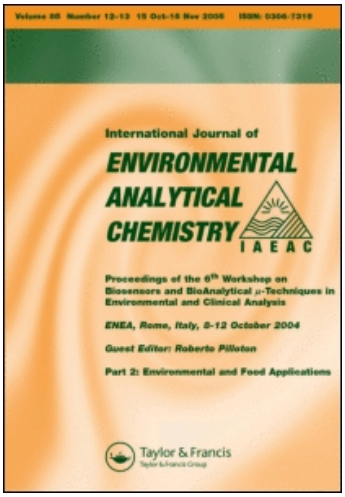

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Pesticide exposure and genotoxicity correlations within a Greek farmers' group

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Humans are exposed to pesticides as a consequence of their use in fields or persistence in a variety of media, including air, water, soil, plants, and animals. The use of pesticides, to which humans are exposed, will possibly increase in the near future. This exposure has been related to several human diseases, including cancer. In the present study, we investigated whether occupational exposure to a complex mixture of pesticides is resulting in increased micronuclei (MN) frequency in peripheral blood lymphocytes. Exposed and control subjects were selected in southern Crete, where intensive use of pesticides is observed. Statistically significant differences in micronuclei frequencies in the studied groups were revealed from the obtained results. Comparison of MN frequencies on control and exposed subjects between smokers and non-smokers did not reveal any statistically significant differences. Further studies in other areas in Greece, enlarging the sample size and covering other farmer groups with different farming activities and levels of exposure, are needed to generalize the findings of this study.

Keywords: Pesticides; Micronucleus assay; Human lymphocytes; Biomonitoring

1. Introduction

Pesticides constitute a heterogeneous class of chemicals representing an important group of environmental pollutants. To fight various pathogens and improve crop production, farmers use them extensively all over the world. Because of increased demand in food production, in recent years their use has dramatically increased. In Europe more than 2 million tonnes are released into the environment, yearly [1]. This widespread use of pesticides, the long and persistent exposure of farmers to them, and their accumulation in fields make the carcinogenic and mutagenic risk assessment a public health concern.

Occupational exposure to pesticides occurs via inhalation or skin contact during the preparation of the solutions to be sprayed or during spraying. The *in vivo*

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potential exposure to these chemicals is very difficult to estimate as human exposure does not occur under controlled conditions.

Crete is the largest island in Greece (8331.23 km^2), with a population of 536,980 inhabitants (1991 Census). The population, being predominantly rural or semi-rural, is mainly involved with farming, in conjunction with business and tourism. Vineyards and olive trees are the main farming activities of inhabitants in the Messara area, southern Crete. One should take into account that the majority of farmers usually, in parallel, keep small fields with other crops. These activities together require different kinds of pesticides, so farmers are widely exposed to different mixtures of pesticides. The main concern in this particular area is farmers' health because of the potential side-effects, especially genotoxicity, from the extensive use of pesticides.

It is commonly accepted that pesticides are the most important method of self-poisoning in the developing world. Three million cases of pesticide poisoning, nearly 220,000 fatal, occur worldwide every year [2]. Acute poisoning with carbamates and organophosphates had been previously reported for Crete [3–5].

While data on the acute toxicity of many of these chemicals are plentiful, knowledge on their delayed effects is limited. The International Agency for Cancer Research (IARC) has reviewed the potential carcinogenicity of a wide range of insecticides, fungicides, herbicides and other similar compounds, of which 50–60 pesticides have been classified as carcinogenic to laboratory animals. Associations with cancer have been reported in human studies for several chemicals such as phenoxy acid herbicides, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), lindane, methoxychlor, toxaphene, and several organophosphates [6].

A great number of tests have been developed to evaluate genetic damage. The micronucleus test in human peripheral blood lymphocytes, first reported by Fenech and Morley [7], has been widely applied in studies of genetic toxicology. Micronuclei (MN) contain acentric chromosome fragments or whole chromosomes, and they are recognized as distinct formations that exist in daughter cells separated from the main nucleus [8, 9]. They are the result of chromosome breakage and/or chromosome loss due to abnormal chromosome distribution during mitosis. The main characteristic of the test is the use of cytochalasin-B, an inhibitor of actin polymerization, which prevents cytokinesis while permitting nuclear division [7, 10]. As a result, binucleated (BN) cells are produced. These cells are scored for the presence of MN. The sensitivity and reliability of the cytokinesis block micronucleus (CBMN) assay in human lymphocytes is an effective tool to measure cytogenetic damage by pesticides in several populations [11–14].

