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Is organic farming safer to farmers' health? A comparison between organic and traditional farming

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HIGHLIGHTS

- There are no previous studies on the impact of organic farming on workers' health.
- Genetic damage and immunological alterations of workers were studied.
- Results confirm increased DNA damage levels in farmers exposed to pesticides.

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ABSTRACT

Exposure to pesticides is a major public health concern, because of the widespread distribution of these compounds and their possible long term effects. Recently, organic farming has been introduced as a consumer and environmental friendly agricultural system, although little is known about the effects on workers' health. The aim of this work was to evaluate genetic damage and immunological alterations in workers of both traditional and organic farming. Eighty-five farmers exposed to several pesticides, thirty-six organic farmers and sixty-one controls took part in the study. Biomarkers of exposure (pyrethroids, organophosphates, carbamates, and thioethers in urine and butyrylcholinesterase activity in plasma), early effect (micronuclei in lymphocytes and reticulocytes, T-cell receptor mutation assay, chromosomal aberrations, comet assay and lymphocytes subpopulations) and susceptibility (genetic polymorphisms related to metabolism - *EPHX1*, *GSTM1*, *GSTT1* and *GSTP1* - and DNA repair–*XRCC1* and *XRCC2*) were evaluated. When compared to controls and organic farmers, pesticide farmers presented a significant increase of micronuclei in lymphocytes (frequency ratio, FR = 2.80) and reticulocytes (FR = 1.89), chromosomal aberrations (FR = 2.19), DNA damage assessed by comet assay (mean ratio, MR = 1.71), and a significant decrease in the proportion of B lymphocytes (MR = 0.88). Results were not consistent for organic farmers when compared to controls, with a 48% increase of micronuclei in lumphocytes frequency ($p = 0.016$) contrasted by the significant decreases of TCR-Mf ($p = 0.001$) and %*T* ($p = 0.001$). Our data confirm the increased presence of DNA damage in farmers exposed to pesticides, and show as exposure conditions may influence observed effects. These results must be interpreted with caution due to the small size of the sample and the unbalanced distribution of individuals in the three study groups.

1. Introduction

The harmful properties of pesticides have been described for the last decades not only in the environment (Werf, 1996) but also on human health (WHO, 1990). Genotoxicity studies conducted

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in pesticide-exposed populations have found inconsistent results (Bolognesi, 2003; Bull et al., 2006), although the reason of this heterogeneity remains largely unknown. Some authors believe that this variation may be attributable to exposure factors, while others suggest that pesticide exposure can induce an adaptive response, which can modulate adverse effects (Pastor et al., 2002). In addition, population sample size, inadequate experimental data and individual susceptibility greatly contribute to discordant results (Au et al., 1998). Regarding the possible effects of pesticide exposure on the human immune system, there are some studies providing evidence that these compounds, although not antigenic themselves, may alter immune functions (Corsini et al., 2008). Data available in the literature is mainly related to immunosuppression with reports on decrease of %$CD26^+$, %$CD4^+$, neutrophil function, decrease of antibody production by B lymphocytes and decrease of natural killer cells activity among other alterations in pesticide-exposed populations (Colosio et al., 1999; Corsini et al., 2008; Li, 2007).

Although many hazardous pesticides have been recently withdrawn from the European market, numerous compounds registered are still used, provoking serious and scientifically documented consequences for human health. In 2009, Pesticides Action Network (PAN) Europe identified several pesticides in use which are classified by different organizations as cancer-causing, toxic to the reproductive system, genotoxic or endocrine disrupting (PAN, 2009). Besides, in the last decades, organic farming became popular among people as there is a widespread belief that organic agricultural systems are friendlier to the environment and consumer than traditional farming systems. Nevertheless, studies that tried to establish a link between organic food consumption and consumers' health were mainly inconclusive as there are a large number of confounding factors that impair any inference (Dangour et al., 2009). Regarding workers' health, outcome from different agricultural systems is limited to a few observation studies of sperm quality (Jensen et al., 1996; Juhler et al., 1999) that also obtained conflicting results.

