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Evaluation of DNA damage in white blood cells of healthy human volunteers using the alkaline comet assay and the chromosome aberration test

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The present study was undertaken to contribute to the characterization of the degree of variability in baseline damage in white blood cells from control population, and to investigate how this variability is associated with external and internal factors. Altogether 170 healthy volunteers, randomly selected from the general population of the Republic of Croatia, participated in the study. Two sensitive tests: the alkaline comet assay and the chromosome aberration test were applied to study the background levels of DNA damage in their white blood cells. The results point to inter-individual differences, indicating different genome sensitivity. As revealed by both assays, the background levels of DNA damage were mostly influenced by smoking habit as well as medical exposure (especially to diagnostic X-rays). Sex and age of subjects did not significantly influence the values of DNA damage recorded in the white blood cells. Although higher levels of DNA damage were recorded in blood samples collected during winter and autumn, they were mostly influenced by medicinal exposure and smoking habit. Statistical evaluation of the data confirmed that a positive correlation exists between DNA migration and the number of long-tailed nuclei found with the comet assay and the total number of chromosome aberrations. The data obtained can serve as control values in forthcoming biomonitoring studies.

Keywords: white blood cells, peripheral blood, DNA damage, alkaline comet assay, lymphocytes, chromosome aberration test

The actual values in genotoxicological monitoring of a human population can partly be influenced by endogenous factors including those of biological origins such as gender, age and white blood cell count. A variety of external factors such as chemicals, physical agents, viruses, life-style (smoking and drinking habits, nutrition), residential and/or working areas and seasonal changes can also influence the levels of genetic damage in white blood cells of individuals (Major *et al.*, 1998).

Knowledge of the normal levels of genetic abnormalities in the general population is essential before information on the levels of similar genetic abnormalities in subjects exposed to potentially genotoxic agents in the work environment can be properly interpreted (Anderson *et al.*, 1988).

Human biomonitoring can be performed using different genetic markers. Chromosomal aberrations (CA) in peripheral blood lymphocytes (PBL) as well as other cytogenetic biomarkers such as sister chromatid exchange (SCE) and micronuclei (MN), have been used for a relatively long time in surveillance of work environments with low-dose exposures to mutagens or carcinogens. Peripheral blood lymphocytes, because of their easy availability, have been traditionally used to monitor the effects of exposure to known or suspected mutagens. This methodology is very well established, and criteria to be used in such studies are well documented (Natarajan *et al.*, 1996; Albertini *et al.*, 2000). It has often been postulated that cytogenetic tests may also serve as biomarkers of an early mutagen effect indicating increased cancer risk (Hagmar *et al.*, 1998).

Abbreviations: CA, chromosome aberrations; DSB, double strand break; LMP, low melting point; LTN, long tailed nuclei; MN, micronuclei; MRI, magnetic resonance imaging; NMP, normal melting point; PBL, peripheral blood lymphocytes; SCE, sister chromatid exchanges; SCGE, single-cell gel electrophoresis.

During the last decade, the single cell gel electrophoresis (SCGE) or comet assay was introduced as a rapid, sensitive and inexpensive technique for qualitative and quantitative assessment of DNA damage in single cells (Rojas *et al.*, 1999; Møller *et al.*, 2000; Singh, 2000; Tice, 2000; Collins, 2004). While biomonitoring studies employing cytogenetic techniques are limited to circulating lymphocytes and involve proliferating cell populations, the comet assay can be applied to proliferating and non-proliferating cells (Kassie *et al.*, 2000). This method, within a short time, has found wide usage in epidemiological and biomonitoring studies in humans, to determine DNA damage, as a result of endogenous factors and lifestyle (Betti *et al.*, 1994; Hellman *et al.*, 1997; Frenzilli *et al.*, 1997; Bajpayee *et al.*, 2002; Morillas *et al.*, 2002; Møller *et al.*, 2002; Speit *et al.*, 2003; Mastaloudis *et al.*, 2004; Hininger *et al.*, 2004; Hoffman & Speit, 2005) as well as due to occupational exposure (Awara *et al.*, 1998; Şardaş *et al.*, 1998; Wojewódzka *et al.*, 1998; Andreoli *et al.*, 1999; Palus *et al.*, 1999; Pitarque *et al.*, 1999; Somorovská *et al.*, 1999; Zhu *et al.*, 1999, 2001; Moretti *et al.*, 2000; Kopjar & Garaj-Vrhovac, 2001; Maluf *et al.*, 2001; Želježić & Garaj-Vrhovac, 2001; Garaj-Vrhovac & Kopjar, 2003) as well as environmental exposures (Šrám *et al.*, 1998; Hellman *et al.*, 1999; Valverde *et al.*, 1999).

The accuracy of any risk assessment, especially in the case of low doses, depends upon both the resolution of the methods being used, and the baseline data obtained in well-selected controls. The use of several, independent end-points improves the resolution of an investigation and provides a more accurate risk assessment (Major *et al.*, 1998).

Here we report data obtained by a two-biomarker approach on peripheral blood samples collected from healthy subjects randomly selected from the general population of the Republic of Croatia. The present study was undertaken to contribute to the characterisation of the degree of variability in baseline damage in white blood cells, to investigate how this variability is associated with external and internal factors, and to help to establish background data which may be of use as control values in future biomonitoring studies.

MATERIALS AND METHODS

Population characteristics. The population under study consisted of 170 blood donors (76 female and 94 male; average age 35 ± 10, age range: 20 to 64 years). They were of mixed social class and occupation (students, teachers, housewives, administrative employees, blue collar workers and unemployed). Most of them donated their blood samples during pre-employment medical check-ups, while others were freely engaged in the study.

The investigation was performed in accordance with high standards of ethics. Informed consent was obtained from all participating subjects prior to the start of the study. Blood donors were also informed about the aim and the experimental details of the study. All of them were healthy at the moment of blood sampling and interviews. Each participant completed a standardised questionnaire, designed to obtain relevant details of current health status, health history and lifestyle. Data on exposure to possible confounding factors: smoking habits, alcohol consumption, medicinal usage, contraception, severe infections or viral diseases during the last six months, recent vaccinations, presence of known inherited genetic disorders and chronic diseases, family history of cancer, exposure to indoor/outdoor pollutants, sunlight exposure, exposure to diagnostic X-rays, plus previous radio- or chemotherapy were collected. Donors who reported alcohol consumption, history of cancer and any cancer therapy, known inherited genetic disorders, treated acute infections, and/or chronic non-infectious diseases, intake of vitamins or intensive sportive activities during the last week were excluded from this study.

Seventy-six subjects were regular smokers (29 female and 47 male subjects), and 94 were non-smokers (47 female and 47 male). One month prior to the blood sampling, 52 subjects were subjected to diagnostic chest X-rays or dental X-rays, usualy during pre-employment medical checkups; two subjects reported medical exposure to magnetic resonance imaging (MRI) a few months ago, while two reported occasional use of antirheumatic drugs through one year prior to the study. Other subjects reported no medicinal treatments. Their dietary habits were not appreciably different. None of them had history of occupational exposure to known genotoxic chemicals.

Blood sampling. Peripheral blood samples (V = 5 ml) were collected under sterile conditions by venipuncture into heparinised tubes (BD vacutainer, Becton Dickinson, NJ, USA) in the morning hours, between 9 and 10 a.m. After collection, all blood samples were randomly coded, cooled at 4°C, transported to the laboratory and processed as quickly as possible (usually within 2 h following the blood sampling).

The alkaline comet assay. The comet assay was carried out under alkaline conditions, basically as described by Singh *et al.* (1988). Two fully-frosted microscopic slides per subject were prepared. Each slide was covered with 1% normal melting point (NMP) agarose (Sigma). After solidification, the gel was scraped off from the slide. The slides were then coated with 0.6% NMP agarose. When this layer

had solidified a second layer containing a whole blood sample (4 μl) mixed with 0.5% low melting point (LMP) agarose (Sigma) was placed on the slides. After 10 min of solidification on ice, slides were covered with 0.5% LMP agarose. Afterwards the slides were immersed for at least 1 h in ice-cold freshly prepared lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris/HCl, 1% Na-sarcosinate (Sigma), pH 10, with 1% Triton X-100 (Sigma) and 10% dimethyl sulfoxide (Kemika) added fresh to lyse cells and allow DNA unfolding. The slides were then randomly placed side by side in a horizontal gel-electrophoresis tank, facing the anode. The unit was filled with freshly prepared electrophoretic buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13.0) and the slides were set in this alkaline buffer for 20 min to allow DNA unwinding and expression of alkali-labile sites. Denaturation and electrophoresis were performed at 4°C under dim light. Electrophoresis was carried out for the next 20 min at 25 V (300 mA). After electrophoresis the slides were washed gently three times at 5-min intervals with a neutralisation buffer (0.4 M Tris/HCl, pH 7.5) to remove excess alkali and detergents. Each slide was stained with ethidium bromide (20 μg/ml) and covered with a coverslip. Slides were stored at 4°C in humidified sealed containers until analysis. To prevent additional DNA damage, handling of blood samples and all steps included in the preparation of slides for the comet analysis were conducted under yellow light or in the dark. Furthermore, to avoid possible position effects during electrophoresis, each replicate was processed in a different electrophoretic run.