The aim of the present study was to evaluate potent cytogenetic damage in farmers occupationally exposed to different mixtures of pesticides. This evaluation was performed by the use of CBMN assay in peripheral blood lymphocytes *in vivo*.

2. Experimental

2.1 *Subjects investigated*

The study was carried out on a group of 11 farmers, occupationally exposed to different mixtures of pesticides, from Messara, a well-developed agricultural region in the south of Crete. Intensive cultivation of vineyards and olive trees is the main activity

Table 1. Characteristics of the studied groups.

	Control	Exposed		
No. subjects	11	11		
Age (years)[a]	42.18 ± 4.21	46.45 ± 3.38	$F = 0.62$; $^*p = 0.44$	
Range	23–67	25–60		
Years of exposure	–	26.45 ± 3.38		
	Smoking habit			
	Non-smokers	Smokers	Non-smokers	Smokers	
Cigarettes/day[a]	6	5 31.40 ± 6.48	6	5 24.00 ± 2.45	$F = 1.14$; $^*p = 0.31$

[a]Mean ± SE. $^*p > 0.05$.

in the area. Farmers' average age was 46.45 ± 3.38 years. They were using and applying pesticides over a period of approximately 25 years. Our control group consisted of 11 healthy donors from the same area, without any indication of previous occupational exposure to pesticides or any other agents suspicious of being genotoxic. Their average age was 42.18 ± 4.21 years. Particular characteristics of our studied groups (control and exposed) are depicted in table 1. Blood specimens were collected in January 2003.

All participants were aware of the aims of the present study. The study was conducted according to the Ioannina University Ethics Committee. All were asked to complete a standardized questionnaire regarding their health condition, use of particular pesticides, or persistent medication. Finally, they were asked to sign an informed consent form according to Ethics Committee. All subjects were divided into smokers and non-smokers, while their main distinction was that they varied according to the use or not of pesticides. The particular pesticides to which the farmers were exposed are listed in table 2.

The farmers studied were spraying from 6–100 acres two to 15 times per year with the below-mentioned pesticides. Half, either smokers or non-smokers, used to take precautions by wearing protective clothing (cloths, masks, and gloves), while the other half did not. Details on the quantities of pesticides used were not given.

2.2 *Lymphocyte cultures – MN analysis*

Blood samples were obtained under sterile conditions in heparinized tubes. Whole blood (0.5 mL) from each donor was added to 6.5 mL of Ham's F10 medium, 1.5 mL of foetal calf serum, and 0.3 mL of phytohaemagglutinin (all media were supplied by Gibco, NY). Cultures were incubated at 37°C in a humidified atmosphere for 72 h. Forty-four hours after initiating cultures, 6 μg mL^{-1} of cytochalasin-B (Sigma) was added to culture medium to block cell division. Cells were collected by centrifugation 72 h post-culture initiation. A mild hypotonic treatment was given at room temperature, followed by fixation with freshly made methanol/acetic acid mixture. Cells were spread onto microscope slides and stained with Giemsa.

To calculate the MN frequency, at least 1000 BN cells were scored for each donor. For scoring MN, standard criteria were used [8, 9]. To determine possible

Table 2. Pesticides used (+) by each farmer of our study group.

	Farmers										
Pesticides	1	2	3	4	5	6	7	8	9	10	11
Abamectin					+						
Cypermethrin			+				+			+	+
Deltamethrin					+						
Dimethoate		+		+							
Fenthion		+		+							
Methamidophos					+						
Methidathion	+			+							
Parathion	+	+	+	+	+	+	+	+	+	+	+
Benomyl					+						
Bordeaux mixture						+		+			
Copper sulphate									+		
Fenbuconazole					+						
Dinocap					+						
Metalaxyl								+	+		
Penconazole	+			+							
Glyphosate		+		+		+					
Paraquat	+		+	+			+			+	+

cytotoxic effects, 2000 cells were counted for the calculation of the Cytokinesis Block Proliferation Index (CBPI), which is given by the equation: $CBPI = M_1 + 2M_2 + 3(M_3 + M_4)/N$, where M_1, M_2, M_3 and M_4 correspond to the numbers of cells with one, two, three, and four nuclei, and N is the total number of cells [15]. Micronucleus size is expressed as the ratio of micronucleus diameter to the cell nucleus diameter. This ratio was determined as follows: small micronuclei $\leq 1/10$, medium micronuclei $1/9 < MN < 1/3$, large micronuclei $\approx 1/3$, of nuclear diameter [16].