The objective of this work was to study genetic and immunological alterations in workers of two different types of agricultural systems (organic and traditional) using a multistage approach in order to integrate information obtained with biomarkers of exposure, effect and susceptibility. Biomarkers of exposure included determination of pesticides in urine, namely pyrethroids, organophosphates and carbamates, excretion of total thioethers in urine and enzymatic activity of plasma cholinesterase. Biomarkers of effect comprised the study of genetic damage with different assays: micronucleus (MN) evaluation (both in lymphocytes and reticulocytes), chromosomal aberrations (CA) test, DNA damage–evaluated by means of comet assay–, and also somatic mutation [T-cell receptor (TCR) mutation assay]. In addition, alterations in the immune system were also assessed using lymphocyte subsets analysis. The potential role of genetic polymorphisms in genes related with the metabolic fate of pesticides (*EPHX1*, *GSTM1*, *GSTT1* and *GSTP1*) and DNA damage repair (*XRCC1* and *XRCC2*) in modulating individual levels of biomarkers related to pesticide effects was also evaluated.

To our knowledge, this is the first study that compares genetic and immunological damage among workers that are involved in the traditional and the organic farming systems.

2. Materials and methods

2.1. Study population

The group of traditional agricultural system consisted of 85 farmers using pesticides (43 males and 42 females) from a main Portuguese agricultural (horticulture) area (Povoa de Varzim and Esposende; within Oporto district). Four months of pesticide exposure was considered the cut-off point for inclusion in exposed group. The group of organic agricultural system was composed of 36 organic farmers not using pesticides (17 males and 19 females) from the same geographical area (Oporto district) and also producing horticultural products. The control group comprised 61 acquaintance non-exposed individuals (26 males and 35 females), living in the same area and with no history of occupational exposure to pesticides or other genotoxic agents. All individuals were Caucasians. Characteristics of the studied groups are presented in Table 1. All subjects were fully informed about the procedures and objectives of this study and each of them signed an informed consent prior to the study. Ethical approval for this study was obtained from the institutional Ethical Board of the Portuguese National Institute of Health.

Table 1
Characteristics of study group.

	Study group		
	Unexposed controls ($n=61$)	Organic farmers ($n=36$)	Pesticide workers ($n=85$)
Age[a] (year)	39.5 ± 12.3 (19–61)	39.6 ± 14.5 (18–68)	40.0 ± 12.2 (18–63)
Gender			
Males	26 (42.6%)	17 (47.2%)	43 (50.6%)
Females	35 (57.4%)	19 (52.8%)	42 (49.4%)
Smoking habits			
Non-smokers	50 (82.0%)	31 (86.1%)	80 (94.1%)
Smokers	11 (18.0%)	5 (13.9%)	5 (5.9%)
Cigarettes/day			
<15	5 (45.5%)	1 (20.0%)	4 (80.0%)
≥15	6 (54.5%)	4 (80.0%)	1 (20.0%)
Task			
Non-applicator			30 (35.3%)
Applicator			55 (64.7%)
Workplace			
Open-field		14 (38.9%)	13 (15.3%)
Greenhouses			6 (7.1%)
Both		22 (61.1%)	66 (77.6%)
Duration of employment[a] (years)	9.5 ± 12.3	22.7 ± 16.2	
Pesticide preparation			
No			30 (35.3%)
Yes			55 (64.7%)
Chemical class of Pesticide (last reported exposure)[b]			
Pyrethroids			6 (7.1%)
Carbamates			20 (23.5%)
Organophosphates			17 (20.0%)
Other			32 (37.6%)
Use of PPE			
No			25 (29.4%)
Yes			60 (70.6%)
Inadequate usage of pesticides			
No			67 (78.8%)
Yes			18 (21.2%)
Season			
Autumn–winter	61 (100%)	26 (72.2%)	46 (54.1%)
Spring–summer	0	10 (27.8%)	39 (45.9%)

[a] Mean ± SD (range); PPE: personal protective equipment.
[b] Ten of the exposed individuals were not able to report the chemical concerning their last exposure.

Individuals included in organic farmers group were all certified organic farmers and therefore working in compliance with EU Regulation 834/2007 and 889/2008 for at least four months; these regulations describe all the necessary requirements for certified production and indicate sensitive issues such as chemicals usage (allowed under authority's control) and land requirements. In the face-to-face interview, all individuals stated that the usage of allowed substances was absolutely exceptional and therefore in this study, we consider organic farmers as not exposed to those products. Organic farmers had no previous history of occupational exposure to pesticides or other genotoxic agents.

In a face to face interview, each subject gave the necessary information on demographic features such as age, gender, smoking habits and also to determine possible additional confounding factors such as X-ray exposure, previous and current medication.