Slides were examined at 250× magnification with a fluorescence microscope (Zeiss, Germany), equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm. A total of 100 comets per subject were scored (50 from each of two replicate slides). Comets were randomly captured at a constant depth of the gel, avoiding the edges of the gel, occasional dead cells and superimposed comets. The microscope was connected to a black and white camera with a computer-based image analysis system (Comet Assay II, Perceptive Instruments Ltd., UK). This image analysis system acquires images, computes the integrated intensity profiles for each cell, estimates the comet cell components, and then evaluates the range of derived parameters. As a measure of DNA damage in this study tail length was used. It was calculated from the centre of the head and presented in micrometers.

Chromosome aberration (CA) test. In order to rigorously control technical conditions, all materials were purchased as single batches prior to the start of the study. The chromosome aberration test was performed in agreement with current IPCH and IAEA guidelines (Albertini *et al.*, 2000; IAEA, 2001). Blood samples were cultivated in Ham's F-10 medium (Sigma) supplemented with 20% fetal calf serum, phytohaemagglutinin, and antibiotics penicillin and streptomycin. Duplicate cultures per subject were set up and incubated at 37 ± 1°C for 48 h. To arrest dividing lymphocytes in metaphase, colchicine (0.004%) was added 3 h prior to the harvest. Cultures were centrifuged at 1000 r.p.m. for 10 min, the supernatant was carefully removed, and the cells were resuspended in a hypotonic solution (0.075 M KCl) at 37°C. After centrifugation for 10 min at 1000 r.p.m., the cells were fixed with a freshly prepared fixative of ice cold methanol/glacial acetic acid (3:1, v/v). Fixation and centrifugation were repeated several times until the supernatants were clear. Cells were pelleted and resuspended in a minimal amount of fresh fixative to obtain a homogeneous suspension. The cell suspension was dropped onto microscope slides and left to air-dry. Slides were stained with 5% Giemsa solution (Sigma). All slides were coded and scored blindly. Two hundred metaphases per subject (100 metaphases from each parallel culture) were analysed for chromosomal aberrations. Structural chromosome aberrations were classified based on the number of sister chromatids and breakage events involved. Only metaphases containing 45–47 centromeres were analysed. Total numbers and types of aberrations, as well as the percentage of aberrant cells per each subject were evaluated.

Statistical analyses. Statistical analyses were carried out using Statistica 5.0 package (StatSoft, Tulsa, USA). Each subject was characterized for the extent of DNA damage by considering the mean (± standard deviation), median, range and dispersion coefficient (H) for the comet tail lengths measured. The dispersion coefficient was calculated as the ratio of the sample variance to the sample mean (Albertini *et al.*, 2000). Moreover, cells were classified as either "undamaged" or "damaged" by considering threshold levels indicating the comets with a long-tailed nucleus (LTN), i.e. the length over the 95th percentile of the distribution of the tail lengths (Betti *et al.*, 1994; Moretti *et al.*, 2000). Multiple comparisons between groups were done by means of multifactor ANOVA with post-hoc Scheffé test as well as using the canonical analysis on the logarithmic transformed data. The level of statistical significance was set at $P < 0.05$.

RESULTS

The alkaline comet assay

Characteristics of the subjects: age, sex, smoking habits, medical exposure, time of the blood sampling, and individual DNA damage data are report-

Table 1. Demographic data of healthy human volunteers and results of alkaline comet assay and chromosome aberration (CA) analysis in their white blood cells

Donor code	Sex age	Smoking habit Medical exposure	Sample Month	DNA migration – comet tail length (μm)						Total number and distribution of CA				Cells with CA (%)
				Mean ± S.D.	Med.	H	Min.	Max.	No. of LTN	B_1	B_2	Ac	Σ	
1	M / 20	S / X	10	14.17 ± 1.03	14.26	0.10	11.67	16.86	2	0	2	0	2	1.0
2	M / 20	S	2	14.38 ± 1.49	14.42	0.15	11.54	17.95	5	0	0	0	0	0
3	M / 21	NS	1	12.65 ± 0.92	12.97	0.07	11.02	15.56	0	0	0	0	0	0
4	F / 21	NS / X	1	13.70 ± 0.94	13.61	0.07	11.67	16.86	2	0	0	0	0	0
5	F / 21	S	12	13.56 ± 1.10	13.61	0.09	11.67	16.21	0	0	0	1	1	0.5
6	M / 21	S	10	14.53 ± 0.75	14.26	0.04	12.97	16.21	0	0	0	0	0	0
7	M / 21	S / X	10	14.05 ± 1.15	14.26	0.09	11.02	15.66	0	1	0	0	1	0.5
8	M / 21	NS / X	2	14.90 ± 1.28	14.74	0.11	12.18	17.31	4	1	0	0	1	0.5
9	M / 22	NS	1	13.89 ± 1.34	14.26	0.13	9.08	16.21	0	0	0	0	0	0
10	M / 22	NS / X	2	14.23 ± 1.34	14.42	0.13	11.54	17.31	1	1	0	0	1	0.5
11	M / 22	NS	12	14.03 ± 1.65	13.46	0.19	11.54	19.87	9	0	0	0	0	0
12	M / 22	NS / X	2	14.62 ± 1.34	14.74	0.12	11.54	17.31	3	0	0	0	0	0
13	M / 23	S	2	15.55 ± 1.34	15.38	0.12	12.18	20.51	11	1	0	0	1	0.5
14	F / 23	NS	3	14.72 ± 1.24	14.91	0.11	11.02	16.86	2	2	0	0	2	1.0
15	M / 23	NS	5	13.02 ± 0.94	12.97	0.07	11.02	14.91	0	0	0	0	0	0
16	M / 23	NS / X	10	13.78 ± 0.79	13.61	0.05	12.32	14.91	0	0	1	0	1	0.5
17	M / 23	S / X	2	15.29 ± 1.75	15.38	0.20	10.90	19.23	19	0	0	0	0	0
18	M / 23	NS / X	2	13.51 ± 1.49	13.46	0.16	10.26	17.95	1	0	1	0	1	0.5
19	M / 24	NS / X	3	15.28 ± 1.64	15.38	0.18	10.90	19.23	14	1	0	0	1	0.5
20	M / 24	NS / X	3	15.38 ± 1.85	15.38	0.22	10.26	20.51	21	2	0	0	2	1.0
21	M / 24	NS	1	14.15 ± 0.92	14.26	0.06	12.32	17.50	2	0	0	0	0	0
22	M / 24	S / X	10	15.04 ± 0.94	15.56	0.06	12.32	16.21	0	0	0	1	1	0.5
23	F / 24	NS	7	13.03 ± 1.25	12.82	0.12	10.90	16.67	0	1	0	0	1	0.5
24	M / 25	S / X	3	15.38 ± 1.92	15.38	0.24	10.90	19.87	18	1	0	0	1	0.5
25	F / 25	S / X	3	15.35 ± 1.54	15.38	0.15	11.54	19.87	12	0	0	0	0	0
26	M / 25	NS	9	12.54 ± 1.13	12.32	0.10	10.37	14.26	0	0	0	1	1	0.5
27	M / 25	NS / X	10	14.48 ± 1.21	14.26	0.10	11.02	16.86	4	1	0	1	2	1.0
28	F / 25	NS	5	12.52 ± 0.84	12.64	0.06	9.72	13.61	0	1	0	0	1	0.5
29	F / 25	NS	5	12.75 ± 0.84	12.97	0.06	11.02	13.61	0	0	0	0	0	0
30	M / 25	S	10	13.98 ± 1.04	13.94	0.08	12.32	16.21	0	2	0	0	2	1.0
31	F / 25	NS	6	13.70 ± 1.43	13.46	0.15	10.26	17.31	1	0	0	0	0	0
32	F / 25	NS	7	13.98 ± 1.55	14.10	0.17	10.26	17.31	2	0	0	0	0	0
33	F / 26	S / X	3	15.91 ± 2.24	16.03	0.31	10.26	19.87	35	0	0	1	1	0.5
34	M / 26	S / X	3	15.21 ± 1.73	15.38	0.20	11.54	19.23	15	2	0	0	2	1.0
35	M / 26	S	10	13.64 ± 0.97	13.61	0.07	12.32	15.56	0	2	0	0	2	
36	M / 26	S / X	10	13.82 ± 1.47	13.61	0.16	11.02	16.86	2	1	0	0	1	0.5
37	M / 26	NS	3	14.60 ± 0.76	14.91	0.04	12.32	15.56	0	0	2	0	2	1.0
38	M / 26	NS	9	12.63 ± 1.34	12.64	0.14	10.37	14.91	0	0	0	0	0	0
39	F / 26	S	11	15.04 ± 0.98	14.91	0.06	12.97	17.50	4	0	0	0	0	0
40	M / 26	NS	5	12.45 ± 0.82	12.32	0.05	10.37	14.26	0	0	0	0	0	0
41	M / 26	S	10	15.00 ± 1.17	14.91	0.09	12.32	16.86	6	2	0	0	2	1.0
42	M / 26	NS / X	4	14.96 ± 1.49	14.74	0.15	10.90	18.59	10	1	0	0	1	0.5