Statistical analysis of MN data was performed by the one-way ANOVA test. The statistical software used for data analysis was the Origin 7.0 (OriginLab Corporation, Northampton, MA).

3. Results and discussion

In the Messara area of southern Crete, the main farming activity is vineyards and olive trees. Farmers are exposed all year round for various periods to different mixtures of pesticides. We aimed to study the possible genotoxic effects on farmers exposed to a complex mixture of pesticides.

Multiple exposures are the rule and not the exception in farming practice: pesticide applicators spray large amounts of agrochemical mixtures, including a significant number of genotoxic compounds. Cytogenetic studies on pesticide sprayers cover a broad range of population employed in the cultivation of different crops and in different applications: grapes, vegetables, cotton, flowers, and tomatoes grown in greenhouses or in the open field. The most commonly used pesticides were chlororganics and, more recently, carbamates, organophosphates, and pyrethroids, which have been reported to have genotoxic effects in experimental studies in bacterial and mammalian systems [17].

Table 3. Frequency of use (%) and classification of pesticides used by Messara area farmers.

Type	Product	Group	Pesticide use	Frequency (%)	US EPA classification (T)	US EPA classification (C)	Md	Cd
Insecticides	Abamectin	Antibiotic	GUP	9.1	IV	E	–	–
	Cypermethrin	Pyrethroid	RUP	36.4	II–III	C	–	Inc
	Deltamethrin	Pyrethroid	NA	9.1	II	NA	–	–
	Dimethoate	Organophosphorus	GUP	18.2	II	C	−/+	Inc
	Fenthion	Organophosphorus	RUP	18.2	II	E	−/+	−/+
	Methamidophos	Organophosphorus	RUP	9.1	I	E	−/+	–
	Methidathion	Organophosphorus	RUP	18.2	I	C	−/+	−/+
	Parathion	Organophosphorus	RUP	100.0	NA	C	−/+	–
Fungicides	Benomyl	Benzimidazole	GUP	9.1	IV	C	−/+	−/+
	Bordeaux mixt.	Copper fungicides	GUP	18.2	I	NA	NA	NA
	Copper sulphate	Copper fungicide	GUP	9.1	I	NA	−/+	Inc
	Fenbuconazole	Conazole fungicide	NA	9.1	NA	C	–	−/+
	Dinocap	Dinitrophenol	GUP	9.1	III	E	−/+	–
	Metalaxyl	Benzeoid	GUP	18.2	III	E	−/+	Inc
	Penconazole	Triazole	NA	18.2	III	NA	–	–
Herbicides	Glyphosate	Organophosphorus	GUP	27.3	II	E	–	–
	Paraquat	Bypiridylic	RUP	54.5	I	E	+	Inc

Freq: % frequency of use; (T): toxicity classification; I: highly toxic; II: moderately toxic; III: slightly toxic; IV: practically non-toxic; (C): carcinogenicity classification: C: possible human carcinogen; E: probably not carcinogenic; RUP: restricted use; GUP: general use; Md: mutagenicity data; Cd: carcinogenicity data; NA: not available; Inc: inconclusive; −: negative effects; +: positive effects.

Our study group was mainly exposed to organophosphates and pyrethroids, which are nowadays commonly used pesticides. The same farmers also used chemicals such as copper and conazole fungicides, abamectin, benomyl, dinocap, metalaxyl, penconazole, and paraquat. Table 2 lists the pesticides used by each farmer, while table 3 lists the frequency of use of the main pesticides used in this region, their classification according to the Environmental Protection Agency (EPA), as well as the available experimental data [18–20].

