Artículo 4

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Occupational Exposure to Pesticides and Cytogenetic Damage: Results of a Hungarian Population Study Using the Micronucleus Assay in Lymphocytes and Buccal Cells

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The frequency of micronuclei (MN) in peripheral blood lymphocytes and in buccal epithelial cells was used as a biomarker of genotoxic effects resulting from occupational exposure to pesticides. In addition, the cytokinesis-block proliferation index (CBPI) was calculated to detect possible variations in the proliferative kinetics of lymphocytes due to pesticide exposure. This study was performed on 84 pesticide-exposed workers and 65 unexposed controls from Hungary. The pesticide-exposed workers, classified as moderately and highly exposed, were also evaluated separately. Statistical evaluation of the cytogenetic biomarkers indicated that there were no significant differences between pesticide-exposed workers and controls, nor between moderately and highly exposed workers. Nevertheless, the statistical analysis revealed that additional factors such as age, sex, ingestion of raw vegetables, and working as a pesticide applicator affected lymphocyte MN frequency. In addition, age, sex, and smoking affected the frequency of MN in buccal cells. Results from the CBPI analysis showed that the proliferation index decreased with pesticide exposure and that this parameter was also affected by smoking and by the gender of individuals. The results of this study indicate no significant increase in MN in this group of Hungarian workers; however, the reduced CBPI in the highly exposed population suggests a possible genotoxic effect of pesticide exposure. Environ. Mol. Mutagen. 40:101–109, 2002. © 2002 Wiley-Liss, Inc.

Key words: biomonitoring; micronucleus test; human lymphocytes; buccal cells; pesticides

INTRODUCTION

Occupational exposure to pesticides has been associated with different health hazards, including a wide range of subclinical and clinical effects. Among the possible effects related to this exposure, genetic damage has important health implications for the induction of cancer, adverse reproductive outcomes, and other chronic illnesses [IARC, 1991; Arbuckle and Sever, 1998; Lander et al., 2000; Meinert et al., 2000; Jenner, 2001; Ji et al., 2001].

At present, many biomarkers are used to determine the exposure levels to genotoxic agents, as well as their related effects. Among these biomarkers the use of micronuclei (MN) by the cytokinesis-block micronucleus assay (CBMN) is increasing, due to its many advantages: reliable identification of cells that have completed only one nuclear division, sensitivity and precision, quickness and simplicity, no special training for MN scoring, the ability to screen large numbers of cells, and good reproducibility [Norppa et al., 1993; Fenech, 1997; Surrallés and Natarajan, 1997]. MN, which appear in the cytoplasm of the divided cells as small additional nuclei, result from chromosome fragments or whole chromosomes that are left behind during mitotic division. Thus, the presence of MN is an indication of exposure to clastogenic and/or aneugenic agents.

MN, both in peripheral blood lymphocytes and in epithelial cells, offer many advantages in the biomonitoring of human populations exposed to suspected genotoxins [Warner et al., 1994; Moore et al., 1996; Vaglenov et al., 1997; Burgaz et al., 1999; Karahalil et al., 1999]. In particular, MN in lymphocytes increase in agricultural workers with occupational exposure to pesticides [Bolognesi et al.,

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1993; Scarpato et al., 1996a,b; da Silva Augusto et al., 1997; Joksic et al., 1997; Calvert et al., 1998; Gómez-Arroyo et al., 2000], although negative results have also been reported in different populations with occupational pesticide exposure [Titenko-Holland et al., 1997; Davies et al., 1998; Venegas et al., 1998; Lucero et al., 2000; Pastor et al., 2001a,b]. Similarly, MN in both buccal and urothelial epithelial cells have been used in the biomonitoring of agricultural workers exposed to pesticides. Although only a few studies have been conducted, data reported using these cells also indicate both positive [Gómez-Arroyo et al., 2000] and negative results [Lucero et al., 2000; Pastor et al., 2001a,b].

The inconsistent responses among studies could reflect different exposure conditions, such as exposure magnitude, the use of protective measures, and the specific genotoxic potential of the pesticides used, which indicate that data obtained from one population cannot be directly used for risk estimations in other exposed groups. These factors make it necessary to study many well-defined populations in order to have a general view of the genetic risk associated with pesticide exposure. Taking into account both the possible genetic risk of pesticide exposure and the advantages of using MN as biomarkers, we report here the results obtained from biomonitoring a population from Hungary that is occupationally exposed to pesticides.

MATERIALS AND METHODS

Study Populations

Between February and November 1998, 155 Hungarians were surveyed by trained interviewers. After obtaining informed consent from the donors an extensive questionnaire was completed that gathered data on potential confounding factors in population monitoring (e.g., age, gender, and lifestyle factors such as smoking, diet, and medication). Interviewers completed an additional questionnaire for the exposed group, inquiring about protective measures, time of pesticide application, particular kind of work, and years of exposure.

A total of 149 people were included in the study. The exposed group consisted of 84 agricultural workers from southeastern Hungary, where many agricultural farms grow vegetables in greenhouses throughout the year. The control group consisted of 65 individuals primarily from the same area, although about one-third were from Budapest. The group of agricultural workers, 58 men and 26 women, was exposed to complex and different mixtures of pesticides. They worked in greenhouses and outdoors as sprayers, harvesters, and in related jobs. The exposed group was divided into a moderately exposed subgroup with 65 individuals (39 men and 26 women) and a highly exposed subgroup (19 men). The criteria for classifying workers as highly exposed were based on the information obtained on recent pesticide exposure comprising a series of signs and symptoms: tiredness, weakness, dizziness, nausea, vomiting, blurred vision, headache, sweating, tearing, diarrhea, tremors, hypotension, and breathing difficulty, whether treatment or hospitalization was needed or not. All highly exposed men developed four or more symptoms and some of them needed first aid or were hospitalized in recent years as a result of overexposure to pesticides. Serum cholinesterase levels, when data were available (58% in the highly exposed subgroup), were also taken into account. A 20% or greater drop in cholinesterase levels was considered indicative of poisoning. All highly exposed persons with available cholinesterase levels had a depressed level, except in one case, the exposed women who worked in greenhouses—the majority of them as harvesters.

The control group of 65 individuals (53 men and 12 women) carried out activities not related to agriculture. They worked in clerical jobs, health care, manufacturing, and in related jobs; most came from the same region where the exposed individuals worked, whereas about one-third were from Budapest.

Lymphocyte Culture, Staining, and Scoring

Blood was obtained from each subject by venipuncture using heparinized vacutainers. In the "B. Johan" laboratory, lymphocyte cultures were initiated by adding 0.5 mL of whole blood to 4.5 mL of RPMI 1640 medium supplemented with 15% heat-inactivated fetal bovine serum, 1% antibiotics (penicillin and streptomycin), and L-glutamine. Lymphocytes were stimulated by 1% of phytohemagglutinin and incubated for 72 hr at 37°C. Two cultures per subject were established. A final concentration of 6 μg/mL of cytochalasin B [Surrallés et al., 1994] was added to the cultures 44 hr later to arrest cytokinesis. At 72 hr of incubation the cultures were harvested by centrifugation at 200*g* for 8 min and treated with a hypotonic solution (2–3 min in 0.075 M KCl at 4°C). Cells then were centrifuged and a methanol-acetic acid (3:1, v/v) solution was gently added. This fixation step was repeated twice and the resulting cells were resuspended in a small volume of fixative solution and dropped onto clean slides. Finally, the slides were sent to the Universitat Autònoma de Barcelona where they were stained with 10% Giemsa in phosphate buffer (pH 6.8) for 10 min and scored.

The frequency of binucleated cells with micronuclei (BNMN) and the total number of MN in lymphocytes (MNL) were determined by blind scoring a total of 1,000 binucleated cells with well-preserved cytoplasm (500 per replicate) for each subject [Surrallés and Natarajan, 1997] on coded slides. In addition, 500 lymphocytes were scored to determine the percentage of cells with one to four nuclei and the cytokinesis-block proliferation index (CBPI) was calculated according to Surrall´es et al. [1995]. In order to minimize variability, the same expert carried out all the microscopic analyses.

Buccal Cell Collection, Staining, and Scoring

Buccal cell samples were obtained by rubbing the inside of the cheeks of study subjects with a toothbrush. The cells were collected in a conical tube containing 20 mL of buffer solution (0.1 M EDTA, 0.01 Tris-HCl, and 0.02 M NaCl, pH 7), and transported to the "B. Johan" laboratory for processing. After three washes in the buffer solution by centrifugation at 400*g* for 10 min, 50 μl of cell suspension were dropped onto preheated (55°C) slides and allowed to air-dry for 15 min on a slide-warmer. The slides were fixed in 80% cold methanol for 30 min, air-dried overnight at room temperature, and stored at −20°C until use. The slides were sent to Barcelona where they were stained with a DNA-specific dye, 4′,6-di-amidino-2-phenylindole dihydrochloride (DAPI). A total of 2,000 cells/donor were scored blind, on coded slides, by one observer using an Olympus BX50 fluorescent microscope. The criteria for MN evaluation were those proposed by Titenko-Holland et al. [1998]. The frequency of mononucleated buccal cells with micronuclei (BCMN) and the total number of micronuclei in buccal cells (MNBC) were determined for each study subject.

Statistical Analysis

Statistical computations were performed using SPSS v. 10.0 software (SPSS, Chicago, IL, USA) and SAS system for Windows, v. 8.0 (SAS Inst., Cary, NC, USA). To detect differences between groups with regard to the mean value of confounding factors (age, alcohol, smoking), the t-test was applied.

The cytogenetic variables BNMN, MNL, and CBPI were first evaluated

TABLE I. Characteristics of the Control and Pesticide-Exposed Hungarian Study Populations

	Control (n = 65)		Exposed (n = 84)	
	No.	Mean ± SE	No.	Mean ± SE
Age (years)	65	45.05 ± 0.96	84	41.98 ± 0.73
Men	53 (81.5%)	44.62 ± 1.14	58 (69%)	41.50 ± 0.95
Women	12 (18.5%)	46.92 ± 1.28	26 (31%)	43.04 ± 1.03
Years of pesticide exposure				18.75 ± 0.89
Nonsmokers	47 (72.3%)		45 (53.6%)	
Exsmokers	5 (7.7%)		9 (10.7%)	
Smokers (cig/day)	13 (20%)	14.15 ± 1.88	30 (35.7%)	18.67 ± 1.87
Ethanol (g/week)		65.58 ± 15.46		93.17 ± 16.18
Coffee (cups/day)		1.86 ± 0.18		2.55 ± 0.24
Tea (cups/day)		0.98 ± 0.16		1.17 ± 0.15
Red meat (times/week)		3.12 ± 0.27		3.82 ± 0.25
White meat (times/week)		2.40 ± 0.22		2.70 ± 0.19
Fish (times/week)		0.54 ± 0.13		0.42 ± 0.07
Raw vegetables (times/week)		5.28 ± 0.31		5.17 ± 0.28
Cooked vegetables (times/week)		4.68 ± 0.34		5.12 ± 0.26
Fruit (g/day)		247.92 ± 26.86		323.94 ± 26.94

SE: standard error.

by using a multiple linear regression analysis (including variables such as exposure, age, sex, number of cigarettes). Due to the lack of adjustment to the model requirements, MNL and BNMN were transformed to normalize distributions and homogenize variances. Thus, the square root transformation was performed to analyze MNL and BNMN data. CBPI values did not require transformation. The models for main variable adjustment took into account all the continuous and dichotomized variables, as well as the interactions between the most important variables studied.

In the multiple linear regression analysis, different methods for variable selection were used (backward and forward). After these analyses, all significant variables were subjected to a final multiple regression analysis. The adequacy of the fit for each model was verified by the analysis of residuals, tolerance limits, and homoscedasticity. This procedure was applied to compare the exposed group with the nonexposed. A general linear model was used to analyze the possible differences between the moderately exposed and the highly exposed groups. A new polychotomic variable (V: v1, moderately exposed men; v2, highly exposed men; v3, moderately exposed women) was included, as well as age, working as a pesticide applicator, and weekly alcohol consumption.

The cytological variables BCMN and MNBC, scored in buccal epithelial cells, were evaluated first by Poisson regression but, due to the high overdispersion, a binomial regression analysis was performed. A backward selection method was used.

P-values correspond to two-sided tests. An alpha error (α) of <0.05 was considered the significance level.