43	M / 26	S / X	4	14.44 ± 1.82	14.74	0.23	10.90	18.59	7	1	0	0	1	0.5
44	M / 27	NS / X	3	14.98 ± 2.02	15.06	0.31	9.62	18.59	16	0	0	0	0	0
45	M / 27	NS	5	12.84 ± 0.75	12.97	0.04	11.02	13.61	0	1	0	0	1	0.5
46	M / 27	NS / X	3	15.07 ± 1.18	14.91	0.09	12.32	17.50	14	1	0	1	2	1.0
47	M / 27	NS	5	12.43 ± 0.76	12.32	0.05	11.02	13.61	0	0	0	0	0	0
48	M / 27	NS / X	2	14.62 ± 1.60	14.74	0.17	10.26	17.95	6	1	0	0	1	0.5
49	F / 27	S	7	13.31 ± 1.43	13.46	0.16	9.62	16.67	0	0	0	0	0	0
50	M / 28	S / X	2	15.33 ± 1.43	15.38	0.13	11.54	18.59	10	1	0	0	1	0.5
51	M / 28	S / X	10	14.20 ± 0.73	14.26	0.04	12.32	14.91	0	0	0	0	0	0
52	M / 28	S / X	10	13.81 ± 0.84	13.94	0.05	11.67	14.91	0	1	1	0	2	1.0
53	M / 28	NS / X	3	14.63 ± 1.78	14.10	0.22	11.54	19.87	11	2	0	0	2	1.0
54	F / 28	NS / X	4	15.18 ± 1.72	14.74	0.19	11.54	19.23	15	2	0	0	2	1.0
55	M / 28	S / X	4	14.79 ± 1.87	14.74	0.24	10.90	19.23	12	0	0	0	0	0
56	F / 29	S	2	15.42 ± 1.32	15.38	0.11	12.18	18.59	12	1	0	0	1	0.5
57	M / 29	S / X	3	15.18 ± 2.02	15.38	0.27	11.54	19.87	20	1	1	1	3	1.5
58	F / 29	NS	5	12.97 ± 0.66	12.97	0.03	11.02	13.61	0	1	0	0	1	0.5
59	M / 29	NS / X	2	14.23 ± 1.29	14.10	0.12	10.90	17.31	1	0	0	1	1	0.5
60	F / 29	NS	7	13.58 ± 1.54	13.46	0.18	9.62	16.67	0	2	0	0	2	1.0
61	F / 29	NS	7	13.65 ± 1.55	13.46	0.18	10.26	17.31	2	0	0	0	0	0
62	M / 29	S / X	4	14.41 ± 1.63	14.74	0.19	10.90	19.23	7	1	0	0	1	0.5
63	F / 30	S	4	14.55 ± 1.53	14.10	0.16	10.90	19.23	6	0	0	0	0	0
64	M / 30	S	4	14.26 ± 1.43	14.10	0.14	10.26	17.95	4	0	0	0	0	0
65	M / 30	NS	10	14.03 ± 0.71	14.26	0.04	12.32	14.91	0	0	0	0	0	0
66	F / 30	NS	1	14.42 ± 1.10	14.26	0.08	12.32	17.50	4	0	0	0	0	0
67	F / 30	NS	3	13.92 ± 0.89	14.26	0.06	12.32	15.56	0	1	0	0	1	0.5
68	M / 30	NS / X	2	14.00 ± 1.42	14.10	0.14	10.91	17.31	3	1	0	0	1	0.5
69	M / 31	S	3	14.89 ± 1.48	14.74	0.15	11.54	19.87	6	0	0	0	0	0
70	F / 31	S	4	13.98 ± 1.31	14.10	0.12	10.26	17.31	1	2	0	0	2	1.0
71	M / 31	S	2	14.29 ± 1.56	14.10	0.17	11.54	17.95	4	0	0	1	1	0.5
72	F / 31	S	7	13.51 ± 1.47	13.46	0.16	10.26	16.67	0	1	0	0	1	0.5
73	F / 32	S / X	3	15.22 ± 1.80	15.38	0.21	10.90	19.23	20	0	0	0	0	0
74	F / 32	NS	5	12.62 ± 0.98	12.97	0.08	9.08	14.26	0	0	0	0	0	0
75	M / 32	NS	3	13.83 ± 1.35	13.46	0.13	10.90	17.31	1	0	0	1	1	0.5
76	M / 32	NS	3	13.08 ± 1.48	12.82	0.17	9.62	17.31	2	0	0	0	0	0
77	M / 32	S / X	2	15.15 ± 1.26	15.06	0.11	12.18	17.31	6	2	0	0	2	1.0
78	F / 32	S	7	14.30 ± 1.30	14.10	0.11	10.90	17.31	1	1	0	1	2	1.0
79	M / 32	S	10	14.05 ± 1.35	14.26	0.13	11.02	16.86	2	2	0	0	2	1.0
80	M / 32	S / X	4	14.97 ± 1.80	14.74	0.22	11.54	18.59	18	0	1	0	1	0.5
81	M / 33	NS / X	2	14.94 ± 1.52	14.74	0.15	10.90	18.59	9	2	0	0	2	1.0
82	M / 33	NS	5	12.76 ± 0.86	12.97	0.06	9.72	13.61	0	1	0	0	1	0.5
83	F / 33	NS	1	13.95 ± 1.03	13.94	0.08	11.67	16.86	2	0	0	0	0	0
84	M / 33	S / X	3	13.17 ± 1.60	12.82	0.19	9.62	16.67	0	1	0	0	1	0.5
85	M / 33	S / X	2	14.60 ± 1.62	14.74	0.18	10.26	17.95	10	1	1	0	2	1.0
86	F / 33	S	6	14.72 ± 1.22	14.74	0.10	11.54	16.67	0	1	0	0	1	0.5
87	M / 34	S / X	2	14.80 ± 1.54	14.74	0.16	11.54	18.59	9	0	1	0	1	0.5
88	M / 35	NS / X	2	14.41 ± 1.59	14.42	0.18	10.90	17.31	3	0	0	1	1	0.5
89	F / 35	NS	3	13.99 ± 1.51	14.10	0.16	10.26	16.67	0	1	0	0	1	0.5
90	F / 35	S	4	14.60 ± 1.35	14.74	0.13	11.54	17.31	3	0	0	1	1	0.5