Blood samples taken from healthy adults and healthy farmers were analysed for micronuclei induction and the calculation of the Cytokinesis Block Proliferation Index (CBPI) under standard criteria [8, 9] in lymphocyte cultures. Table 4 lists data for BNMN cells and MN per 1000 BN cells for each donor. Data analysis of control subjects did not indicate statistically significant differences in the MN induction ($F = 0.00$, $p = 0.99$) between smokers (6.20 ± 1.66) and non-smokers (6.20 ± 1.01) as well as in the BNMN induction ($F = 0.00$, $p = 0.99$) between smokers (6.20 ± 1.66) and non-smokers (6.20 ± 1.01). Possible variations in the proliferative kinetics of lymphocytes were evaluated by calculating the CBPI. Analysis of the observed CBPI between smokers (1.64 ± 0.04) and non-smokers (1.63 ± 0.02) in control subjects revealed no statistically significant differences ($F = 0.04$, $p = 0.85$). The present data are in accordance with those reported recently by the HUMN collaborative group studying the smoking effect on spontaneous micronuclei frequencies in human lymphocytes [21].

Statistical analysis of farmers' data revealed no significant differences in the induction of MN ($F = 0.02$, $p = 0.89$) between smokers (8.60 ± 1.50) and non-smokers (8.83 ± 0.87), as well as of BNMN ($F = 0.42$, $p = 0.53$) between smokers (7.40 ± 1.56) and non-smokers (8.50 ± 0.85). Analysis of the observed CBPI between smokers

Table 4. Frequencies of micronucleated binucleated cells (BNMN) and micronuclei (MN) as revealed after Giemsa staining, in control and exposed subjects.[a]

		BNMN		MN		CBPI	
	Donors	Control	Exposed	Control	Exposed	Control	Exposed
A	1st	7	7	7	7	1.73	2.17
	2nd	3	8	3	9	1.66	2.03
	3rd	3	13	3	14	1.60	2.32
	4th	6	5	6	8	1.72	2.26
	5th	12	4	12	5	1.50	2.16
Total – A		31	37	31	43	–	–
MF (‰) ± SE		6.20 ± 1.66	7.40 ± 1.56	6.20 ± 1.66	8.60 ± 1.50	1.64 ± 0.04	2.19 ± 0.05
B	6th	3	7	3	7	1.62	2.13
	7th	7	12	7	12	1.58	2.28
	8th	4	10	4	11	1.72	2.19
	9th	10	8	10	8	1.65	2.19
	10th	7	7	7	8	1.65	2.01
	11th	6	7	6	7	1.58	2.01
Total – B		37	51	37	53	–	–
MF (‰) ± SE		6.20 ± 1.01	8.50 ± 0.85	6.20 ± 1.01	8.83 ± 0.87	1.63 ± 0.02	2.14 ± 0.04
Total – A + B		68*	88*	68*	96*	–	–
MF (‰) ± SE		6.20 ± 0.88	8.00 ± 0.82	6.20 ± 0.88	8.73 ± 0.79	1.64 ± 0.02	2.16 ± 0.03

[a]A: smokers; B: non-smokers; BNMN: micronucleated binucleated cells; MN: micronuclei; CBPI: Cytokinesis Block Proliferation Index; MF (‰) ± SE: mean frequencies (‰) ± standard error; *11,000 binucleated cells (BN) cells were counted in total for each studied group.

(2.19 ± 0.05) and non-smokers (2.14 ± 0.03) in exposed subjects revealed no statistically significant differences ($F = 0.64$, $p = 0.44$). The effect of smoking on MN frequency remains a controversial issue. The present results are in accordance with earlier studies that reported no significant correlation between MN frequency and smoking habit [14, 22, 23].

Our observation of decreased lymphocyte CBPI in control subjects (1.64 ± 0.02) compared with farmers (2.16 ± 0.03) could be the result of individual responses to culture conditions which could correlate with a difference of lymphocyte proliferation in our studied groups.

Figure 1 presents data on the size ratio of MN. To distinguish between clastogenic and aneugenic activity due to exposure to the particular pesticides, we compared MN sizes calculated. Small MN have a greater possibility of containing acentric chromosome fragments indicating a clastogenic effect, while large MN may possibly contain whole chromosomes, indicating an aneugenic effect [24–26].

No statistically significant differences on the size ratio of small MN ($F = 0.26$, $p = 0.62$), medium MN ($F = 0.31$, $p = 0.59$), and large MN ($F = 0.02$, $p = 0.88$) were detected in control smokers and non-smokers. Similarly, in farmers, the observed differences on the size ratio of small MN ($F = 0.11$, $p = 0.75$), medium MN ($F = 0.02$, $p = 0.90$) and large MN ($F = 0.98$, $p = 0.34$) were not statistically significant between smokers and non-smokers.