RESULTS

Agricultural Workers and Controls

The main characteristics of the 149 volunteers participating in the study, 84 exposed workers and 65 controls, are shown in Table I. In this table potential confounding factors are also indicated. Some of these factors were different in the exposed population and the controls. For example, the mean age was slightly higher in the controls than in the exposed group ($P = 0.011$). There were also differences in smoking habits between the two groups: the exposed group had more smokers and smoked more cigarettes per day than the nonexposed group ($P = 0.008$). For the statistical analysis, we considered exsmokers as people who had stopped smoking between 1 and 5 years before sampling. If they had never smoked or had quit smoking for more than 5 years, they were considered nonsmokers. Differences were also observed in the cups of coffee drunk per day ($P = 0.031$). The remaining variables were similar for both groups. Other data extracted from the questionnaire revealed that the majority of the population did not follow any specific kind of diet; they consumed mainly seed oil and margarine as oils. Concerning exposure to well-known mutagenic agents, almost 90%, both controls and farmers, had received diagnostic X-rays in the last 3 years, most of them because of mandatory chest X-ray screening for tuberculosis in Hungary.

Compared to controls, the exposed individuals were subjected to a variety of pesticides, including insecticides, fungicides, and herbicides applied on vegetables like tomato and green pepper in greenhouses and on crops like corn and wheat in open fields. The application of pesticides was mainly carried out below the head (43.9%), which could reduce the contact with mucous membranes (eyes, mouth, nose). This group showed a mean of 18.75 ± 0.89 (range 1–38) years of exposure to pesticides, indicating that the population had been chronically exposed. A great percentage of workers asserted the use of protective measures and almost 85% reported using agrochemical products stored in a specific safe location.

Detailed mean values of cytogenetic damage and the CBPI, by groups and sex, are shown in Table II. Table III shows a summary of the results obtained in the multiple linear regression analysis of the lymphocyte data. A total of

TABLE II. Mean Values (±SE) of the Cytogenetic Parameters Evaluated in Pesticide-Exposed and Control Study Groups*

	Control		Exposed	
	No.	Mean ± SE	No.	Mean ± SE
MNL	53	10.30 ± 0.97	76	10.22 ± 0.81
Men	41	9.07 ± 1.01	50	10.64 ± 1.04
Women	12	14.50 ± 2.19	26	9.42 ± 1.27
BNMN	53	9.15 ± 0.91	76	9.30 ± 0.70
Men	41	8.07 ± 0.96	50	9.46 ± 0.88
Women	12	12.83 ± 2.09	26	9.00 ± 1.16
CBPI	53	1.51 ± 0.02	76	1.32 ± 0.01
Men	41	1.52 ± 0.03	50	1.27 ± 0.02
Women	12	1.48 ± 0.03	26	1.41 ± 0.02
MNBC	64	2.36 ± 0.66	74	1.76 ± 0.29
Men	52	2.67 ± 0.80	55	1.91 ± 0.37
Women	12	1.00 ± 0.39	19	1.32 ± 0.36
BCMN	64	1.98 ± 0.51	74	1.62 ± 0.23
Men	52	2.25 ± 0.62	55	1.76 ± 0.29
Women	12	0.83 ± 0.30	19	1.21 ± 0.33

MNL, total number of micronuclei in lymphocytes (‰); BNMN, binucleated lymphocytes with micronuclei (‰); CBPI, cytokinesis block proliferation index for lymphocytes; MNBC, micronuclei in mononucleated buccal cells; BCMN, buccal cells with micronuclei.
*A total of 1,000 cells per donor were scored for BNMN and MNL; 2,000 cells per donor were scored for BCMN and MNBC.

129 samples were available for the study. These results do not show any effect of exposure on the lymphocyte cytogenetic variables studied (BNMN and MNL). However, from the list of variables included in the analysis, before and after the population was matched for age, it was observed that age and the ingestion of raw vegetables had a significant effect on BNMN and MNL. Whereas increasing age was positively related with MN, the consumption of raw vegetables had an inverse relationship. We created a new variable, the interaction between exposure and gender, represented as exposure*gender, that allowed studying the relationship between these variables. The interaction exposure*gender was significant, showing that nonexposed women had the highest frequency of MN.

Multiple linear regression analysis of the CBPI indicated that exposure to pesticides had a significant negative relationship, that is, exposed workers had lower levels of CBPI than nonexposed controls, with exposed men having the lowest CBPI, as reflected in the interaction exposure*gender. The number of cigarettes per day also showed a negative correlation with CBPI.

For the buccal cell study, 138 samples were scored and the data indicated that the frequency of MN in the exposed group did not increase. The results of the negative binomial regression analysis of buccal cells, evaluating both BCMN and MNBC, are presented in Table IV. Only a slight influence of the variables studied was detected, although the number of cigarettes was positively related to buccal MN frequency. Age showed a tendency to be positively related and the interaction age*gender showed that, for the same age, men had a higher number of MN in buccal cells than women.

Workers: Moderately and Highly Exposed

The exposed individuals were classified into two groups: a moderately and a highly exposed group. The moderately exposed group was made up of both men and women, whereas the highly exposed group contained only men.

Table V shows the main characteristics of the two groups. Both were matched for age, cigarette smoking, and years of exposure to pesticides. Slight differences were detected in the total hours of pesticide application per year ($P = 0.050$) and grams of alcohol (ethanol) consumed per week ($P = 0.062$).

Both groups of farmers usually worked inside greenhouses (56%); 31.6% of the highly exposed group worked as applicators, compared to 16.9% of the moderately exposed. The main type of crop cultivated by this population was vegetables (64.3%; 67.7% in moderately exposed and 52.6% in highly exposed). Workers applied pesticides mainly at a level below the head. Moreover, 79.7% of the moderately exposed and 100% of the highly exposed workers stated that they had used protective measures, principally gloves, and a combination of these with boots and masks. It is important to note that all highly exposed men indicated that they had experienced pesticide poisoning via dermal penetration (58.8%) or inhalation (41.2%) and 52.6% of them had required hospitalization.

Table VI shows the mean values of the cytogenetic variables studied and the CBPI. Data are presented for both exposure groups and sexes. The analysis shows that highly exposed individuals have elevated levels of MN for both lymphocytes and buccal cells, but these levels do not attain statistical significance. In Table VII the results obtained in the ANOVA analysis indicate that there are no significant differences between the two exposed groups. From the covariates introduced, only age and pesticide application activity show a positive relationship with an increase of both MNL and BNMN. When CBPI is analyzed, only covariates v1 and v2 show any effect, indicating a lower CBPI index for men. For the study of epithelial buccal cells, 74 samples were available. The results of the negative binomial regression analysis (Table VIII) show no increase in MN in the highly exposed group. Only cigarette smoking, among all the included factors, shows a positive relationship with MN.

DISCUSSION

The results obtained in this study show that there is no exposure-related induction of chromosome damage, as indicated by the MN assay, in either peripheral lymphocytes or buccal epithelial cells. Although several studies have also reported negative results with cytogenetic biomarkers in

TABLE III. Summary of the Results Obtained in the Multiple Linear Regression Analysis of the Whole Hungarian Study Population; Analysis of Lymphocyte Data

	No.	B	Beta	*P*	Tolerance	R^2
MNL	129					0.116
Intercept		1.725	—	0.005	—	
Gender		0.747	0.285	0.041	0.374	
Age		0.037	0.236	0.007	0.962	
Raw vegetables		−0.077	−0.173	0.048	0.959	
Exposure*Gender		−0.698	−0.232	0.095	0.374	
BNMN	129					0.101
Intercept		1.551	—	0.007	—	
Gender		0.330	0.133	0.125	0.976	
Age		0.037	0.251	0.004	0.979	
Raw vegetables		−0.079	−0.186	0.033	0.965	
CBPI	129					0.353
Intercept		1.517	—	0.000	—	
Exposure		−0.214	−0.570	0.000	0.783	
No. cigarettes/day		−0.003	−0.180	0.017	0.933	
Exposure*Gender		0.126	0.257	0.002	0.814	

B, nonstandardized coefficient; Beta, standardized coefficient; R^2, coefficient of determination. MNL, total number of micronuclei in lymphocytes (‰); BNMN, binucleated lymphocytes with micronuclei (‰); CBPI, cytokinesis block proliferation index for lymphocytes.

TABLE IV. Results of Negative Binomial Regression Analysis of Buccal Cell Data From the Whole Study Population

	N	B	*P*	Scale deviance	Value/DF
MNBC	138			141.628	1.056
Intercept		−0.675	0.288		
Age		0.030	0.028		
No. cigarettes/day		0.029	0.018		
Age*Gender		−0.014	0.032		
BCMN	138			142.767	1.065
Intercept		−0.531	0.382		
Age		0.024	0.061		
No. cigarettes/day		0.024	0.037		
Age*Gender		−0.013	0.031		

MNBC, micronuclei in mononucleated buccal cells; BCMN, buccal cells with micronuclei; B, nonstandardized coefficient; DF, degrees of freedom.

humans exposed to pesticides [Hoyos et al., 1996; Davies et al., 1998; Venegas et al., 1998; Pastor et al., 2001a,b], many others have detected cytogenetic effects, indicating that MN frequency is a useful indicator of the association between chromosome damage and pesticide exposure [da Silva Augusto et al., 1997; Gómez-Arroyo et al., 2000; Figgs et al., 2000; Garaj-Vrhovac and Zeljezic, 2001]. Our negative findings suggest that the Hungarian population studied may have been exposed to pesticides with a low genotoxic potential, or that the use of protective measures led to a decrease in the level of exposure. In addition, Au et al. [1999], who investigated the genetic susceptibility to environmental toxic agents, suggested that the work conditions may select workers who are resistant to the toxic effect; therefore, they may have less cytogenetic damage than expected.

In agreement with previous reports [Barale et al., 1998; Davies et al., 1998; Fenech, 1998a; Falck et al., 1999], we found that age was strongly and positively associated with MN. In our study, the oldest individuals correspond to the control women, which could explain the high levels of MN in this group since it has been established that women have a higher frequency of MN than men [Barale et al., 1998; Davies et al., 1998; Falck et al., 1999]. Although a tendency to a higher frequency of MN in women was observed in our study, it did not reach statistical significance for BNMN. But when the interaction exposure*gender was calculated, a marginal significance was found, showing that the control women have the most cytogenetic damage.

From the multivariate analysis of the overall population, diet was an important confounding factor. The frequency of BNMN was inversely related to the ingestion of raw vegetables. Several studies of MN have shown that vegetarians have lower levels of cytogenetic damage than nonvegetarians [Davies et al., 1998; Dhawan et al., 2001]. This could be a consequence of ingesting lower levels of some muta-

TABLE V. Characteristics of the Two Pesticide-Exposed Groups

	Moderately exposed No. = 65		Highly exposed No. = 19	
	No.	Mean ± SE	No.	Mean ± SE
Age (years)		42.28 ± 0.85		40.95 ± 1.41
Men	39 (60%)	41.77 ± 1.24	19 (100%)	40.95 ± 1.41
Women	26 (40%)	43.04 ± 1.03	—	
Years of pesticide exposure		19.34 ± 1.06		16.74 ± 1.51
Hours of application/year		113.76 ± 16.08		182.31 ± 31.96
Nonsmokers	38 (58.8%)		7 (36.8%)	
Exsmokers	5 (7.7%)		4 (21.1%)	
Smokers (cig/day)	22 (33.8%)	18.77 ± 2.12	8 (42.1%)	18.38 ± 4.13
Ethanol (g/week)		70.54 ± 14.45		170.58 ± 48.68
Coffee (cups/day)		2.66 ± 0.30		2.16 ± 0.34
Tea (cups/day)		1.20 ± 0.17		1.05 ± 0.31
Red meat (times/week)		3.66 ± 0.28		4.37 ± 0.50
White meat (times/week)		2.66 ± 0.20		2.84 ± 0.49
Fish (times/week)		0.38 ± 0.08		0.53 ± 0.14
Raw vegetables (times/week)		5.08 ± 0.33		5.47 ± 0.55
Cooked vegetables (times/week)		5.05 ± 0.30		5.37 ± 0.56
Fruit (g/day)		348.09 ± 30.36		241.32 ± 55.67

TABLE VI. Mean Values of the Cytogenetic Damage in the Two Pesticide-Exposed Groups*

	Moderately exposed		Highly exposed	
	No.	Mean ± SE	No.	Mean ± SE
MNL	57	9.63 ± 0.82	19	12.00 ± 2.08
Men	31	9.81 ± 1.09	19	12.00 ± 2.08
Women	26	9.42 ± 1.27	—	—
BNMN	57	8.88 ± 0.75	19	10.58 ± 1.67
Men	31	8.77 ± 0.99	19	10.58 ± 1.67
Women	26	9.00 ± 1.16	—	—
CBPI	57	1.33 ± 0.02	19	1.30 ± 0.03
Men	31	1.25 ± 0.02	19	1.30 ± 0.03
Women	26	1.41 ± 0.02	—	—
MNBC	56	1.59 ± 0.24	18	2.28 ± 0.94
Men	37	1.73 ± 0.31	18	2.28 ± 0.94
Women	19	1.32 ± 0.36	—	—
BCMN	56	1.52 ± 0.22	18	1.94 ± 0.68
Men	37	1.68 ± 0.28	18	1.94 ± 0.68
Women	19	1.21 ± 0.33	—	—

MNL, total number of micronuclei in lymphocytes (‰); BNMN, binucleated lymphocytes with micronuclei (‰); CBPI, lymphocyte cytokinesis block proliferation index (‰); MNBC, micronuclei in mononucleated buccal cells; BCMN, buccal cells with micronuclei.
*A total of 1,000 cells per donor were scored for BNMN and MNL; 2,000 cells were scored for BCMN and MNBC.

gens such as benzo(a)pyrene and other food mutagens that are present in cooked meat. In addition, a diet rich in vegetables increases the levels of antioxidants and vitamins (potentially antimutagenic micronutrients), which could reduce genetic damage [Loprieno et al., 1991; Duthie et al., 1996]. It should be noted that Fenech found that differences in chromosomal damage between vegetarians and nonvegetarians were dependent on the age of the study group [Fenech and Rinaldi, 1995; Fenech, 1998b].