91	M / 35	NS	3	14.93 ± 1.35	14.74	0.12	12.18	18.59	7	0	0	0	0	0
92	M / 35	NS	3	13.13 ± 1.34	12.82	0.14	9.62	16.67	0	0	0	0	0	0
93	F / 35	NS	7	14.04 ± 1.32	14.10	0.12	10.26	17.31	1	1	0	0	1	0.5
94	F / 35	NS	12	13.95 ± 1.63	14.10	0.19	10.26	18.59	4	1	0	1	2	1.0
95	F / 35	NS	7	14.44 ± 1.24	14.74	0.11	10.90	17.31	1	1	0	0	1	0.5
96	M / 35	NS / X	4	14.89 ± 1.62	14.74	0.18	11.54	18.59	11	1	0	0	1	0.5
97	M / 36	S / X	4	15.71 ± 1.92	16.03	0.23	10.90	19.23	26	0	0	0	0	0
98	F / 36	S	12	14.60 ± 1.42	14.42	0.14	10.90	17.31	4	1	0	0	1	0.5
99	M / 36	NS / X	2	14.63 ± 1.73	14.74	0.20	10.26	19.23	10	0	0	1	1	0.5
100	M / 36	S	4	14.72 ± 1.54	14.74	0.16	11.54	17.95	8	0	0	0	0	0
101	M / 36	S	3	15.27 ± 1.41	15.06	0.13	12.18	19.23	10	1	0	0	1	0.5
102	F / 36	NS	7	12.68 ± 1.55	12.82	0.19	9.62	16.67	0	0	0	1	1	0.5
103	M / 36	NS / X	9	15.47 ± 1.44	15.38	0.13	12.82	17.95	18	0	0	0	0	0
104	M / 37	S / X	2	14.94 ± 1.53	14.74	0.16	11.54	18.59	12	2	0	0	2	1.0
105	M / 37	S	3	14.70 ± 1.45	14.74	0.14	10.90	17.95	7	1	0	0	1	0.5
106	F / 37	NS	6	14.82 ± 1.38	14.74	0.13	12.18	17.95	3	0	1	0	1	0.5
107	M / 37	S / X	4	14.54 ± 1.41	14.74	0.14	10.26	17.95	5	1	0	1	2	1.0
108	F / 38	NS	2	12.10 ± 1.49	12.32	0.18	9.72	17.50	2	0	0	0	0	0
109	M / 38	S / X	10	14.28 ± 0.67	14.26	0.03	11.67	14.91	0	2	1	0	3	1.5
110	M / 38	S	3	14.69 ± 1.54	14.74	0.16	10.90	17.95	9	1	0	0	1	0.5
111	F / 38	NS	4	14.88 ± 1.39	14.74	0.13	12.18	17.95	6	0	0	1	1	0.5
112	M / 38	S	4	13.99 ± 1.36	14.10	0.13	10.90	17.95	1	0	0	1	1	0.5
113	F / 38	NS / X	4	14.20 ± 1.77	14.74	0.22	10.26	17.95	5	1	1	0	2	1.0
114	M / 39	NS	3	15.26 ± 1.24	15.38	0.10	12.18	17.31	6	1	0	0	1	0.5
115	M / 39	NS	3	12.73 ± 1.58	12.82	0.20	9.62	16.03	0	2	0	0	2	1.0
116	M / 39	NS / AD	3	15.39 ± 1.41	15.38	0.13	12.18	17.95	16	1	1	0	2	1.0
117	F / 39	NS	6	14.81 ± 1.22	14.74	0.10	12.18	17.95	1	0	0	0	0	0
118	M / 40	NS	3	14.74 ± 1.25	14.74	0.11	12.18	17.31	2	1	0	1	2	1.0
119	F / 41	NS	4	14.72 ± 1.41	14.74	0.13	11.54	17.95	5	0	0	1	1	0.5
120	M / 41	S	3	13.28 ± 1.46	13.46	0.16	10.26	17.31	1	0	0	0	0	0
121	F / 41	NS	4	15.04 ± 1.45	15.38	0.14	9.62	17.95	7	1	0	0	1	0.5
122	M / 41	S / MRI	3	15.17 ± 1.66	15.38	0.18	11.54	19.23	18	2	0	0	2	1.0
123	F / 41	S	6	14.81 ± 1.27	14.74	0.11	10.90	17.95	2	0	0	0	0	0
124	F / 41	S	7	14.31 ± 1.42	14.10	0.14	10.26	17.31	1	1	0	0	1	0.5
125	M / 42	NS / X	3	15.55 ± 1.65	15.38	0.18	12.18	19.87	17	0	0	1	1	0.5
126	F / 42	S	4	14.15 ± 1.21	14.10	0.10	11.54	16.67	0	1	0	0	1	0.5
127	M / 42	NS / X	2	14.04 ± 1.42	14.10	0.14	10.26	16.67	0	0	0	0	0	0
128	M / 42	S	5	14.65 ± 1.62	14.74	0.18	10.90	17.95	1	0	0	0	0	0
129	M / 43	S	3	13.09 ± 1.39	12.82	0.15	10.26	17.31	1	1	0	0	1	0.5
130	F / 43	S	6	14.67 ± 1.46	14.74	0.15	10.90	17.95	4	1	0	0	1	0.5
131	F / 44	S	4	14.72 ± 1.64	14.74	0.18	10.90	19.87	8	0	0	1	1	0.5
132	M / 44	NS	9	12.67 ± 1.25	12.64	0.12	10.37	14.91	0	1	0	0	1	0.5
133	M / 44	S	4	14.90 ± 1.72	14.74	0.20	10.26	21.15	13	0	1	1	2	1.0
134	F / 45	S	4	13.37 ± 1.19	13.46	0.11	10.26	17.31	1	1	0	0	1	0.5
135	F / 45	NS	10	14.54 ± 1.65	14.74	0.19	10.26	17.31	7	1	0	1	2	1.0
136	F / 45	NS	6	15.04 ± 1.40	15.38	0.13	11.54	17.95	6	1	0	0	1	0.5
137	F / 45	S	7	14.09 ± 1.39	14.10	0.14	10.26	17.31	1	0	0	0	0	0
138	F / 45	NS	7	14.22 ± 1.29	14.10	0.12	10.90	17.95	1	0	0	1	1	0.5

139	M / 45	S	10	14.88 ± 1.72	14.74	0.20	10.26	19.87	9	4	0	1	5	2.5
140	F / 46	S	4	14.06 ± 1.51	14.10	0.16	10.26	17.31	1	1	1	0	2	1.0
141	M / 46	S	3	14.24 ± 1.36	14.10	0.13	11.54	17.95	5	2	0	0	2	1.0
142	M / 46	S / X	10	14.57 ± 0.74	14.91	0.04	11.67	15.56	0	2	0	1	3	1.5
143	F / 47	S	4	13.94 ± 1.38	14.10	0.14	10.90	16.67	0	2	0	0	2	1.0
144	M / 47	S / MRI	4	15.08 ± 1.51	14.74	0.15	11.54	18.59	11	1	0	0	1	0.5
145	F / 47	NS	7	12.83 ± 1.32	12.82	0.14	9.62	16.03	0	1	0	0	1	0.5
146	F / 47	S	6	14.73 ± 1.39	14.74	0.13	10.90	17.31	2	0	0	1	1	0.5
147	F / 48	S	4	13.81 ± 1.41	13.78	0.14	10.90	16.67	0	1	0	1	2	1.0
148	F / 49	NS	5	14.07 ± 1.54	14.10	0.17	10.90	16.67	0	0	0	0	0	0
149	F / 49	NS	3	13.94 ± 1.33	14.74	0.13	10.90	17.31	2	0	0	0	0	0
150	F / 49	NS	4	14.41 ± 1.36	14.74	0.13	11.54	17.95	4	1	0	0	1	0.5
151	F / 49	S	7	13.81 ± 1.41	13.78	0.14	10.90	16.67	0	0	0	0	0	0
152	M / 50	NS	5	12.52 ± 0.91	12.32	0.07	9.72	13.61	0	1	0	0	1	0.5
153	F / 50	NS	10	14.86 ± 1.52	14.74	0.15	10.26	17.95	9	2	0	0	2	1.0
154	F / 50	NS	6	14.76 ± 1.37	14.74	0.13	10.90	17.95	5	1	0	0	1	0.5
155	F / 51	NS	4	14.62 ± 1.50	14.74	0.15	11.54	18.59	8	1	0	0	1	0.5
156	F / 51	S	4	14.04 ± 1.39	14.10	0.14	10.90	17.95	2	1	0	1	2	1.0
157	M / 51	NS	5	14.00 ± 1.47	14.10	0.15	10.90	16.67	0	0	0	0	0	0
158	F / 51	NS	6	14.15 ± 1.44	14.10	0.15	10.90	17.31	1	1	1	0	2	1.0
159	F / 51	NS	7	14.02 ± 1.57	14.10	0.18	10.26	17.95	1	1	0	0	1	0.5
160	F / 52	NS	6	13.22 ± 1.40	13.14	0.15	10.26	16.67	0	0	0	0	0	0
161	F / 52	NS / AD	12	15.06 ± 1.42	15.38	0.13	12.18	17.95	9	0	0	0	0	0
162	F / 53	NS	6	13.93 ± 1.59	14.10	0.18	9.62	16.67	0	1	0	0	1	0.5
163	F / 53	NS	6	13.86 ± 1.43	13.78	0.15	10.90	17.31	2	0	0	0	0	0
164	F / 53	NS	6	15.10 ± 1.43	15.38	0.14	11.54	17.95	8	1	0	0	1	0.5
165	F / 54	S	4	14.17 ± 1.31	14.10	0.12	11.54	17.31	1	1	0	0	1	0.5
166	F / 56	S	6	13.61 ± 1.54	13.46	0.17	10.26	17.31	1	1	0	0	1	0.5
167	M / 58	NS	5	13.81 ± 1.51	14.10	0.17	10.90	17.31	2	1	0	0	1	0.5
168	F / 59	NS	4	13.76 ± 1.16	14.10	0.10	10.90	17.31	1	0	0	0	0	0
169	F / 61	NS	6	15.03 ± 1.28	14.74	0.11	12.18	17.31	1	1	0	0	1	0.5
170	F / 64	NS	6	14.35 ± 1.39	14.74	0.13	10.90	17.31	1	0	0	0	0	0

F, female subject; **M,** male subject; **NS,** non-smoker; **S**-smoker; **X,** diagnostic exposure to X-rays; **MRI,** diagnostic exposure to magnetic resonance imaging; **AD,** antirheumatic drugs; **Med.,** median; **H,** dispersion coefficient; **LTN,** long tailed nuclei; **CA,** chromosome aberrations $\mathbf{B_1}$, chromatid break; $\mathbf{B_2}$, chromosome break; **Ac,** acentric fragment.

ed in Table 1. Comparisons of group mean values are reported in Tables 2 and 3.