However, the total mean frequencies between controls (2.63 ± 0.41) and farmers (5.00 ± 0.52) reveal statistically significant differences on the size ratio of small MN ($F = 12.66$, $p = 0.002$), which indicates a possible clastogenic effect associated with pesticide exposure in the studied farmers.

The clastogenic effect appears to be cumulative for continuous exposure to pesticide mixtures. People chronically exposed are more susceptible to the clastogenic action

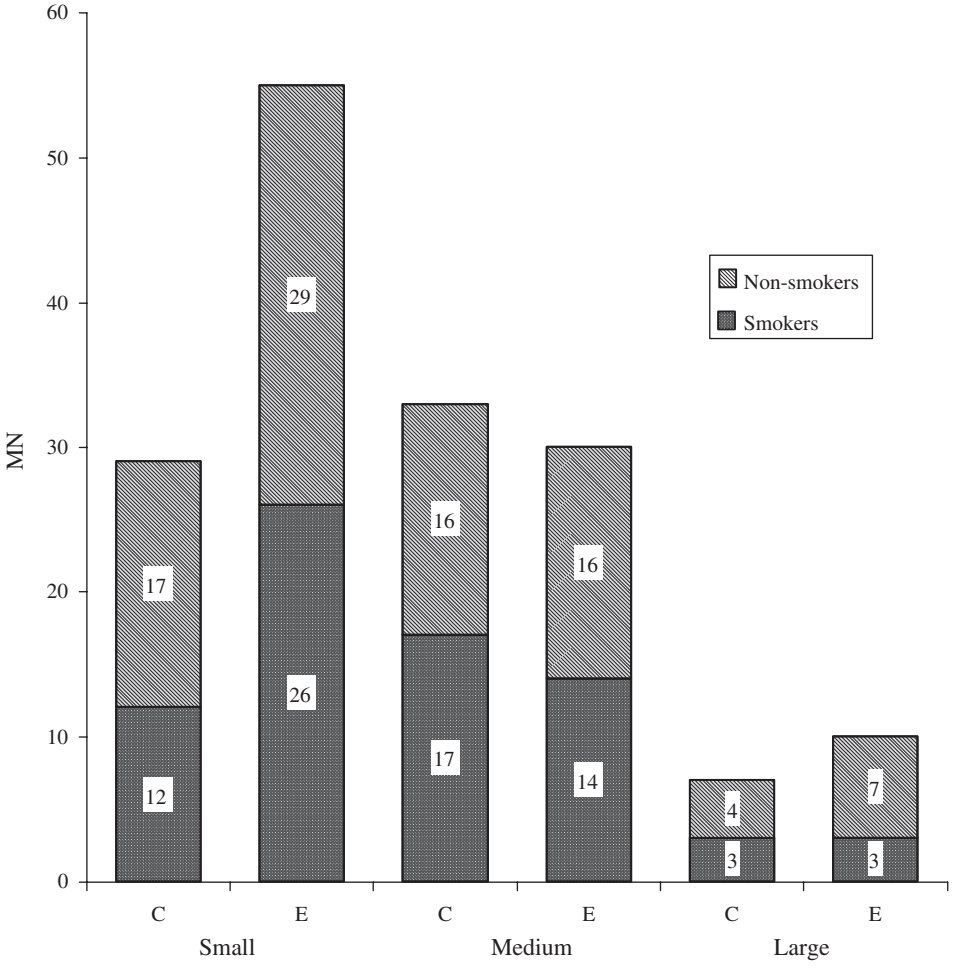

Figure 1. Frequencies of micronuclei (MN) per size, as revealed after Giemsa staining, in control (C) and exposed (E) subjects.

of pesticides. Increased chromosomal damage, measured as chromosome aberrations or MN frequency, associated with years of employment has been demonstrated in populations of farmers as a result of continuous exposure to a complex mixture of pesticides [17]. A comparison of the total mean frequencies between controls and farmers reveals that there are no statistically significant differences on the size ratio in medium-sized ($F = 0.11$, $p = 0.74$) and large ($F = 0.61$, $p = 0.44$) micronuclei, indicates an absence of aneugenic activity associated with pesticide exposure.