The analysis of the groups of exposed workers revealed that the applicators of pesticides had an increase in cytogenetic damage when compared to agricultural workers who were not applicators. As similar results were found in other studies, it is possible that this occupation results in more direct contact with the pesticides. Thus, Falck et al. [1999] indicated that MN increased especially in greenhouse workers involved in pesticide spraying. A high percentage of the highly exposed individuals worked exclusively or partially as applicators, and although they asserted that they had used protective measures, all had been intoxicated by pesticides in the years immediately preceding the current investigation.

Analysis of data from all study subjects indicated that pesticide exposure is capable of altering cell proliferation kinetics, suggesting that exposure to pesticides induces both a cell cycle delay and a decrease of the lymphocyte proliferation index (CBPI). As indicated by the interactions evaluated in the study for the whole population, exposed men had the lowest proliferation index. When only exposed individuals were considered, men also showed a significant decrease in CBPI. Other studies (e.g., Amorin et al. [2000]) also found a decrease in the mitotic index in men. Cell proliferation delay due to pesticide exposure has also been observed [Rupa et al., 1991; Pasquini et al., 1996]; nevertheless, our results do not agree with Gómez-Arroyo et al. [2000], who found an acceleration of the cell cycle and an increase of the mitotic index in exposed workers. The fact that exposed men had lower lymphocyte proliferation may be related to their different types of jobs (mainly applicators of pesticides) compared to women (mainly harvesters). It has been hypothesized that chronic exposure to toxins, such as pesticides, induces a response that increases apoptosis sensitivity and/or extends cell cycle delay that enables cells to repair their DNA [Kirsch-Volders and Fenech, 2001].

There are few data on the effects of tobacco on lympho-

TABLE VII. Summary of the Results of ANOVA Analysis of Lymphocyte Data From the Two Pesticide-Exposed Populations

	B	*P*	R^2
MNL			0.124
Intercept	0.860	0.326	
Age	0.047	0.029	
Applicator	0.681	0.055	
Alcohol	−0.0003	0.709	
V			
v1	−0.110	0.738	
v2	0.270	0.481	
v3	0	—	
BNMN			0.130
Intercept	1.053	0.238	
Age	0.042	0.039	
Applicator	0.722	0.030	
Alcohol	−0.0004	0.592	
V			
v1	−0.223	0.467	
v2	0.139	0.697	
v3	0		
CBPI			0.247
Intercept	1.488	0.001	
Age	−0.001	0.547	
Alcohol	0.000	0.597	
N° cigarettes	−0.002	0.121	
V			
v1	−0.151	0.000	
v2	−0.116	0.010	
v3	0		

No. = 76 B, nonstandardized coefficient; R^2, coefficient of determination; v1, moderately exposed men; v2, highly exposed men; v3, moderately exposed women. MNL, total number of micronuclei in lymphocytes (‰); BNMN, binucleated lymphocytes with micronuclei (‰); CBPI, cytokinesis block proliferation index for lymphocytes.

TABLE VIII. Results of Negative Binomial Regression Analysis of Buccal Cell Data From the Two Pesticide-Exposed Groups

	N	B	*P*	Scale deviance	Value/DF
MNBC	74			78.878	1.095
Intercept		0.312	0.065		
No. cigarettes/day		0.029	0.014		
BCMN	74			80.620	1.119
Intercept		0.270	0.092		
No. cigarettes/day		0.025	0.023		

MNBC, micronuclei in mononucleated buccal cells; BCMN, buccal cells with micronuclei; B, nonstandardized coefficient; DF, degrees of freedom.

cyte proliferation. In the present study, tobacco was negatively related to CBPI. This observation is consistent with the findings of Amorin et al. [2000] who found that smokers had a significantly lower mitotic index compared to ex- and nonsmokers and McCue et al. [2000], who demonstrated that cigarette smoke inhibits lymphocyte proliferation.

When data from all study subjects were evaluated, the MN frequencies in epithelial buccal cells significantly increased with the age of the subjects, as occurred with the lymphocytes. Nevertheless, other studies [Sarto et al., 1987; Burgaz et al., 1999] did not find any relation between age and MN in buccal epithelial cells. We also found that smoking increased the frequency of MN for both the whole population and the exposed groups. Several studies have also reported a significant increase in the frequency of micronucleated buccal mucosal cells for smokers [Sarto et al., 1987; Piyathilake et al., 1995; Özkyl et al., 1997], which agrees with our results.

The analysis of the buccal cell data for all the 138 individuals indicated that the interaction age*gender was statistically significant. This means that at the same age men have a greater number of MN than women. The previous studies indicate that MN in buccal cells show no differences between sexes [Kayal et al., 1993; Gómez-Arroyo et al., 2000]. Thus, the increases observed could be due to a sum of different factors (e.g., drinking, smoking) not uniformly distributed between sexes.

In summary, this work shows that there were no significant increases in the frequency of MN among pesticide-exposed workers as a whole or when exposed workers were divided into moderately exposed and highly exposed groups. Several confounding variables, however, showed significant associations with MN frequency. Finally, CBPI values decreased in the highly exposed workers, which is a possible genotoxic effect of pesticide exposure, but only when the levels were particularly high.

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Artículo 5

Biomonitoring of four European populations occupationally exposed to pesticides: Use of micronuclei as biomarkers

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Mutagenesis (en prensa)

Biomonitoring of four European populations occupationally exposed to pesticides: Use of micronuclei as biomarkers

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Abstract

This paper presents the results obtained in the framework of an EU-research project, aimed at investigating the relationship between occupational exposure to pesticides and the induction of cytogenetic damage. Populations from Greece, Spain, Poland and Hungary, all of them characterised by an intensive agriculture activity, were the subject of the study. A total of 239 agricultural workers and 231 unexposed controls were examined for cytogenetic effects in lymphocytes of peripheral blood and exfoliated cells of the oral mucosa. The frequency of micronuclei (MN) was evaluated in both cell types, and their relationship with different confounding factors (e.g. sex, country, smoking habit, etc.) was determined. The cytokinesis-block proliferation index (CBPI) was also calculated to detect possible variations in the proliferative kinetics of lymphocytes due to pesticide exposure. The results obtained indicate that there are no increases in the MN frequencies in the agricultural workers when compared to the controls for either lymphocytes or buccal cells. However, exposed individuals showed a significant decrease in the cytokinesis-block proliferation index (CBPI) when compared to controls. When the effect of the different confounding factors was evaluated, age was positively related with MN in lymphocytes, and the Polish population showed a MN frequency significantly higher than those observed in the other populations. For the buccal cells, the Spanish population showed a higher MN frequency, attaining significant differences in comparison to other populations. Finally, the CBPI was found to be inversely influenced by age, and Hungarian exposed men were the group that showed the lower values.

Introduction

As it is well known, pesticides are extensively used all over the world and, in the last years, their use has increased spectacularly. Large amounts of these chemicals are released into the environment and many of them affect non-target organisms, being a potential hazard to human health. Pesticide exposure is ubiquitous, due not only to agricultural pesticide use or contamination of foods, but also to the extensive use of these products in and around residences. The individuals occupationally exposed to pesticides (as field workers, mixers, loaders, applicators, etc.) which are in direct contact with these chemicals may provide a good opportunity to study their adverse health consequences.

At present there are registered 834 active pesticide substances in the European Union (Report from the Commission to the European Parliament and the Council, 2001) and some of them have been classified as possible or probable mutagens and/or carcinogens by the International Agency for Research on Cancer (IARC, 1990, 1991).

Thus, exposure to pesticides has been associated with an increase in the incidence of non-Hodgkin's lymphoma (Hardell *et al.,* 1999; Zheng *et al.,* 2001), multiple myeloma (Khuder and Mutgi, 1997), soft tissue sarcoma (Kogevinas *et al.,* 1995), lung sarcoma (Blair *et al.,* 1983), pancreatic, stomach, liver, bladder, and gallbladder cancer (Ji *et al.,* 2001; Shukla *et al.,* 2001), Parkinson disease (Jenner, 2001; Sherer *et al.,* 2001), Alzheimer disease (Gauthier *et al.,* 2001), and reproductive outcomes (Arbuckle *et al.,* 2001), among others.

In view of these findings, the detection of populations at risk constitutes a very important topic. In this context, it must be pointed out that cytogenetic markers such as chromosomal aberrations (CA), sister chromatid exchanges (SCEs), micronuclei (MN) and recently single cell gel electrophoresis (SCGE), have been extensively used for detection of early biological effects of DNA damaging agents. Regarding pesticide exposure, many reports dealing with CA (Arm, 1999; Au *et al.,* 1999; Antonucci and de Styllos Colus, 2000; Zeljezic and Garaj-Vrhovac, 2001), SCE (De Ferrari *et al.,* 1991; Garaj-Vrhovac and Zeljezic, 2001; Shaham *et al.,* 2001) and SCGE (Zeljezic and Garaj-Vrhovac, 2001; Garaj-Vrhovac and Zeljezic, 2000) found significant increases in these biomarkers, providing suggestive evidence of genotoxic effects induced by pesticides.

MN are formed by the condensation of acentric chromosomal fragments or by whole chromosomes, lagging behind the cell division. This is the only biomarker that allows the evaluation of both clastogenic and aneuploidogenic effects in a vast range of cells, since they are detected in interphase. The sensitivity and reliability of the MN assay in human lymphocytes, by blocking the cytokinesis

(CB) with cytochalasin-B (Cyt-B), has shown to be an effective tool to measure cytogenetic damage of pesticides in several populations (Bolognesi *et al.,* 1993; da Silva Augusto *et al.,* 1997; Joksić *et al.,* 1997; Falck *et al.,* 1999). In addition, this assay also allows the detection of effects on cell proliferation and cytotoxicity. Moreover, MN can be evaluated in different kinds of cells, that not necessarily had to be divided in vitro (as epithelial cells); thus, the analysis of MN in exfoliated buccal cells has demonstrated to be a sensitive method for monitoring genetic damage in human populations (Sarto *et al.,* 1990; Karahalil *et al.,* 1999). Nevertheless, few studies with pesticide exposed populations have been carried out using buccal cells, and, from the available data, only one has found a positive relationship with exposure (Gómez-Arroyo *et al.,* 2000).

In the present study, to asses whether the prolonged exposure to complex mixtures of pesticides leads to an increase in cytogenetic damage, human peripheral lymphocytes and buccal epithelial cells were analysed by using the micronucleus assay. This is a large study where agricultural workers from four different European countries were included. We previously evaluated these populations in separate studies (Lucero *et al.,* 2000; Pastor *et al.,* 2001a,b; 2002) in the frame of a European project. This paper does not try to sum up the results already reported, but aims to present a global analysis of the populations included in the project, the goal of which was to determine if pesticide exposure is reflected in an increase of cytogenetic damage.

Materials and methods

Subjects studied

A total of 478 individuals from four European countries were selected for the study. 247 were agricultural workers exposed to pesticides and 231 controls. Their origins were: 50 exposed and 66 controls came from an area outside Athens, called Nea Makri, in Greece; 63 exposed and 51 controls from the province of Almería in Spain; 50 exposed and 49 controls from Małopolska, a region of Southern Poland; and 84 exposed and 65 controls from South East Hungary. With the exception of Greece and Hungary (with 20 and 26 exposed and 25 and 12 control women, respectively), the rest of the population studied were composed by men.