The results reported in Table 1 point to an inter-individual diversity among the subjects studied. As shown in Table 1, individual values of DNA migration in white blood cells were in the range 12.10 ± 1.49 to 15.91 ± 2.24. Average value of DNA migration in the whole group studied was 14.25 ± 0.80 μm and the median was 14.31 μm (Table 1). The number of long-tailed nuclei (LTN), e.g. comets with tail length exceeding the 95th percentile for the considered parameter, is also reported. Cells with tail length values below the cut-off (16.67 μm) were classified as "undamaged", and those with higher values as "damaged". Scoring 100 comets per subject, 5 LTN are expected on average, and 8 LTN per subject with a probability of 5%. As reported in Table 1, the distribution of "damaged" cells was clearly wider in subjects exposed to diagnostic X-rays and smokers compared to other subjects. The average number of LTN in the whole group was 4.75 (range: 0–35). Altogether 128 subjects (75%) had no or less than 8 LTN per 100 comets scored, while other 42 subjects (25%) having 8 or more LTN in 100 comets were considered as "outliers". The distribution of the "outliers" with respect to their smoking status or medicinal exposure is shown on Fig. 1. It has to be pointed out that among non-smokers without any medical exposure only four subjects were "outliers" and they usually had 8 or 9 LTN per 100 comets (Table 1).

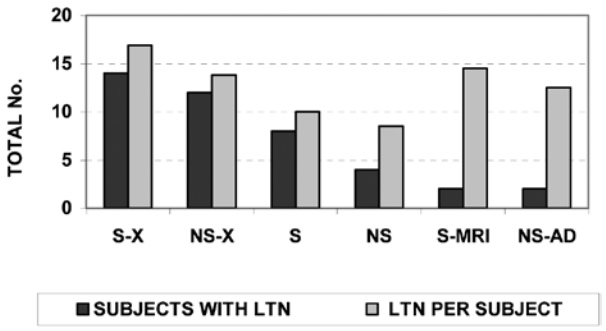

Figure 1. The distribution of "outliers" (i.e. subjects with 8 and more long tailed nuclei — LTN per 100 comets) with respect to their smoking status or medical exposure.
NS, non-smoker; S, smoker; X, diagnostic exposure to X-rays; MRI, diagnostic exposure to magnetic resonance imaging; AD, antirheumatic drugs.

Individual differences in the level of primary DNA damage could be also seen when analyzing the values of dispersion coefficient (H). High value of H indicates an increase in the proportion of cells with a high extent of damage (Table 1).

When multifactor ANOVA on logarithmically transformed data was applied for tail lengths, no statistically significant differences between individuals as related to their age were found out. Male subjects had a little higher, but not statistically significant, mean value of comet tail length compared to female subjects. However, their average number of LTN (5.89 LTN per 100 comets) was significantly increased compared to female subjects (3.33 LTN per 100 comets) (Table 2).

Smoking habit significantly influenced the levels of primary DNA damage. Smokers had an average tail length of 14.49 ± 0.63 μm and 5.95 LTN per 100 comets. Both parameters were significantly increased with respect to non-smokers (average tail length of 14.05 ± 0.88 μm and 3.78 LTN per 100 comets) (Table 2).

Although only two subjects reported diagnostic exposure to MRI, or occasional intake of antirheumatic drugs, the levels of primary DNA

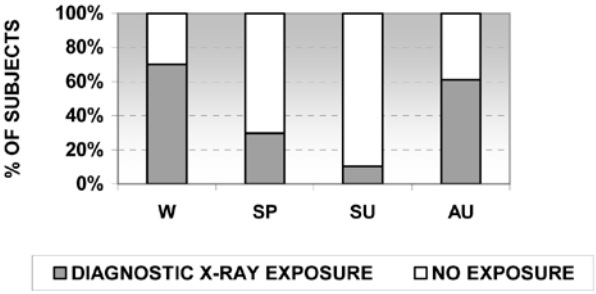

Figure 2. The distribution of subjects with diagnostic X-ray exposure dependent on the season of blood sampling.
W, winter; SP, spring; SU, summer; AU, autumn.

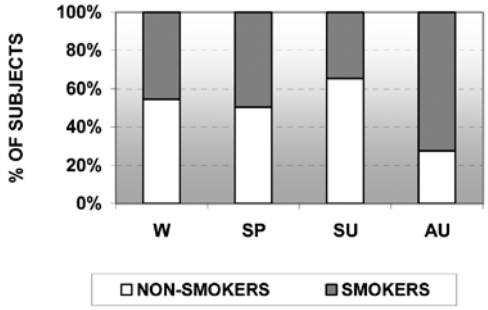

Figure 3. The distribution of smoking and non-smoking subjects with respect to the season of blood sampling.
W, winter; SP, spring; SU, summer; AU, autumn.

damage in their white blood cells were also significantly increased (Table 2). Diagnostic X-ray exposure most efficiently enhanced the levels of primary DNA damage: the mean tail length in 52 subjects with medical exposure was 14.69 ± 0.60 μm, while 8.92 LTN per 100 comets were recorded (Table 2).

Although significant differences between mean tail lengths and the number of LTN recorded in blood samples collected in winter and summer were observed, statistical analysis confirmed that they were mostly influenced by diagnostic X-ray exposure and smoking habits. As shown on Fig. 2, the most of blood samples of subjects with diagnostic X-ray exposure were collected in winter, while the most of blood samples collected in summer were donated by non-smoking subjects (Fig. 3).

These observations prompted us to separately analyse the subpopulation without any diagnostic exposure (n=114) with idea to evaluate the effects of gender, age, smoking, and season of blood sampling on the level of primary DNA damage in their white blood cells. The results of this evaluation are reported in Table 3. They confirmed that smoking significantly influenced the levels of primary DNA

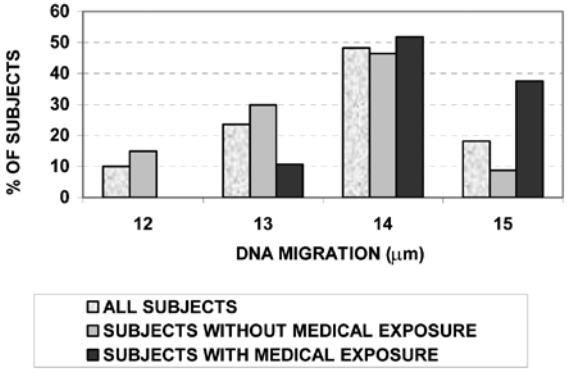

Figure 4. The distribution of the mean values of DNA migration recorded in white blood cells of 170 healthy blood donors.
The values obtained for the whole study group *vs.* subgroups with or without medical exposure are compared.

Table 2. Results of alkaline comet assay and analysis of structural chromosome aberrations (CA) in white blood cells of 170 healthy blood donors, expressed as group mean values.

DNA migration was evaluated by measuring 100 comets per subject, while incidence of CA was evaluated by analysing 200 metaphases per subject.