Comparing the main frequencies of the size ratio in small, medium, and large MN for smokers and non-smokers between controls and farmers, similar results were obtained. Statistically significant differences were observed on the size ratio in small MN, between control and exposed smokers ($F = 6.03$, $p = 0.04$) as well as non-smokers ($F = 5.54$, $p = 0.04$). However, non-statistically significant differences were observed on the size ratio in medium and large MN, between control and exposed smokers ($F = 0.21$, $p = 0.66$) as well as non-smokers ($F = 0.00$, $p = 1.00$). In addition, a statistical

comparison between control and exposed smokers ($F=0.00$, $p=1.00$) as well as between control and exposed non-smokers ($F=0.92$, $p=0.36$) revealed no significant differences.

In a mammography screening programme among the female population in Crete, it was revealed that women exposed to greenhouse pesticides developed a significantly higher risk for fibroadenoma, ductal hyperplasia, sclerotic adenosis, fibrohyperplastic disease, cystic disease, and inflammatory mastitis than non-exposed women. In addition, no significant differences were detected in the rate of fibrocystic changes, lipoma, and malignant tumors. In the mean time, risk markers for subsequent invasive breast-cancer development showed a higher incidence in exposed women [27]. However, it should be mentioned that the polymorphic TTTA tetranucleotide observed in the fourth intron in the CYP19 gene, an important enzyme of oestrogen biosynthesis associated with breast-cancer susceptibility [28], was not found to vary significantly among exposed and non-exposed women from the same island [29].

Table 5 lists the overall statistical analysis results for our studied groups. An analysis was carried out between control and exposed smokers as well as non-smokers and total control and exposed subjects. The analysis revealed that among smokers, there are no statistically significant differences in the BNMN induction ($F=0.28$, $p=0.61$) or in the MN induction ($F=1.15$, $p=0.31$). In the non-smoking group, the analysis did not reveal any statistically significant differences in the BNMN induction ($F=3.12$, $p=0.11$) or in the MN induction ($F=3.98$, $p=0.07$). Statistically significant differences in the induction of MN ($F=4.63$, $p=0.04$) but not of BNMN ($F=2.28$, $p=0.15$) were observed after analysis in the overall groups.

Studies on micronucleus frequencies in Italian, Yugoslavian, and Greek farmers, occupationally exposed to complex mixtures of pesticides, have reported significant differences in MN frequency between control and exposed farmers [14, 30, 31]. Our data accord with these reports. In addition, 15 out of 17 studies give positive results in the induction of chromosome aberrations (CA), sister chromatid exchanges (SCE), or MN frequency with a range of 1.12–15.8 increments. Our data revealed a 1.41-fold increment. Experimental evidence shows that occupational exposure to mixtures of pesticides has been associated with an increase in genotoxic damage [17].

Table 5. Comparison of micronucleated binucleated cells (BNMN) and micronuclei (MN) mean frequencies (‰) ± standard error, as revealed after Giemsa staining, in control and exposed human lymphocyte cultures from smokers and non-smokers.

	BNMN	MN
Control/smokers	6.20 ± 1.66	6.20 ± 1.66
Exposed/smokers	7.40 ± 1.56	8.60 ± 1.50
	$F=0.28$, $p=0.61$	$F=1.15$, $p=0.31$
Control/non-smokers	6.20 ± 1.01	6.20 ± 1.01
Exposed/non-smokers	8.50 ± 0.85	8.83 ± 0.87
	$F=3.12$, $p=0.11$	$F=3.98$, $p=0.07$
Total-control	6.20 ± 0.88	6.20 ± 0.88
Total-exposed	8.00 ± 0.82	8.73 ± 0.79
	$F=2.28$, $p=0.15$	$F=4.63$, $p=0.04$[a]

[a] $p<0.05$.

4. Conclusions

The main results of our study can be summarized as follows:

- Statistically significant differences in MN frequencies among total control subjects and exposed farmers were observed.
- The MN size ratio indicates a possible clastogenic effect as a result of pesticide exposure.
- Smoking appears not to induce higher MN rates in either exposed or control subjects.

Taking into account that Crete is the largest island in Greece with particular farming activities, it is not easy to draw final lines of evidence on the overall situation among farmers. Further studies in other areas in Greece covering groups with other farming activities and levels of exposure are needed to generalize the findings of the present study.

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