Prior to the study, all the individuals signed an informed consent and filled in a detailed questionnaire inquiring information about possible confounding factors such as age, gender, smoking and drinking habits, vaccination, medication, X-ray examinations and diet. In the case of the exposed group, occupational activity, years of agrochemical exposure, main pesticides used, kind of crops, protective measures used, etc., were also recorded. The main characteristics of the studied population are listed in Table I. It is necessary to emphasise that due to extrinsic factors some data were missing, thus the final number of analysed individuals was lower, remaining 457 individuals for MN scoring in lymphocytes and 441 individuals for MN analysis in buccal cells.

With regard to the smoking habit, individuals were classified as non-smokers, when they had never smoked or had quit smoking more than five years ago, as ex-smokers if they had stopped smoking between one and five years before sampling, and current smokers. A particular characteristic of the Greek population is that it was constituted only by non or ex-smokers.

All the agricultural workers were regularly exposed to complex mixtures of pesticides that differ depending of the region, climate and kind of crop. Nevertheless, carbamates, organophosphorus and pyrethroids were the families of pesticides mostly used (Table II). The farmers worked mainly in greenhouses, although Polish and Hungarian cohorts also worked in open fields. The principal crops were vegetables and ornamental plants. Pesticide application was usually carried out from above the head in Greece, Spain and Poland, and under the head in Hungary. Almost 80% of the pesticide-exposed workers asserted to use some kind of protection during the preparation and application of pesticides; in Spain and Poland they used usually more than one protective measure (gloves, breathing masks, glasses, impermeable boots, etc.). In spite of that, 21.5% of them had suffered recent pesticide intoxication. Most of these intoxications were by dermal contact and inhalation, and manifested as dermatitis, eczema and irritability of mucous membranes (eyes, nose).

Regarding to the control individuals, they carried out clerical and health care jobs, in the same village or region where the exposed ones came from. None of them had recent exposures to

agrochemicals or other suspected genotoxic agents, and had no previous occupational exposure to genotoxicants.

Taking into account the heterogeneity of the populations studied, in this work we have carefully considered a wide range of external confounding factors that might influence the results. It should be noted that if we look at the previous studies, some variables have not been included in spite of their relevance (eg. X-ray, miscarriages…), due to the lack of data from some of the populations.

The data reported here correspond to blood and buccal samples collected during 1998.

Lymphocyte cultures, staining and BNMN scoring

Blood samples were obtained from each subject by venipuncture in heparinized vacutainers. Samples from Nea Makri (Greece) and Almería (Spain) were sent within 24 hr to the Universitat Autònoma de Barcelona (Spain) where they were immediately processed. Lymphocyte cultures from samples collected in Małopolska (Poland) were set up in the laboratory of the Department of Environmental and Radiation Biology (DERB) of the H. Niewodniczański Institute of Nuclear Physics (Kraków), and the blood samples from Hungary were processed in the B. Johan National Center for Epidemiology in Budapest. The same standardised protocol was used in all participating laboratories.

Lymphocyte cultures were set up by adding 0.5 mL of whole blood to 4.5 mL of RPMI 1640 medium supplemented with 15% heat-inactivated foetal calf serum, 1% antibiotics (penicillin and streptomycin) and L-glutamine. Lymphocytes were stimulated by 1% of phytohaemagglutinin and incubated for 72 hr at 37ºC. Two cultures per subject were established. A final concentration of 6 μg/mL of cytochalasin-B (Surrallés *et al.,* 1994) was added to the cultures 44 hr later to arrest cytokinesis. At 72 hr of incubation, the cultures were harvested by centrifugation at 800 rpm for 8 min and treated with a hypotonic solution (2-3 min in 0.075 M KCl at 4ºC). Cells were centrifuged thereafter and a methanol: acetic acid (3:1 v/v) solution was gently added. This fixation step was repeated twice and the resulting cells were resuspended in a small volume of fixative and dropped onto clean slides.

The slides of all samples were stained and scored in the laboratory of mutagenesis, Department of Genetics and Microbiology, Universitat Autònoma de Barcelona. They were stained with 10% Giemsa in phosphate buffer (pH 6.8) for 10 min. Following the criteria proposed by Fenech (1993), to determine the frequency of binucleated cells with micronuclei (BNMN) and the total number of MN in lymphocytes (MNL), a total of 1000 binucleated cells with well preserved cytoplasm (500 per replicate) were scored per subject on coded slides. In addition, 500 lymphocytes were scored to determine the percentage of cells with one to four nuclei, and the cytokinesis-block proliferation index (CBPI) was calculated according to Surrallés *et al.* (1995). To avoid differences by observers, the same individual carried out all the microscopic analysis.

Buccal cells procedure, staining and MN scoring

Buccal cell samples were obtained by rubbing the inside of the cheeks with a toothbrush. The cells were collected in a conical tube containing 20 mL of buffer solution (0.1 M EDTA, 0.01 Tris-HCl and 0.02 M NaCl, pH 7), and immediately transported to the correspondent laboratory for further processing (Athens, Barcelona, Kraków and Budapest). After three washes in the buffer solution followed by centrifugation at 1500 rpm during 10 min, 50 μL of an adequate cell suspension density was dropped onto preheated (55ºC) slides and allowed to air-dry for 15 min on a slide-warmer. The slides were fixed in 80% cold methanol for 30 min and air-dried overnight at room temperature. Next the slides were sent to Barcelona where they were stored at –20 ºC until use, and stained with a DNA specific stain, namely 1μg/mL of 4',6-di-amidino-2-phenylindole dihydrochloride (DAPI) that avoids possible scoring artefacts. A total of 2000 cells/donor were scored, on coded slides, by one scorer under an Olympus BX50 fluorescent microscope. The criteria for MN evaluation were those suggested by Titenko-Holland et al., (1998). The frequency of mononucleated buccal cells with micronuclei (BCMN) and the total number of micronuclei in buccal cells (MNBC) were determined for each studied subject.

Statistical method

The statistical computations were performed using the SPSS version 10.0 software (SPSS, Chicago, IL, USA) and the SAS system for Windows, version 8.0 (SAS, Cary, NC, USA). The Student's *t*-test, the analysis of variance and the Chi-square test were used to compare means and frequencies for demographic, dietary and habit factors, between populations, exposures and sexes.

The cytogenetic variables BNMN and CBPI were analysed using a generalized linear model (GLZ). All variables that could have any influence in the results were included in the analysis (age, sex, exposure, country, diet, cigarettes, etc.). *Post hoc* comparisons using the Tukey's correction were also done. The BNMN data were transformed by a square root to achieve all the requirements of the method. The cytological variable BCMN, scored in buccal cells, was first studied by Poisson regression but due to the high over-dispersion found, a negative binomial regression analysis was finally carried out. A backward selection method was used in all cases (BNMN; CBPI and BCMN) as an exploratory method. The most important variables as well as the main interactions were taken into account.

The type III sum of square method was used because it is a test of effects after controlling for all other factors and, in addition it is easily interpreted. *P* values correspond to two-sided tests and an alpha error (α) < 0.05 was considered the significance level.

Results

The main characteristics of the farm workers and controls from the four studied populations are presented in Table I. This table also indicates those dietary characteristics that can act as potential confounding factors on the micronuclei frequency analysis.

Regarding the average of age, it can be observed a slight statistically significant difference (P = 0.004) between controls and exposed; nevertheless the little average difference does not represent biological significance. Differences between sexes were only appreciated in the exposed individuals, where women are older than men (P = 0.002). Significant age differences between countries were also observed in the analysis of exposed men, the Spanish agricultural workers being the youngest ($P \leq$ 0.001).

Two different groups can be established with regard to the years of occupational exposure to pesticides. On the one hand, the Mediterranean subjects (Greece and Spain) and, on the other, those from Middle-Europe (Poland and Hungary). The average period working in agriculture for the farmers of the first group was about 9 years and, therefore, they were exposed for fewer years than those of the second group, who had been occupationally exposed to chemical pesticides for 16 to 18 years. Such difference is highly statistically significant ($P \leq 0.001$).

In relation to the alcohol consumption (gr/week), the values obtained for the controls and exposed indicated a similar behaviour; nevertheless, differences were clearly detected between populations and between males and females. Thus, in the Greek population the alcohol consumption in controls is higher than in exposed farmers, whilst in the Hungarian population the tendency is the opposite. Differences were observed between men and women ($P < 0.001$), being men those who drink more.

Tobacco is a well-known factor that can influence the level of genotoxic damage. The Greek population did not include current smokers, thus an elevated number of ex-smokers was studied. This lead to statistically significant differences when compared to the rest of populations for both controls and exposed. Nevertheless, all smokers consume similar number of cigarettes per day. Among the smokers, there are more men than women, both in control (P = 0.0003) and in exposed (P = 0.002).

Taking into account that the studied populations belong to different countries, which can be expected to have different dietary habits, the role of several dietary factors on the MN frequency has been studied. Thus, the frequency of ingestion of red meat per week could be differentiated in two groups, the Mediterranean population (Greece and Spain) and the Middle-European (Poland and Hungary). This second group was a greater consumer of red meat. A significant increased consumption of red meat was found in exposed *vs* controls (P = 0.007). On the other hand, fish intake follows the opposite, being Greek and Spanish populations being those with a high fish intake. It is remarkable the low level of fish consumption in the Hungarian population. Concerning the ingestion of raw

vegetables per week, only a significant difference was found between Spanish and Polish controls ($P = 0.005$); meanwhile the consumption of cooked vegetables was very heterogeneous and significant differences were found between populations, and between controls and exposed ($P = 0.015$). Finally, the fruit consumption per week was not different between the four populations, including men and women, with the exception of exposed men, where Hungarians had greater significant consumption of fruit than Spanish ($P < 0.0001$) and Polish ($P = 0.029$).

As can be observed in table II, each population uses their own pesticides. Different factors have influenced the choice of each product (kind of crop, weather conditions, pests, etc.) and, although slight differences can be appreciated in the compounds used, generally the main chemical families that the pesticides belong to are the same. Carbamates are used approximately with the same frequency in each population; pyrethroids differed specially between Mediterranean and Middle European countries, the latter with a high percentage of use. Regarding to organophosphorus, the only difference was found in Hungary where the percentage of use was higher. The use of antibiotics in Spain must be mentioned.

Table III shows the means of the cytogenetic variables evaluated. All the statistical analysis takes into account dietary, demographic, and tobacco and alcohol habits. However, due to the lack of significance of some variables, they were not taken into account (backward method) although others remained in the study for their apparent interest. The results of the GLZ final models for BNMN and CBPI are summarised in table IV. It is observed that exposure to pesticides does not induce any significant increase in the frequency of BNMN (Figure 1); nevertheless the age shows a strong and positive significant effect over BNMN ($P < 0.0001$, B = 0.014), that means that the frequency of MN increases with the age of individuals. It must be mentioned that all figures show the lsmeans or least-squares means, corresponding to the mean adjusted for the other terms in the model.

There were differences between populations but due to the fact that two of them included women, we created a new variable that includes both the country and the sex of individuals, called CS (country-sex). CS was introduced in the model as a random factor (like the other variables). CS seemed to have a significant influence on BNMN. The Polish population (all men) is the group with higher levels of BNMN, showing significant differences with respect to Greeks, Spanish and Hungarians (men and women) (Figure 2). Greek women showed higher levels of cytogenetic damage than Spanish ($P = 0.01$); Hungarian men had the lowest damage level being significantly lower to the levels found in Greek men and women ($P = 0.02$; $P = 0.001$). There were no significant differences between men and women in the Greek and Hungarian populations and also between women.

Other variables were the interactions that we considered of interest to be studied because they could contribute to a better understanding of the results. When the interaction CS*Exposure was taken into account, the Polish population continued showing significant BNMN differences regarding all the possible combinations (control, exposed, men, women) with the exception of Greek and Hungarian control women (Figure 3). No differences were found between controls and agricultural workers from Poland. The CS*Age interaction, in the BNMN model, indicated that the age effect was more accentuated in women from Greece.

Table IV also shows the results of the proliferation index values (CBPI). Thus, the group occupationally exposed to pesticides showed a significantly lower CBPI, regarding to the non exposed (Figure 4). On the other hand, CBPI was inversely correlated with age. In the final model selected for the analysis of CBPI, CS and the interaction CS*Exposure were also included. Two blocks were clearly differentiated; on one side, Greeks and Spanish, on the other, Polish and Hungarians, according to the sex and the geographic area. Differences between men were found for the different populations, and also for women. Mediterranean people showed greater significant levels of CBPI than Middle-European people ($P < 0.0001$, for all possible combination cases) (Figure 5); although the Hungarian CBPI levels were significant lower than those of Polish (men, $P < 0.0001$; and women, $P = 0.0017$). In Greek and Hungarian populations, no differences between sexes were found. The results from the interaction CS*Exposure showed, as indicated before, significant differences between countries. Greeks and Spanish had higher CBPI values than Polish and Hungarians, independent of sex ($P < 0.0001$) (Figure 6). Greek control men showed the higher CBPI values, being significant different from to Greek exposed men ($P = 0.0005$) and to Spanish control men ($P = 0.02$). Hungarian exposed men, whose had the lowest CBPI levels, showed significant differences compared to Polish (controls and exposed, $P < 0.0001$) and Hungarian controls (men, $P < 0.0001$; and women, $P = 0.001$)

and Hungarian exposed women (P = 0.01). Hungarian exposed women also showed significant differences compared to Polish (exposed, P = 0.009; and control, P = 0.0001).