Subgroups	No. of subjects	DNA migration (μm) Mean ± S.D. (median) Range	No. of LTN Range	Structural CA (Mean/Range) B_1	B_2	Ac	Σ	% of cells with CA
SEX								
Female	76	14.16 ± 0.75 (14.15) 12.10–15.91	3.33 0–35	0.62 0–2	0.05 0–1	0.18 0–1	0.86 0–2	0.43 0–1
Male	94	14.32 ± 0.85 (14.51) 12.43–15.71	5.89[a] 0–26	0.72 0–4	0.15 0–2	0.18 0–5	1.05 0–3	0.53 0–2.5
SMOKING HABITS								
Non–smokers	94	14.05 ± 0.88 (14.06) 12.10–15.55	3.78 0–21	0.57 0–2	0.09 0–2	0.16 0–1	0.82 0–2	0.41 0–1
NS–F	47	14.05 ± 0.78 (14.04) 12.10–15.18	2.79 0–15	0.62 0–2	0.06 0–1	0.13 0–1	0.81 0–2	0.40 0–1
NS–M	47	14.06 ± 0.97 (14.15) 12.43–15.55	4.77 0–21	0.53 0–2	0.11 0–2	0.19 0–1	0.83 0–2	0.41 0–1
Smokers	76	14.49 ± 0.63[b] (14.56) 13.09–15.91	5.95[b] 0–35	0.80 0–4	0.13 0–2	0.21 0–1	1.14[b] 0.5	0.57[b] 0–2.5
S–F	29	14.36 ± 0.65 (14.30) 13.31–15.91	4.21 0–35	0.62 0–2	0.03 0–1	0.28 0–1	0.93 0–2	0.47 0–1
S–M	47	14.58 ± 0.61 (14.65) 13.09–15.71	7.02 0–26	0.91 0–4	0.19 0–2	0.17 0–1	1.28 0–5	0.64 0–2.5
AGE (years)								
20–29	62	14.20 ± 0.92 (14.23) 12.43–15.91	5.45 0–35	0.63 0–2	0.13 0–2	0.13 0–1	0.89 0–3	0.44 0–1.5
30–39	55	14.31 ± 0.79 (14.44) 12.10–15.71	5.22 0–26	0.64 0–2	0.13 0–1	0.20 0–1	0.96 0–3	0.48 0–1.5
40–49	34	14.30 ± 0.68 (14.36) 12.67–15.55	3.82 0–18	0.79 0–4	0.06 0–1	0.32 0–1	1.18 0–5	0.59 0–2.5
50–64	19	14.15 ± 0.66 (14.04) 12.52–15.10	2.74 0–9	0.74 0–2	0.05 0–1	0.05 0–1	0.84 0–2	0.42 0–1
TIME OF BLOOD SAMPLING								
Winter (I–III)	62	14.49 ± 0.82[c] (14.63) 12.10–15.91	7.40[d] 0–35	0.68 0–2	0.11 0–2	0.16 0–1	0.95[c] 0–3	0.48[c] 0–1.5
Spring (IV–VI)	63	14.17 ± 0.81 (14.35) 12.43–15.71	3.94 0–26	0.59 0–2	0.10 0–1	0.16 0–1	0.84 0–2	0.42 0–1
Summer (VII–IX)	20	13.66 ± 0.76 (13.73) 12.54–15.47	1.45 0–18	0.55 0–2	–	0.20 0–1	0.75 0–2	0.38 0–1
Autumn (X–XII)	25	14.32 ± 0.47 (14.20) 13.56–15.06	2.84 0–9	1.00 0–4	0.20 0–2	0.28 0–1	1.48[d] 0–5	0.74[d] 0–2.5
MEDICINAL EXPOSURE								
None	114	14.01 ± 0.79 (14.05) 12.10–15.55	2.54 0–13	0.63 0–4	0.05 0–2	0.18 0–1	0.86 0–5	0.43 0–2.5
X–Rays	52	14.69 ± 0.60[e] (15.20) 13.17–15.91	8.92[e] 0–35	0.75 0–2	0.21[e] 0–2	0.21 0–1	1.17 0–3	0.59 0–1.5
MRI	2	15.23 ± 0.23[e] (15.23) 15.06–15.23	12.50[e] 9–16	0.5 0–1	0.5[e] 0–1	–	1.00 0–2	0.50 0–1
AD	2	15.13 ± 0.06[e] (15.13) 15.07–15.17	14.50[e] 11–18	1.5 1–2	–	–	1.50[e] 1–2	0.75[e] 0.5–1
All subjects	**170**	**14.25 ± 0.80 (14.31)** **12.10 – 15.91**	**4.75** **0–35**	**0.68** **0–4**	**0.11** **0–2**	**0.18** **0–1**	**0.96** **0–5**	**0.48** **0–2.5**

F, female subject; M, male subject; NS, non–smoker; S, smoker; X, diagnostic exposure to X–rays; MRI, diagnostic exposure to magnetic resonance imaging; AD, antirheumatic drugs; LTN, long tailed nuclei; CA, chromosome aberrations; B_1, chromatid break; B_2, chromosome break; Ac, acentric fragment. Multiple comparisons were made using multifactor ANOVA with post–hoc Scheffé test; significantly increased values ($P<0.05$) were: [a]with regard to female subjects; [b]with regard to non–smokers; [c]with regard to samples collected in summer; [d]with regard to samples collected in all other seasons; [e]with regard to subjects without any medical exposure.

damage, while gender and age did not significantly contribute to the pattern of DNA migration in white blood cells. Although the highest levels of primary DNA damage were recorded in blood samples collected in autumn, they were mostly influenced by smoking status.

Table 3. Results of alkaline comet assay and analysis of structural chromosome aberrations in white blood cells of 114 healthy blood donors, without any medicinal exposure, expressed as group mean values.

DNA migration was evaluated by measuring 100 comets per subject, while incidence of CA was evaluated by analysing 200 metaphases per subject.

Subgroups	No. of subjects	DNA migration (μm) Mean ± S.D. (median) Range	No. of LTN Range	Structural CA (Mean/Range) B_1	B_2	Ac	Σ	Structural CA (Mean / Range)
SEX								
Female	69	14.09 ± 0.71 (14.07) 12.10–15.42	2.25 0–12	0.64 0–2	0.04 0–1	0.19 0–1	0.87 0–2	0.43 0–1
Male	45	13.90 ± 0.91 (14.03) 12.43–15.55	2.98 0–13	0.62 0–4	0.07 0–2	0.16 0–1	0.84 0–5	0.42 0–2.5
SMOKING HABITS								
Non-smokers	67	13.80 ± 0.86 (13.95) 12.10–15.26	1.96 0–9	0.52 0–2	0.06 0–2	0.13 0–1	0.72 0–2	0.36 0–1
NS–F	47	14.00 ± 0.78 (14.02) 12.10–15.10	2.33 0–9	0.60 0–2	0.05 0–1	0.14 0–1	0.79 0–2	0.40 0–1
NS–M	47	13.45 ± 0.88 (13.11) 12.43–15.26	1.29 0–9	0.38 0–2	0.08 0–2	0.13 0–1	0.58 0–2	0.29 0–1
Smokers	47	14.32 ± 0.58[a] (14.30) 13.09–15.55	3.36[a] 0–13	0.79 0–4	0.04 0–1	0.23 0–1	1.06[a] 0.5	0.53[a] 0–2.5
S–F	29	14.23 ± 0.54 (14.16) 13.31–15.42	2.12 0–12	0.69 0–2	0.04 0–1	0.27 0–1	1.00 0–2	0.50 0–1
S–M	47	14.43 ± 0.62 (14.53) 13.09–15.55	4.90 0–13	0.90 0–4	0.05 0–1	0.19 0–1	1.14 0–5	0.57 0–2.5
AGE (years)								
20–29	30	13.68 ± 0.92 (13.65) 12.43–15.55	1.87 0–12	0.53 0–2	0.07 0–2	0.07 0–1	0.67 0–2	0.33 0–1
30–39	37	14.09 ± 0.79 (14.26) 12.10–15.27	2.86 0–10	0.54 0–2	0.03 0–1	0.22 0–1	0.78 0–2	0.39 0–1
40–49	29	14.20 ± 0.65 (14.24) 12.67–15.04	2.90 0–13	0.76 0–4	0.07 0–1	0.31[b] 0–1	1.14 0–5	0.57 0–2.5
50–64	18	14.10 ± 0.64 (14.03) 12.52–15.10	2.39 0–9	0.78 0–2	0.06 0–1	0.06 0–1	0.89 0–2	0.44 0–1
TIME OF BLOOD SAMPLING								
Winter (I–III)	29	14.13 ± 0.88 (14.24) 12.10–15.55	3.55[c] 0–12	0.55 0–2	0.07 0–2	0.10 0–1	0.72 0–2	0.36 0–1
Spring (IV–VI)	52	14.03 ± 0.80 (14.11) 12.43–15.10	2.33 0–13	0.54 0–2	0.08 0–1	0.17 0–1	0.79 0–2	0.39 0–1
Summer (VII–IX)	19	13.56 ± 0.65 (13.65) 12.54–14.44	0.58 0–2	0.58 0–2	–	0.21 0–1	0.79 0–2	0.39 0–1
Autumn (X–XII)	14	14.34 ± 0.50[c] (14.29) 13.56–15.04	3.86[c] 0–9	1.21[d] 0–4	–	0.29 0–1	1.50[e] 0–5	0.75[e] 0–2.5
All subjects	**114**	**14.01 ± 0.79 (14.05)** **12.10 – 15.55**	**2.54** **0–13**	**0.63** **0–4**	**0.05** **0–2**	**0.18** **0–1**	**0.86** **0–5**	**0.43** **0–2.5**

F, female subject; M, male subject; NS, non-smoker; S, smoker; X, diagnostic exposure to X–rays; MRI, diagnostic exposure to magnetic resonance imaging; AD, antirheumatic drugs; LTN, long tailed nuclei; CA, chromosome aberrations; B_1, chromatid break B_2, chromosome break; Ac, acentric fragment. Multiple comparisons were made using multifactor ANOVA with post–hoc Scheffé test; significantly increased values ($P<0.05$) were: [a]to non–smokers; [b]with regard to other subgroups based on the age; [c]with regard to samples collected in spring and summer; [d]with regard samples collected in spring and winter; [e]with regard samples collected in winter.

