDTL as a measure of recovery after the *in vitro* challenge assay, two donors appeared to be less efficient in repair capacity at the 120 min repair time point. Interestingly, two CS individuals showed elevated DNA damage 5 min after repair incubation when considering %RDTDNA as a measure of repair capacity. These CS also showed an abnormal repair capacity at 10 min, suggesting abnormal single-strand break repair. One of the ENS individuals appeared to have a high repair capacity at all repair time intervals. %RDTDNA was shown to have a higher ICC. Therefore, %RDTDNA is more reliable for the estimation of global repair capacity. Thus, importance should be given to our observation that the level of %RDTDNA found in the exposed group was much lower than in controls (Table 4). Does this mean that people exposed to chronic low level IR have more efficient repair than the controls? If so, this suggests that these exposed individuals might not be sensitive to the challenge dose of 2 Gy γ-rays.

The global repair phenotype assay, although performed for only a relatively small number of workers, confirms the first study on IR by Leprat *et al*. (1998) and leads to the recommendation that this assay be used for the estimation of global repair phenotype with respect to IR. These conclusions are also supported by a similar work performed by Schmezer *et al*. (2001) using the Comet assay to study *in vitro* challenge with bleomycin. However, one should consider the inter-individual variation in PHA stimulation as a confounding

Table VIII. Determinants of genotoxocity residual DNA damage parameters for all workers

Genotoxicity parameters	Independent variables	Partial r^2	Slope	R^2	*p*-value
Log TM (μm)[a]	I1: Interaction	0.1495	−0.0019	0.1495	0.0563
Log MN Monos (‰)	Age	0.1102	0.0389		0.0009
	I3: Interaction	0.0946	0.0319	0.2048	0.0401
Log MNBN (‰)	Age	0.1450	0.0142		0.0044
RDTL (%)	I2: Interaction	0.2106	−55.3245	0.2106	0.0317

[a]The analysis was only done for exposed population.
The relative influence of the different independent variables (cumulative dose (mSv), exposure status (exposed vs. controls), smoking status (smokers vs. non smokers) and age), I1 (interaction between smoking status and cumulative dose) and I2 (interaction between exposure and smoking), I3 (interaction between exposure and age) on the level of log TL, log TDNA, log TM, logMNMonos, log MNBN, log NDJ, %RDTL, %RDTNA and % RDTM was tested by stepwise multivariate linear regression analysis (SAS package). The significance level for entry into the model was 0.25; all variables left in the model are significant at the 0.15 level. TL, TDNA, TM, MNMonos, MNBN, NDJ, %RDTL, %RDTDNA and %RDTM are respectively: tail length, tail DNA, tail moment, ‰ micronucleated mononucleates, ‰ micronucleated binucleates, % non-disjunction, % residual tail length, % residual tail DNA and % residual tail moment.

factor in the assay. The extent of activation is going to influence the extent of expression of DNA repair proteins. Moreover, we cannot conclude from our results whether an association exists between repair phenotype and repair genes. Moreover, smoking might have an impact on the susceptibility to radiation effects and might be responsible for differences among individuals in the risks of radiation-induced health effects. This confounder makes biomarker measurements difficult to interpret except on a population basis.

In conclusion, a positive correlation between mechanistically related biomarkers has confirmed the relevance of these biomarkers to assess genotoxic effects after exposure to IR. Neither exposure to chronic IR nor smoking induced a statistically significant increase in genotoxic effects in the control group versus the group of exposed workers; multivariate regression analysis confirmed that age positively influenced MNMonos and MNBN and indicated that %RDTL was negatively influenced by the interaction between smoking and exposure status. Use of the Comet assay for the estimation of global repair phenotype with respect to IR is recommended because it is simple, fast and differences in *in vitro* repair capacity can be detected.

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