Regarding the buccal cells with micronuclei (BCMN), no differences were found between the agricultural workers and controls (Table V, Figure 7). The CS variable revealed us that the Spanish population differs significantly from the other populations (Figure 8). Differences were also observed between Hungarian control men and women (P = 0.049) (Table V). Similar differences were found when exposure and CS were studied together confirming that Spanish, independently of their exposure, showed higher significant levels of MN in buccal cells than the rest of the populations studied (Figure 9).

Alcohol did not influence the frequency of BCMN not even when included as interaction with the gender (data no reported here). No differences were obtained when the other variables were introduced.

It must be recalled that to study the possible effect of tobacco, the data from Greece had to be removed from the analysis since this population lacks smokers. Thus, when in the GLZ analysis of BNMN, BCMN and CBPI the smoking habit is included, the results did not change their significance. Consequently, the smoking habit did not affect the parameters evaluated (BNMN, P = 0.513; BCMN, P = 0.180; CBPI, P = 0.303).

Discussion

In summary, the results of this study indicate that the four populations of agricultural workers occupationally exposed to pesticides do not reveal a significant induction of cytogenetic damage, as measured by the MN assay in both lymphocytes and buccal epithelial cells. Although several studies have also reported a lack of cytogenetic effects as consequence of occupational exposure to pesticides (Hoyos *et al.,* 1996; Davies *et al.,* 1998; Venegas *et al.,* 1998; Lander *et al.,* 2000; Pastor *et al.,* 2001a,b, 2002), many others have demonstrated the induction of cytogenetic damage, indicating that the micronuclei frequency is a highly effective biomarker in revealing the association between chromosome damage and pesticide exposure (da Silva Augusto *et al.,* 1997; Gómez-Arroyo *et al.,* 2000; Figgs *et al.,* 2000; Garaj-Vrhovac and Zeljezic, 2000, 2001; Shaham *et al.,* 2001).

In our study, a high heterogeneity between the different populations studied was observed, for both the frequencies of BNMN and MNL (Table III). This heterogeneity is in agreement with the results of other studies, which also showed differences in the MN frequencies. Although the reported average baseline frequency of micronuclei in human lymphocytes was 7.8 ± 5.2 per 1000 cells (ranging from 3 to 23), with age and sex but not smoking as main confounding factors (Surrallés and Natarajan, 1997), the average in our control group was higher (12.25 ± 0.60). However, this value does not differ too much from those reported by Venegas *et al.,* (1998) (BNMN, 10.69 ± 2.08), and it is clearly lower than the values found by Titenko-Holland *et al*., (1997) (BNMN, 18.7 ± 7.6) and by Davies *et al*., (1998) (BNMN, 21.76 ± 1.50).

In principle, these differences can be attributed to methodological aspects, samples manipulation and/or scoring. However, in our study, the samples from Greece and Spain, that are the ones which showed high differences in the BNMN frequency, were both cultured in Barcelona and scored by the same person in a blind study. When comparing with other biomonitoring studies carried out in Barcelona, the levels of BNMN in controls were 20.81 (Pitarque *et al.,* 1996), 22.14 (Gutiérrez *et al.,* 1997) and 10.55 (Pitarque *et al.,* 1999); corroborating once more the existence of inter-individual and inter-population differences.

As previously reported (Fenech and Morley, 1986; Migliore *et al.,* 1991; Fenech *et al.,* 1994; Davies *et al.,* 1998; Fenech, 1998; Falck *et al.,* 1999), age was strongly associated in a positive way with MN frequency (Table IV). Our overall data show a significant increase of 0.014 MN/1000 binucleated lymphocytes per year. In our study the controls were also age debalanced regarding exposed, since they are a little bit older, and this could influence the results. On the other hand, the Spanish group, that is the youngest, showed lower levels of BNMN, which supports the previous findings.

Polish individuals, irrespective of being controls or exposed, showed significant greater levels of BNMN. At first sight, it can be seen (Table III) that the BNMN and MNL levels of Polish were higher

than the rest; with only the exception of Greek and Hungarian control women, who also showed high BNMN and MNL levels. This could be associated with age, but Polish did not stand out for this characteristic (Table I). This hypothesis only would explain the case of the Hungarian women because they were the oldest; nevertheless this assumption is not congruent since Greek control women had more cytogenetic damage and they were younger than Hungarian. Control women did not show any other remarkable characteristic, since all of them had common occupations, basically housewives and administrators, and none of them had been chronically exposed to genotoxic agents.

Why Polish people had greater BNMN values? This higher frequency does not refer only to the pesticide occupationally exposed individuals, but also to the controls; thus, intrinsic factors may be acting. The Polish group did not have a remarkable difference concerning alcohol consumption, pesticides used and smoking habit with respect to the other groups, although differences regarding Mediterranean people were observed in red meat consumption, the Polish being the ones who consumed more red meat. Likely, other genetic and/or environmental factors may account for the observed BNMN values in the Polish group.

With regard to the buccal cell study, a lack of increase of BCMN in the agricultural workers occupationally exposed to pesticide exposed was found. To our knowledge, there is only one study in which a significant increase of MN in buccal cells was found (Gómez-Arroyo *et al.,* 2000); nevertheless, these authors found high levels of cytogenetic damage both in controls and exposed (3.8 and 10 MN in 1000 cells, respectively). A wide variation in the amount of buccal cells with MN has been reported elsewhere. Thus, a high variation has been found in different control populations, the values ranging from 0.3-0.4 ‰ cells with MN (Sarto *et al.,* 1987,1990; Tolbert *et al.,* 1992; Rosin *et al.,* 1994; Karahalil *et al.,* 1999) to 2.7 ‰ (Livingston *et al.,* 1990); 4.7 ‰ (Stich and Rosin, 1983); and 8.4 ‰ (Özkul *et al.,* 1997).

If we look at the differences between countries, taking into account the sex (CS), the Spanish men (both controls and exposed) appeared as those who had the greater levels of BCMN. Why Spanish? The only remarkable aspects that can affect the frequency of BCMN, which differed from the rest of populations studied, are the alcohol and the cigarette consumption. Some studies have found a relationship between alcohol consumption and alterations in the normal oral mucosa (apoptosis, reduction of the area, keratinization, etc.) as well as increases of MN in the epithelial buccal cells (Kassie *et al.,* 2001). On the other hand, some studies (Surrallés *et al.,* 1997; Bloching *et al.,* 2000) found that alcohol did not influence the frequencies of MN.

Smoking is reported to increase the MN frequency in buccal cells (Sarto *et al.,* 1987; Piyathilake *et al.,* 1995; Kiilunen *et al.,* 1997). However, other studies found that smoking is not reflected in an increase of MN in buccal cells (Machado-Santelli *et al.,* 1994; Torres-Bugarín *et al.,* 1998; Burgaz *et al.,* 1999). From our data it is clear that the Spanish group had the highest percentage of smokers (60.7% of the controls, 55.5% of the exposed); since the Polish group had 57% and 36%, and the Hungarians 20% and 35.7%, for controls and exposed, respectively. Thus, although in our study smoking is a suggestive factor to explain the high frequency of BCMN in the Spanish group, the statistical analysis do not indicate that micronuclei formation is influenced by smoked cigarettes (P = 0.18, having into account only smokers; data not shown) and/or alcohol consumption.

An interesting finding in the overall population studied is that pesticide exposure seems to be capable of inducing alterations on the cell proliferation kinetics, suggesting that such exposure induces both a cell cycle delay and a reduction in the proliferation of lymphocytes (CBPI). As indicated by the interactions, the exposed Hungarian men, having the lowest proliferation index, differed significantly from the rest. Other studies (Amorim *et al.,* 2000), have also found a decrease of the mitotic index in men; although in our case this decrease was related to the pesticide exposure. Cell proliferation delay due to pesticide exposure has also been previously reported (Rupa *et al.,* 1991; Pasquini *et al.,* 1996); nevertheless other authors observed no such delay or even an acceleration of the cell cycle. The fact that there were the exposed men who showed lower CBPI levels may be related to the different type of activities they carried out (mainly application of pesticides) when compared to women (mainly harvesters). It must also be indicated that the sample size was unbalanced for sexes.

On the other hand, a relevant aspect found in this study is the significant difference between the Mediterranean and the Middle-Europe countries. The first ones (Greece and Spain) have similar CBPI values, being greater than in the others (Poland and Hungary). Furthermore, the Hungarian agricultural workers were those exposed for more years to pesticides.

To explain the cell cycle delay, it has been hypothesised that chronic low level exposure to toxins, such as pesticides, may induce an adaptive response related to an increase in apoptosis sensitivity and/or a more extended cell cycle delay that enables appropriate repair (Kirsch-Volders and Fenech, 2001). Another fact to be considered is the negative effect of tobacco on lymphocyte proliferation (Amorin *et al.,* 2000; McCue *et al.,* 2000); nevertheless, our results showed that smokers do not have different CBPI when compared to non-smokers or ex-smokers. Finally, we found that age was significantly and negatively associated with CBPI, inducing a decrease of the cell proliferation index with age. Studies on cell proliferation kinetics have also found a negative correlation of the replication index and cell proliferation rate with age (Lazutka *et al.,* 1994).

From the present study, based on four European populations, we can conclude that occupational exposure to pesticides, related with the particular agricultural activities of these areas, does not increase the level of cytogenetic damage when evaluated by the micronucleus assay using peripheral blood lymphocytes and buccal epithelial cells. These results might be surprising taking into account that the four agricultural groups were selected for their high and continued exposure to pesticides, most of them working in greenhouses. It is important to emphasise the working conditions of the individuals studied: 80% of the agriculturals reported the used of protective measures. This fact, together with the relatively low genotoxic potency of the pesticides used (Table II), might be the reason of the lack of a detectable increase in the MN frequency of the agricultural workers. However, an effect of the exposure was observed in the CBPI, indicating some cytotoxicity due to exposure. Perhaps the mode of action of the chemicals involved is not over DNA, but may be other targets.

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Table I. Age, life style and dietary characteristics of the populations analysed in the study