Distribution of subjects with respect to the extent of DNA migration in their white blood cells is displayed on Fig. 4, where differences between whole group *vs*. subgroups with or without medicinal exposure are shown.

Chromosome aberration test

Individual results on the frequencies of chromosome aberrations (CA) recorded in peripheral blood lymphocytes (PBL) are summarized in Table 1, while comparisons of group mean values are reported in Tables 2 and 3.

As reported in Table 1, an inter-individual diversity among the subjects studied was observed. Individual values for the total number of CA in PBL were in the range of 0–5 CA per 200 cells, with an average of 0.96 CA per 200 cells. Total percentage of aberrant cells was in the range of 0–2.5%, with an average of 0.48%. Only three types of structural

chromosome aberrations were observed. The most frequent aberration type were chromatid breaks. Their incidence was determined as a mean frequency of 0.68 per 200 cells. The mean yield of acentric fragments was 0.18 per 200 cells, while chromosome breaks were determined with a mean frequency of 0.11 per 200 cells (Table 2).

All comparisons between subgroups were made by multifactor ANOVA with post-hoc Scheffé test. As reported in Table 2, smoking habit significantly influenced the total number as well as the percentage of CA recorded in PBL. Although male subjects had a little higher total number of CA compared to female subjects, this difference was not statistically significant (Table 2). Age significantly influenced the total number of acentric fragments only in PBL of subjects aged between 40 and 49 years (Table 2). Diagnostic X-ray exposure significantly enhanced total number of chromosome breaks, total number of structural CA in PBL, as well as percentage of aberrant cells. The effects of other medical exposures should be carefully evaluated because in the present study only two subjects reported medicinal exposure to MRI, or occasional intake of antirheumatic drugs.

Moreover, significant variations between total number of CA and the percentage of aberrant cells recorded in blood samples collected in different seasons were observed. The highest total number of CA and the percentage of aberrant cells were recorded in blood samples taken in autumn and in winter (Table 2). However, statistical analysis confirmed that they were mostly influenced by diagnostic X-ray exposure and smoking habits.

The results of the separate study of the subpopulation without medical exposure (n=114) are reported in Table 3. They confirmed that smoking significantly influences the total number as well as the percentage of CA recorded in PBL. Age also significantly influenced the total number of acentric fragments in PBL of subjects aged between 40 and 49 years (Table 3). Significantly increased incidence of chromatid breaks, total number of CA and the percentage of aberrant cells were recorded in blood samples taken in autumn, but they were mostly influenced by smoking habit (Table 3).

The results of statistical analyses showed that the total number of chromatid breaks, chromosome breaks and acentric fragments were in a positive correlation with the total number of CA as well as with the total percentage of aberrant cells.

Moreover, they indicate that a positive correlation also exists between the increased DNA migration (expressed both as mean tail length and total number of LTN) and the total number of CA, as well as with the total percentage of aberrant cells.

DISCUSSION

Assessment of normal levels of DNA damage in the general population is essential for the proper interpretation of data obtained by monitoring of populations occupationally or accidentally exposed to known or potentially genotoxic agents. Although many biomonitoring studies indicate that the baseline genetic damage in white blood cells is affected by various endogenous and external factors, it is not clear how an individual's inborn genetic constitution may influence the yield of such damage.

Over the years our laboratory has accumulated a lot of data on cytogenetic biomarkers and alkaline comet assay in general and various exposed human populations (Kašuba *et al.*, 1995; Garaj-Vrhovac *et al.*, 1997; 1999; Garaj-Vrhovac, 1999; Rozgaj *et al.*, 1999; Garaj-Vrhovac & Kopjar, 2000; 2003; Kopjar & Garaj-Vrhovac, 2001; Rozgaj *et al.*, 2001; Želježić & Garaj-Vrhovac, 2001; 2002; Kašuba *et al.*, 2002). However, from time to time the values of these biomarkers have to be re-evaluated, especially for the general population, to establish the upper level of normal variability so that positive effect of exposure can be more readily recognized.

In the study presented here, two different biomarkers, one of exposure (the alkaline comet assay), and the other of effect (chromosome aberration test) were used to evaluate the baseline DNA damage in white blood cells of healthy blood donors randomly selected from the Croatian general population. The main aim of this study was to investigate the association between the values of the two biomarkers and several external and internal factors, as well as their mutual relationships.

Our goal was to investigate a common "healthy" population. For that reason, prior to the final selection of subjects who participated in the study, we intentionally excluded subjects with any infection, chronic disease or cancer history as well as those with known exposure to confounding factors such as high alcohol consumption, vitamin and antibiotic intake as well as subjects involved in intensive sportive activities.

In spite of the very rigorous procedures (i.e. exactly the same conditions of all steps of the procedures and a very good reproducibility of the assays employed), we observed an inter-individual variability among the subjects studied. Variability is a typical feature of biological systems, extensively reported by various authors when using the comet assay and cytogenetic endpoints (Anderson *et al.*, 1993; Betti *et al.*, 1994; Kašuba *et al.*, 1995; Hellman *et al.*, 1997; Wojewódzka *et al.*, 1998; Landi *et al.*, 1999; Stephan & Pressl, 1999; Morillas *et al.*, 2002; Garaj-Vrhovac & Kopjar, 2003).

Heterogeneity in the level of DNA damage recorded in our study could be in part attributed to individual genome sensitivity. The DNA damage detected by alkaline comet assay represents a steady state between induction of lesions and their repair. Therefore a low damage level as assessed experimentally in an individual may be the result of an actual low number of lesions or of a high efficiency of repair (Somorovská *et al*., 1999; Wojewódzka *et al*., 1999).

Although in many subjects a positive correlation between increased DNA migration and total number of structural chromosome aberrations was observed, it was difficult to explain some individual cases, where such a correlation between both biomarkers did not exist. This observation raises the general question of the relationship between the induction of DNA damage in resting lymphocytes, and its subsequent fixation in genetic alterations after stimulation. When considered together, our data suggest that DNA damage induced *in vivo* in circulating lymphocytes can be largely repaired, thus escaping fixation, as has been also reported earlier (Betti *et al*., 1994; Andreoli *et al*., 1999; Mayer *et al*., 2002). As we observed, the levels of DNA damage detected by chromosome aberration analysis in some subjects could be low, or there would be no detectable increase in cytogenetic biomarkers, even in the presence of a sizeable amount of primary DNA damage recorded *in vivo*. These observations can be easily explained if we consider that the data of the comet assay were based on responses in white blood cells, while the data gathered in the cytogenetic tests are obtained exclusively with proliferation-stimulated lymphocytes. Because white blood cells are a heterogeneous mixture of cells, as regarding their life-span and sensitivity, some differences may be due to different cell populations being compared. Despite the risk of reduced sensitivity, we prefer the use of whole blood samples for the alkaline comet assay. Many other investigators also report the use of whole leukocyte fractions or whole blood when studying induced or basal levels of DNA damage in the comet assay. The use of whole blood is easier and avoids the possibility of inducing additional (e.g. oxidative) DNA damage during the process of separating the various cell types from each other, which in many cases will be hard to control for (Hellman *et al.*, 1997; Speit *et al*., 2003).

The difference between the results in the comet assay and the cytogenetic tests is basically due to variations in the type of DNA alterations that the test system detects: the comet assay detects repairable DNA lesions or alkali-labile sites while cytogenetic tests detect fixed mutations which persist for at least one mitotic cycle (Kassie *et al*., 2000). Many of primary induced DNA lesions are successfully repaired in a few minutes (4–15 min) (Tice, 1995) to a couple of hours (2–3 h) (Singh *et al*., 1988) after infliction. However, the increased levels of primary DNA damage in some subjects could be also attributed to endogenous factors, especially intracellular oxidative stress pronounced after exposure to genotoxic agents (for example tobacco smoke or diagnostic X-rays). This could give an increased steady-state DNA damage, high enough to be detected by the sensitive comet assay. DNA modification might give rise to alkali labile sites that are converted into single strand breaks during alkaline electrophoresis. On the other hand, if base damage is located close together (< 10 bp apart) on opposite DNA strands, simultaneous excision of such modified bases can lead to the formation of DSB, the supposed initial lesion in the formation of chromosomal aberrations (CA). Incompletely repaired or unrepaired DSB are converted into chromosome and chromatid breaks (Pfeiffer *et al*., 2000) that may be visualized on metaphase preparations.