		Greece		Spain		Poland		Hungary		Overall	
		n	Mean ± SE	*n*	Mean ± SE	*n*	Mean ± SE	*n*	Mean ± SE	*N*	Mean ± SE
Age	C	66	43.94 ± 1.11	51	38.53 ± 1.35	49	38.53 ± 1.56	65	45.05 ± 0.96	231	41.91 ± 0.64
	♂	41	45.88 ± 1.23	51	38.53 ± 1.35	49	38.53 ± 1.56	53	44.62 ± 1.14	194	41.75 ± 0.70
	♀	25	40.76 ± 2.01	-	-	-	-	12	46.92 ± 1.28	37	42.76 ± 1.48
	E	50	42.98 ± 1.60	60	32.78 ± 1.15	50	39.14 ± 1.40	84	41.98 ± 0.73	244	39.34 ± 0.63
	♂	30	42.47 ± 2.31	60	32.78 ± 1.15	50	39.14 ± 1.40	58	41.50 ± 0.95	198	38.41 ± 0.72
	♀	20	43.75 ± 2.06	-	-	-	-	26	43.04 ± 1.03	46	43.35 ± 1.05
Years of pesticide exposure	C	-	-	-	-	-	-	-	-	-	-
	E	50	8.62 ± 1.13	63	9.82 ± 1.03	50	16.28 ± 1.10	84	18.75 ± 0.89	247	13.92 ± 0.58
Alcohol (gr/week)	C	66	119.25 ± 23.1	50	113.58 ± 11.2	49	99.41 ± 16.3	65	65.58 ± 15.5	230	98.62 ± 9.06
	E	50	51.56 ± 10.47	62	107.29 ± 14.1	50	127.48 ± 17.7	84	93.17 ± 16.2	246	95.24 ± 7.92
Nº of non-smokers[1]	C	45	68.2 %	14	27.5 %	20	40.8 %	47	72.0 %	126	54.5 %
	E	33	66.0 %	26	41.3 %	27	54.0 %	45	53.6 %	131	53.0 %
Nº of ex - smokers[1]	C	21	31.8 %	6	11.8 %	1	2.0 %	5	7.7 %	33	14.3 %
	E	17	34.0 %	2	3.2 %	5	10.0 %	9	10.7 %	33	13.4 %
Nº of smokers (cig/day)	C	0	-	31	18.58 ± 1.63	28	18.07 ± 1.50	13	14.15 ± 1.88	72	17.58 ± 0.97
	E	0	-	35	18.94 ± 1.79	18	18.00 ± 1.63	30	18.67 ± 1.87	83	18.6 ± 1.06
Red meat[2]	C	66	1.63 ± 0.13	18	2.03 ± 0.32	49	3.31 ± 0.25	65	3.12 ± 0.27	198	2.57 ± 0.13
	E	50	1.66 ± 0.13	34	2.21 ± 0.29	50	3.84 ± 0.20	84	3.82 ± 0.25	218	3.08 ± 0.13
Fish[2]	C	66	1.31 ± 0.11	26	2.33 ± 0.25	49	1.08 ± 0.11	65	0.54 ± 0.13	206	1.14 ± 0.07
	E	50	1.23 ± 0.12	35	1.62 ± 0.20	50	1.18 ± 0.10	84	0.42 ± 0.07	219	0.97 ± 0.06
Raw vegetables[2]	C	65	4.86 ± 0.40	26	6.37 ± 0.26	49	4.24 ± 0.36	65	5.28 ± 0.31	205	5.04 ± 0.19
	E	50	4.26 ± 0.37	39	5.40 ± 0.33	50	4.90 ± 0.32	84	5.17 ± 0.28	223	4.94 ± 0.17
Cooked vegetables[2]	C	66	1.91 ± 0.20	30	5.08 ± 0.44	49	3.39 ± 0.36	65	4.68 ± 0.34	210	3.56 ± 0.18
	E	50	2.72 ± 0.32	38	3.70 ± 0.39	50	4.40 ± 0.32	84	5.12 ± 0.26	222	4.17 ± 0.17
Fruit (gr/ day)	C	64	308.59 ± 34.9	47	239.04 ± 30.7	49	212.96 ± 22.1	65	247.92 ± 26.9	225	255.7 ± 15.0
	E	49	243.88 ± 21.1	46	145.22 ± 29.5	50	200.80 ± 23.8	84	323.94 ± 26.9	229	244.02 ± 14.3

[1]: %; [2]: times per week.

Table II. Pesticides used by the studied groups, with indication of their frequency of use (%) and EPA classification by carcinogenicity[a]

	Pesticide Group	Nea Makri GREECE	Almería SPAIN	Małoposka POLAND	South East HUNGARY	EPA Classification
INSECTICIDES						
Abamectin	Antibiotic		35.9	4.0		nd
Acephate	Organophosphorus			4.0		C
Acetamiprid	Nicotinoid			6.0		nd
Acrinathrin	Pyrethroid		17.2			D
Alphamethrin	Pyretrhoid			4.0	21.8	nd
Bifenthrin	Pyrethroid			6.0		C
Buprofezin	IGR	4.7	4.7			evidences
Carbosulfan	Carbamate			22.0		nd
Cyromazine	IGR	12.5	12.5			E
Clorpyrifos+cypermethrin	Organophosphorus/Pyretroid			4.0		E/ C
Deltamethrin	Pyrethroid			38.0	35.6	nd
Diazinon	Organophosphorus			6.0		not likely
Dichlorvos	Organophosphorus	8.0	3.2		39.1	C
Dimethoate	Organophosphorus			38.0	28.7	C
Endosulfan	Organochlorine	20.3	20.3			not likely
Fenazaquin	Unclassified			4.0		nd
Fenvalerate	Pyrethroid			12.0		E
Formetanate	Formamidine		9.4			E
Imidacloprid	Nicotinoid	50.0	50.0			E
Lambda-cyhalothrin	Pyrethroid			16.0	25.3	D
Malathion	Organophosphorus	8.0	12.5			evidences
Methamidophos	Organophosphorus	25.0	34.4	4.0		E
Methomyl	Carbamate	30.0	50.0	26.0	13.8	E
Oxamyl	Carbamate	14.1	14.1			E
Permethrin	Pyrethroid	10.0	4.7	4.0		C
Pirimicarb	Carbamate			8.0		nd
Pyriproxyfen	IGR	14.1	14.1			E
Tebufenozide	IGR		4.7			E
Tralometrin	Pyrethroid	15.6	15.6			nd
FUNGICIDES						
Benomyl	Benzimidazole/Carbamate			4.0	26.4	C
Bupirimate	Pyrethroid			8.0		nd
Captan	Dicarboximide			6.0		B2
Carbendazim	Benzimidazole	3.1	3.1			nd
Cymoxanilo	Aliphatic nitrogen	14.1	14.1			not likely
Clorothalonil	Aromatic			10.0		likely
Copper oxychloride	Copper			6.0		D
Copper sulphate	Copper				10.3	D
Dichlofluanid	Phenylsulfamide			4.0		nd
Diethofencarb	Carbamate	3.1	3.1			nd
Iprodion	Dicarb/Imidazole			10.0	13.8	likely
Mancozeb	Dithiocarbamate	20.0	12.5		17.2	B2
+oxadixyl	Oxazole			4.0		C
Metiram	Dithiocarbamate			4.0		B2
Nuarimol	Pirimidine		3.1			nd
Fosetyl-alumium	Organophosphorus	6.2	6.2			not likely
Procymidone	Dicarboximide	10.9	10.9			B2
Propamocarb	Carbamate	3.1	3.1	10.0		not likely
Propineb	Dithiocarbamate	7.8	7.8			nd
Thiophanate-methyl	Benzimidazole/Carbamate			9.0		likely
Triforine	Unclassified			9.0		nd
Vinclozolin	Dicarboximide			10.0		C
Zineb	Dithiocarbamate				14.9	nd
HERBICIDES						
Diquat dibromid	Quaternary ammonium				12.6	E
Glyphosate	Organophosphorus				16.1	E
Rimsulfuron	Sulfonylurea				10.3	E
BACTERICIDES						
Kasugamycin	Antibiotic	2.0	4.7			nd
Frequency of utilization of the most used groups (%)	**Carbamates**	50.3	70.2	66.0	40.2	
	Pyretroids	25.6	37.5	92.0	82.7	
	Organophosphorus	47.2	56.3	56.0	83.9	
	Antibiotics	2.0	40.6	4.0	0.0	

IRG- insect growth regulator.

[a]*Chemicals evaluated for carcinogenic potential. Science information management branch. Health effects division. Office of pesticide program. U.S. Environmental Protection Agency (May, 2002)*:
A- human carcinogen; **B-** probable human carcinogen: **B1**, limited evidence of carcinogenicity from epidemiologic studies, **B2**, sufficient evidence from animal studies; **C-** possible human carcinogen; **D-** not classifiable as to human carcinogenicity; **E-** evidence of non carcinogenicity for humans; **nd-** no data avalaible; **evidences-** suggestive evidence of carcinogenicity, but not sufficient to assess human carcinogenic potential; **likely-** likely to be carcinogenic to humans; **no likely-** not likely to be carcinogenic to humans.

Table III. Mean values (± SE) of the cytogenetic parameters evaluated in the populations studied (‰)*

		Greece		Spain		Poland		Hungary		Overall	
		n	Mean ± SE	*n*	Mean ± SE	*n*	Mean ± SE	*n*	Mean ± SE	*N*	Mean ± SE
BNMN	Control	66	14.42 ± 1.29	50	7.34 ± 0.64	49	17.67 ± 1.14	53	9.15 ± 0.91	218	12.25 ± 0.60
	♂	41	12.90 ± 1.20	50	7.34 ± 0.64	49	17.67 ± 1.14	41	8.07 ± 0.96	181	11.56 ± 0.59
	♀	25	16.92 ± 2.77	-	-	-	-	12	12.83 ± 2.09	37	15.59 ± 2.00
	Exposed	50	11.12 ± 0.82	63	8.70 ± 0.74	50	18.28 ± 1.28	76	9.30 ± 0.70	239	11.40 ± 0.49
	♂	30	10.90 ± 0.98	63	8.70 ± 0.74	50	18.28 ± 1.28	50	9.46 ± 0.88	193	11.72 ± 0.57
	♀	20	11.45 ± 1.45	-	-	-	-	26	9.00 ± 1.16	46	10.07 ± 0.92
MNL	Control	66	16.38 ± 1.50	50	8.00 ± 0.71	49	20.10 ± 1.34	53	10.30 ± 0.97	218	13.82 ± 0.69
	♂	41	14.68 ± 1.44	50	8.00 ± 0.71	49	20.10 ± 1.34	41	9.07 ± 1.01	181	13.03 ± 0.68
	♀	25	19.16 ± 3.16	-	-	-	-	12	14.50 ± 2.19	37	17.65 ± 2.26
	Exposed	50	12.22 ± 0.93	63	9.59 ± 0.87	50	20.16 ± 1.39	76	10.22 ± 0.81	239	12.55 ± 0.55
	♂	30	12.33 ± 1.20	63	9.59 ± 0.87	50	20.16 ± 1.39	50	10.64 ± 1.04	193	13.03 ± 0.64
	♀	20	12.05 ± 1.51	-	-	-	-	26	9.42 ± 1.27	46	10.57 ± 0.98
BCMN	Control	56	0.87 ± 0.10	45	1.54 ± 0.26	48	0.96 ± 0.18	64	0.99 ± 0.25	213	1.06 ± 0.10
	♂	34	0.78 ± 0.11	45	1.54 ± 0.26	48	0.96 ± 0.18	52	1.12 ± 0.31	179	1.12 ± 0.12
	♀	22	1.00 ± 0.17	-	-	-	-	12	0.41 ± 0.15	34	0.79 ± 0.13
	Exposed	47	0.72 ± 0.12	58	1.78 ± 0.27	49	0.79 ± 0.11	74	0.81 ± 0.12	228	1.03 ± 0.09
	♂	28	0.69 ± 0.16	58	1.78 ± 0.27	49	0.79 ± 0.11	55	0.88 ± 0.14	190	1.10 ± 0.10
	♀	19	0.76 ± 0.16	-	-	-	-	19	0.60 ± 0.16	38	0.68 ± 0.11
MNBC	Control	56	1.00 ± 0.12	45	1.45 ± 0.25	48	1.05 ± 0.20	64	1.18 ± 0.33	213	1.18 ± 0.12
	♂	34	0.86 ± 0.14	45	1.45 ± 0.25	48	1.05 ± 0.20	52	1.33 ± 0.40	179	1.22 ± 0.14
	♀	22	1.20 ± 0.21	-	-	-	-	12	0.50 ± 0.19	34	0.95 ± 0.16
	Exposed	47	0.77 ± 0.12	58	1.89 ± 0.30	49	0.94 ± 0.17	74	0.88 ± 0.14	228	1.12 ± 0.10
	♂	28	0.75 ± 0.17	58	1.89 ± 0.30	49	0.94 ± 0.17	55	0.95 ± 0.18	190	1.20 ± 0.12
	♀	19	0.81±0.18	-	-	-	-	19	0.65 ± 0.17	38	0.73 ± 0.12
CBPI	Control	66	1.88 ± 0.02	50	1.82 ± 0.02	49	1.62 ± 0.03	53	1.51 ± 0.02	218	1.72 ± 0.01
	♂	41	1.92 ± 0.02	50	1.82 ± 0.02	49	1.62 ± 0.03	41	1.52 ± 0.03	181	1.72 ± 0.01
	♀	25	1.83 ± 0.02	-	-	-	-	12	1.48 ± 0.03	37	1.72 ± 0.03
	Exposed	50	1.76 ± 0.02	63	1.86 ± 0.02	50	1.57 ± 0.02	76	1.32 ± 0.01	239	1.61 ± 0.01
	♂	30	1.75 ± 0.03	63	1.86 ± 0.02	50	1.57 ± 0.02	50	1.27 ± 0.02	193	1.61 ± 0.02
	♀	20	1.78 ± 0.03	-	-	-	-	26	1.41 ± 0.02	46	1.57 ± 0.03

* A total of 500 cells per donor were scored for CBPI.

BNMN, binucleated lymphocytes with micronuclei; MNL, total number of micronuclei in lymphocytes; BCMN, buccal cells with micronuclei; MNBC, micronuclei in buccal cells; CBPI, cytokinesis block proliferation index.