Biomonitoring studies with a combination of cytogenetic tests and comet assay are of special interest because they enable comparison of the relative sensitivity of the two test systems and may also give a clue about the fraction of DNA damage detected in the comet assay that will lead to fixed mutations (Kassie *et al*., 2000). As a biomarker, the comet assay reflects the current exposure (over the previous few weeks) and the actual levels of DNA damage present in white blood cells at the moment of blood sampling. Cytogenetic biomonitoring, on the other hand, provides additional information on the DNA damage levels, especially on past exposures. Humans are exposed to a variety of natural or synthetic genotoxic substances, able to modify the baseline levels of DNA damage. During the life-time everyone "accumulates" some level of radiation exposure, mainly due to background radiation in the environment, diagnostic exposures, or small amounts of radioisotopes ingested with food. From the studies on subjects occupationally exposed to ionizing radiation it is known that the consequence of an *in vivo* exposure to ionizing radiation also might be complex unstable chromosome aberrations, such as dicentric or ring chromosomes (Bender *et al*., 1988a; Natarajan, 1993; Hagelström *et al*., 1995). However, these aberration types were not observed in our study; on the contrary, most of chromosome aberrations detected in peripheral blood lymphocytes were of chromatid-type. Such aberration type is mostly induced by chemical mutagens and carcinogens in lymphocytes during S-phase or post-replicative stages (Bender *et al*., 1988a; Natarajan, 1993).

The results on the effects of different confounding factors obtained in the present study are in good agreement with previous observations.

Taken together, sex and age did not significantly influence the levels of primary DNA damage and incidence of structural chromosome aberration in white blood cells. Preceding biomonitoring studies with the comet assay and chromosome aberrations reported contradictory data on the effects of gender. Some authors observed differences between male and female subjects (Anderson *et al*., 1988; Betti *et al*., 1994; Wojewódzka *et al*., 1998; Mendoza-Núñez *et al*., 2001; Bajpayee *et al*., 2002), while others found similar levels of primary DNA damage in both sexes (Bender *et al*., 1988b; Anderson *et al*., 1993; Bonassi *et al*., 1995; Kašuba *et al*., 1995; Frenzilli *et al*., 1997; Major *et al*., 1998; Pitarque *et al*., 1999; Stephan & Pressl, 1999; Zhu *et al*., 1999). In our study female and male subjects had comparable levels of primary DNA damage and structural chromosome aberrations in their white blood cells. Careful evaluation of the data showed that the higher number of long tailed nuclei (LTN), as observed in male subjects, was caused mainly by smoking habit and diagnostic X-ray exposure. This observation was confirmed when subjects with any kind of medical exposure were excluded from the assessment.

Previous biomonitoring studies with the comet assay and chromosome aberrations also reported contradictory data on possible age-related increase of DNA damage in white blood cells. Some authors reported age-related increase of primary DNA damage as detected by the alkaline comet assay (Singh *et al*., 1991; Piperakis *et al*., 1998; Moretti *et al*., 2000; Maluf *et al*., 2001; Mendoza-Núñez *et al*., 2001). Based on the observations of other authors, the age of the individual appears to have no significant effect on the mean basal level of DNA damage (Betti *et al*., 1994; Frenzilli *et al*., 1997; Awara *et al*., 1998; Wojewódzka *et al*., 1998; Palus *et al*., 1999; Pitarque *et al*., 1999; Zhu *et al*., 1999; 2001). A similar situation was observed in our study. Previously reported data on the age-related incidence of chromosome aberrations are also contradictory (Bolognesi *et al*., 1997). While some authors did not find a significant increase of the frequency of chromosome aberration with age (Anderson *et al*., 1988; 1993; Kašuba *et al*., 1995), others reported significant age dependency of the number of acentric fragments in peripheral blood lymphocytes (Bender *et al*., 1988b; Stephan & Pressl, 1999). Our results are in good agreement with those observations. The restricted number of subjects older than 50 years included in our study makes it impossible to draw a firm conclusion about an association between the age and the number and incidence of distinct types of structural CA in their lymphocytes. Nevertheless, it is noteworthy that the incidence of chromatid breaks, acentric fragments and corresponding number of total CA gradually increases with age in subjects aged from 20 to 49 years, and this increase was statistically significant for acentric fragments in subgroup aged 40-49 years as compared to other subgroups analyzed.

The values of both biomarkers studied indicate that the most effective confounding factors that modulate the baseline damage in white blood cells were smoking habit and medical exposure. The data on the smoking-induced DNA damage in biomonitoring studies are still controversial. Some studies clearly confirmed the genotoxic effects of tobacco smoke, as well as its modifying effect of genome damage induced by other agents (Betti *et al*., 1994; Frenzilli *et al*., 1997; Piperakis *et al*., 1998; Palus *et al*., 1999; Stephan & Pressl, 1999; Zhu *et al*., 1999; 2001; Hininger *et al*., 2004), while others reported no significant increase of the values of the biomarkers evaluated (Anderson *et al*., 1988; 1993; Bender *et al*., 1988b; Kašuba *et al*., 1995; Šrám *et al*., 1998; Pitarque *et al*., 1999; Wojewódzka *et al*., 1999; Maluf *et al*., 2001; Speit *et al*., 2003; Hoffman & Speit, 2005). It is likely that the design of study as well as differences in other lifestyle factors contribute to the inconsistent observations. In our investigation smokers represent 45% of the population studied, and smoking was found to be a confounding factor independently of other endogenous or external factors. Evaluation of the comet assay data indicates that smokers had significantly increased levels of primary DNA damage and a higher number of long tailed nuclei as compared to non-smokers. Moreover, the observed increases were in a positive correlation with the total number of chromosome aberrations and the percentage of cells with aberrations. We also confirmed the modifying effect of smoking on the genome damage in white blood cells of subjects with diagnostic exposure to X-rays.

Diagnostic exposure to X-rays was also found to be a confounding factor that significantly enhanced the levels of DNA damage in white blood cells of subjects involved in our study. Although many investigators usually exclude subjects with diagnostic X-ray exposure from their studies, we decided to evaluate their baseline DNA damage. In this research 30% of subjects were exposed to diagnostic X-rays (chest or dental) up to one month prior to the study, mostly during the pre-employment medical check-ups. Although many authors reported that the doses of radiation used in medical diagnosis are relatively low — the dose of radiation from a chest X-ray is about 0.020–0.025 mSv, while dental X-ray exposes a subject to doses ranged about 5 μSv to 0.03 mSv (Butler *et al*., 1985; Benke, 1995; Rehani, 2000; Berrington De González & Darby, 2004) — they might increase the baseline levels of DNA damage, as detectable with the sensitive alkaline comet assay. This assumption was confirmed in our study where subjects with diagnostic X-ray exposure

had slightly increased DNA migration and a higher number of LTN as compared to subjects without any medicinal exposure. Moreover, increased levels of primary DNA damage were in a positive correlation with the total number of chromosome aberrations in peripheral blood lymphocytes as well as with the total number of cells with CA. Diagnostic X-ray exposure in healthy subjects was also in a positive correlation with the incidence of chromosome breaks in peripheral blood lymphocytes. Despite the increased DNA migration and elevated incidence of structural chromosome aberration in peripheral lymphocytes of subjects who reported medicinal exposure to MRI, or occasional intake of antirheumatic drugs, the effects of these medical exposures should be carefully evaluated because the number of subjects who reported them was too small.

Some authors reported seasonal variation in the results of the comet assay, with more damage detected in samples obtained during summer months (Betti *et al*., 1995; Frenzilli *et al*., 1997; Møller *et al*., 1998; 2000; 2002). However, we were not able to confirm those observations. When the whole group was studied, the highest primary DNA damage (expressed both as DNA migration and the number of LTN) was recorded in samples collected in winter. However, careful statistical evaluation indicated that this was mostly influenced by medicinal X-ray exposure and smoking habits. Namely, the majority of blood samples of subjects with diagnostic X-ray exposure, and a lot of samples of smokers were collected in winter. When subjects with any kind of medical exposure were excluded from the study, the highest primary DNA damage was recorded in samples collected in autumn. In these samples we found significantly increased DNA migration and the number of LTN, as well as an increased incidence of chromatid breaks, total number of CA as well as the percentage of cells with CA. However, it is difficult to say whether the DNA damaging effects could be accounted by sunlight exposure only because during the autumn the prevalence of samples donated by smokers was also observed. One of the main reasons why we did not find a significant modifying effect of sunlight exposure was the small number of blood samples analysed during the summer season, while the second was the prevalence of samples donated by non-smokers throughout this season.

Despite their limitations, the results obtained in the present study revealed a lot of background data that may be of value in future genotoxicological monitoring in the Republic of Croatia. They also confirmed that the alkaline comet assay and chromosome aberration test are sensitive biomarkers that have to be further evaluated and standardized for the assessment of DNA damage in human biomonitoring studies as well as in cases of accidental exposures.

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