Table IV. Results for BNMN and CBPI in the final GLZ models

	BNMN Significance	CBPI Significance
Exposure	0.225	< 0.0001
Age	< 0.0001	0.0001
CS	< 0.0001	< 0.0001
CS*Exposure	0.018	< 0.0001
CS*Age	0.015	-
Models	$R^2 = 0.299$	$R^2 = 0.610$
	$P < 0.0001$	$P < 0.0001$

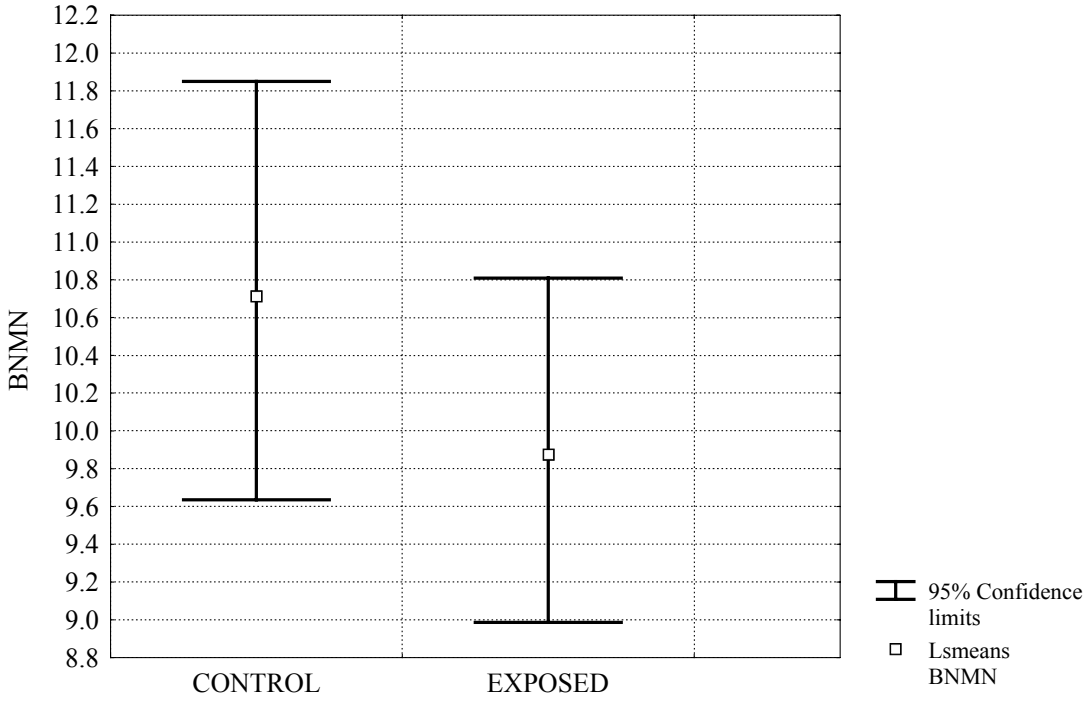
$N = 454$
CS, country-sex.

Table V. Binomial negative regression results for the buccal cells with micronuclei (BCMN)

		BCMN		
		DF	Significance	Value/DF
Exposure		1	0.717	
Alcohol		1	0.856	
CS		5	< 0.0001	
CS*Exposure		5	0.755	
Model	Deviance	426		0.949

$N = 439$
DF, degree of freedom; CS, country-sex.

Fig. 1.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12.0
11.8
11.6
11.4
11.2
11.0
10.8
10.6
10.4
10.2
10.0
9.8
9.6
9.4
9.2
9.0
8.8
BNMN
CONTROL
EXPOSED
95% Confidence limits
Lsmeans BNMN

Fig. 2.

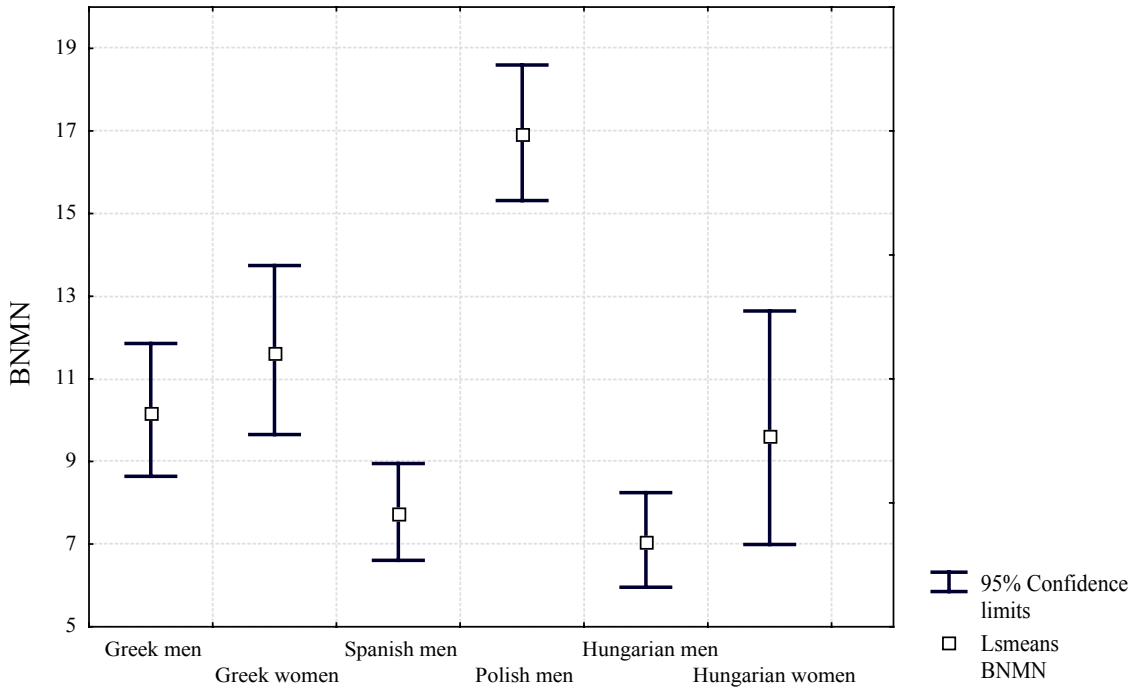
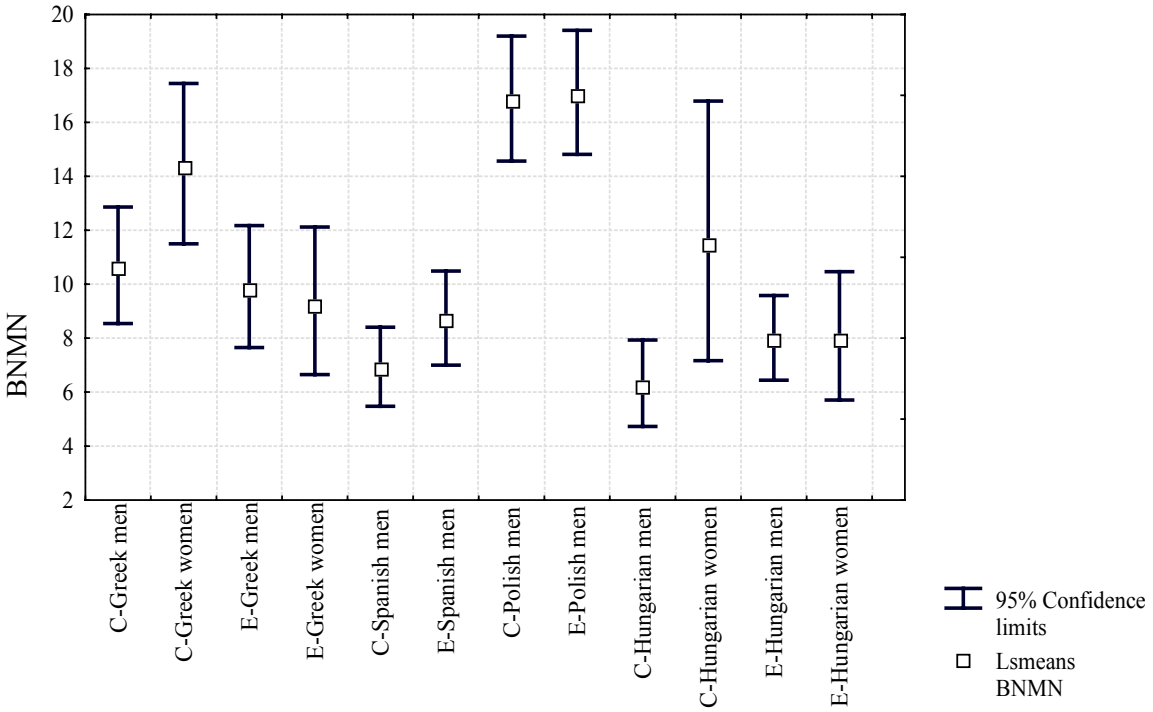

19
17
15
13
11
9
7
5
BNMN
Greek men
Greek women
Spanish men
Polish men
Hungarian men
Hungarian women
95% Confidence limits
Lsmeans BNMN

Fig. 3.



C, controls; E, exposed to pesticides

Fig. 4.

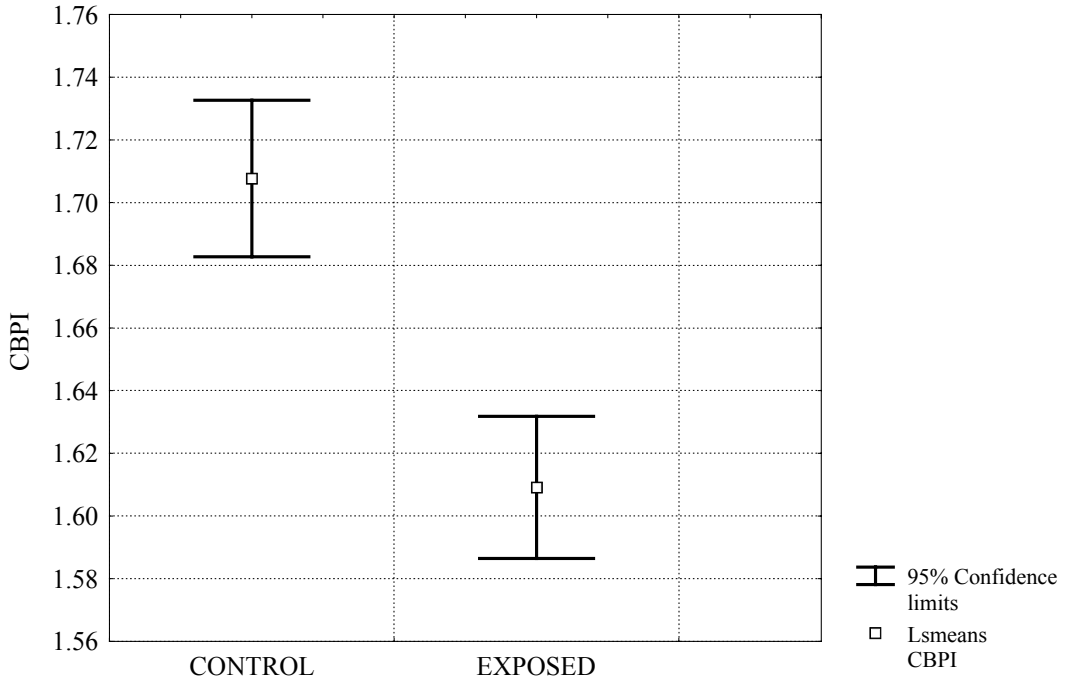


Fig. 5.

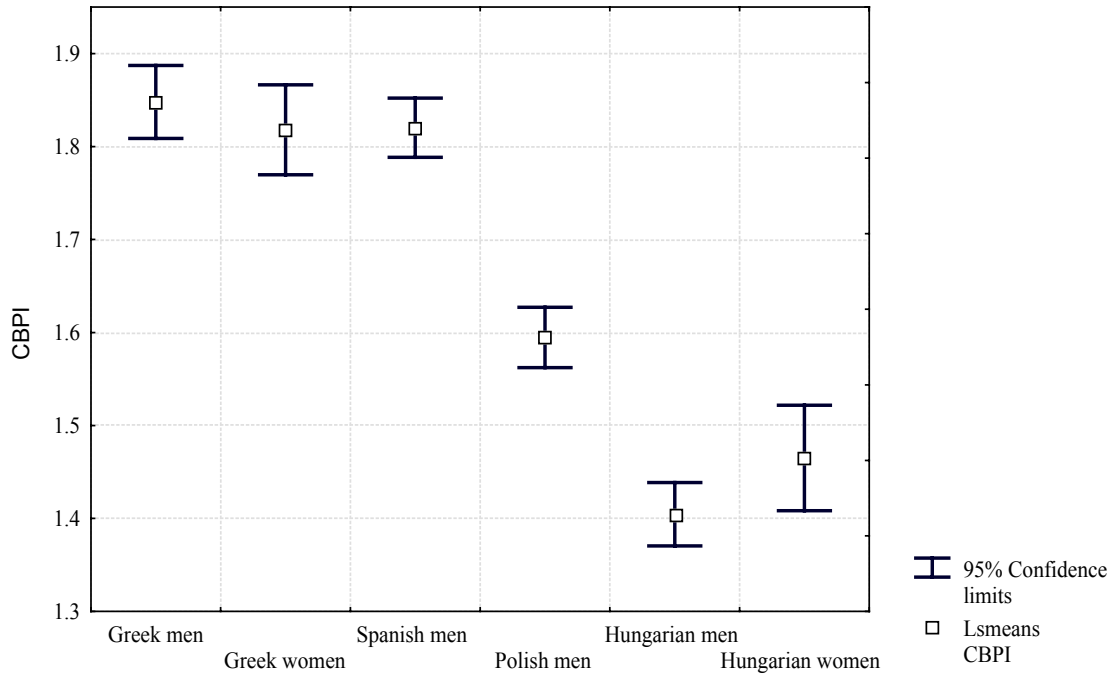
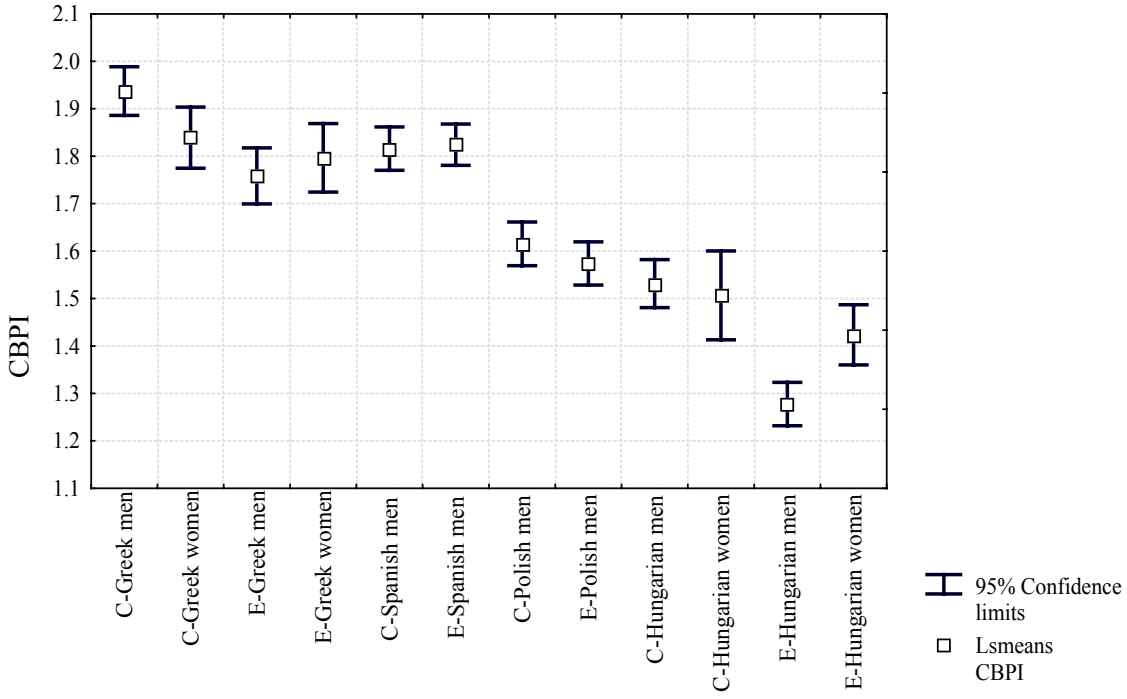


Fig. 6.



C, control; E, exposed

Fig. 7.

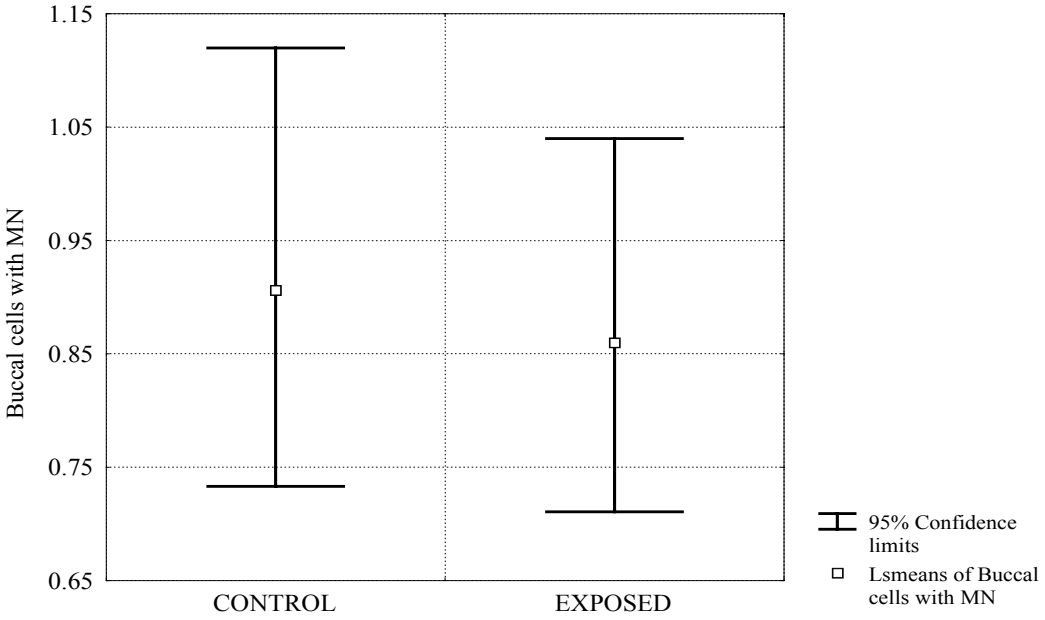

1.15
1.05
0.95
0.85
0.75
0.65
Buccal cells with MN
CONTROL
EXPOSED
95% Confidence limits
Lsmeans of Buccal cells with MN

Fig. 8.

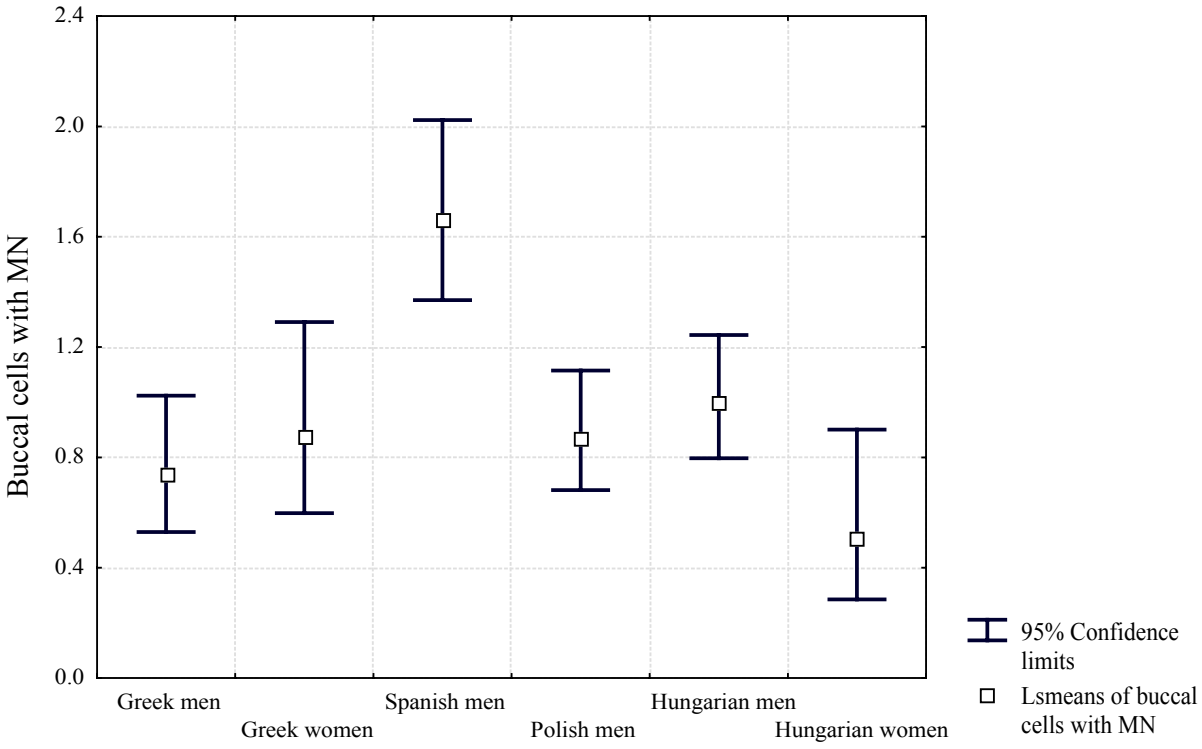
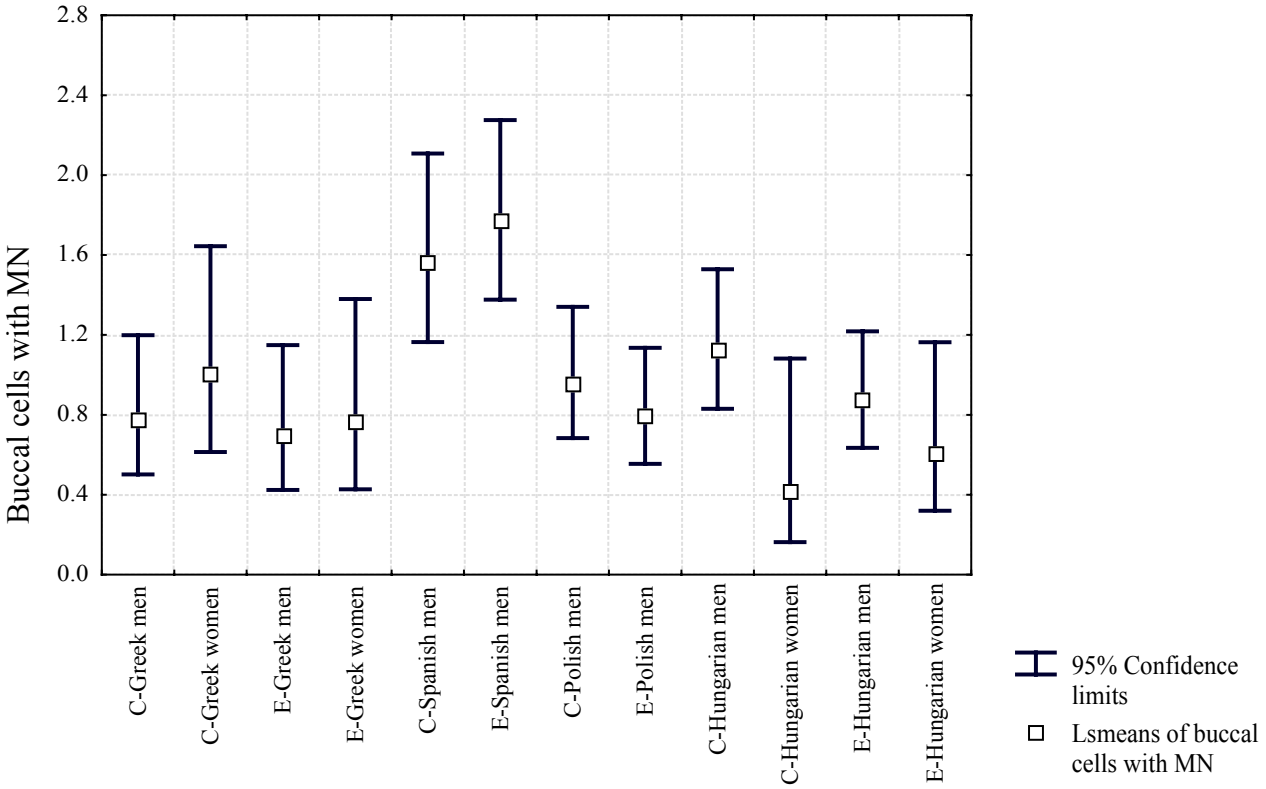

2.4
2.0
1.6
1.2
0.8
0.4
0.0
Buccal cells with MN
Greek men
Greek women
Spanish men
Polish men
Hungarian men
Hungarian women
95% Confidence limits
Lsmeans of buccal cells with MN

Fig. 9.


Buccal cells with MN
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
C-Greek men
C-Greek women
E-Greek men
E-Greek women
C-Spanish men
E-Spanish men
C-Polish men
E-Polish men
C-Hungarian men
C-Hungarian women
E-Hungarian men
E-Hungarian women
95% Confidence limits
Lsmeans of buccal cells with MN

Artículo 6

A follow-up study on micronucleus frequency in Spanish agricultural workers exposed to pesticides

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Mutagenesis, 17 (2002) 79-82