Exposed subjects also gave information concerning work tasks, years of employment, workplace, occurrence of previous intoxications (those resulting of pesticide exposure that required medical treatment) and details on their last exposure to

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Table 2
List of pesticides reported as used by exposed subjects and their classification regarding carcinogenicity (US EPA) and acute hazard (WHO).

Pesticide Compound	Chemical class	US EPA	WHO
Mancozeb	dithiocarbamate	B2	U
Azoxystrobin	Strobin	not likely	U
Folpet	Thiophthalimide	B2	U
propineb	dithiocarbamate		U
Cymoxanil	Unclassified	Inadq. data	III
Mefenoxam	Xylylalanine	not likely	
tolylfluanid	Sulfonamides	likely	U
Carbendazim	Benzimidazole	C	U
Propamocarb	Other Carbamate	not likely	U
Fluazinam	2,6-Dinitroaniline	suggestive	
Fenhexamid	Anilide	not likely	U
Chlorpyrifos	Organophosphate	E	II
Cyhalothrin, lambda	Pyrethroid	D	II
Dimethoate	Organophosphate	C	II
Methiocarb	N-Methyl Carbamate	D	IB
Diazinon	Organophosphate	not likely	II
Buprofezin	Unclassified	suggestive	U
Cypermethrin, alpha	Pyrethroid		II
Paraquat dichloride	Bipyridylium	E	II
Pirimicarb	carbamate	likely	II

WHO hazard classification: IB-Highly hazardous; II-Moderately hazardous; III-Slightly hazardous; U-Unlikely to pose an acute hazard in normal use; US EPA classification: B2-Probable human carcinogen; C-possible human carcinogen; D-not classifiable as to human carcinogenicity; E-evidence of non-carcinogenicity for humans.

pesticides (time since last exposure and chemical used). Not all the individuals included in the exposed group were involved in pesticide application, nor in pesticide preparation; nevertheless, all were exposed to these compounds either by preparing the mixtures for application, by applying the compounds themselves, by providing assistance during applications or during maintenance activities. All individuals included in the exposed group dealt regularly with a wide variety of chemicals and the majority of them were in contact with pesticides a few days before sample collection (the list of pesticide compounds reported by exposed group is presented in Table 2; based on their chemical structure and biological action, chemicals were categorized in four classes: pyrethroids, carbamates, organophosphates and other compounds).

2.2. Sample collection

Blood samples were obtained by venipuncture into different anticoagulant tubes according to the assays to perform. A spot urine sample (approximately 50 mL) was collected simultaneously. Both blood and urine samples were collected in the morning period and in the workplaces throughout one year. Since this may constitute a bias factor as pesticide applications are more frequent in spring and summer, this was taken into account in result analysis. Samples were transported to the laboratory within 3 h and processed immediately for the different assays. All samples were coded and analyzed under blind conditions. The same procedure was followed in the three studied groups.

2.3. Biomarkers of exposure

2.3.1. Urinary Pyrethroids (PYR) determination

For this immunoassay, a commercially available ELISA kit (PN 500201, Abraxis), primarily designed for the analysis of PYR in water, was modified for urinary PYR determinations.

Our primary modifications to the kit procedure were the preparation of standard curves in urine diluted with methanol (1:1) and then in kit diluent (1:10). Stored, frozen urine samples were thawed, vortexed, and an aliquot at the target dilution prepared. To each tube it was transferred 250 μL of diluted sample or standard. The samples and standards were then analyzed as per kit instructions. Concentrations obtained were corrected with the corresponding creatinine value.

Creatinine was determined using CREAJ Gen2 kit (PN 04810716190, Roche Diagnostics) on COBAS INTEGRA 800 according to manufacturer instructions.

2.3.2. Urinary Organophosphates (OP) and Carbamates (CRB) determination

In this determination a colorimetric kit commercially available for quantification of OP and CRB in water (PN 550055, Abraxis) was adapted in order to measure these compounds in urine. Briefly, an aliquot of urine was diluted 1:50 in water and then 1:1 in methanol (final dilution of 1:100). Diazinon standards prepared in diluted pooled urine ranging from 0.20 to 12.50 ng/mL were used to obtain the calibration curve (four parameter logistic curve). Analysis followed the instructions specified in kit insert and obtained OP/CRB concentrations were corrected with the corresponding creatinine value (determined as described above).

2.3.3. Urinary Thioethers (THIO) determination

The procedure was based on what was previously described by Vainio et al. (1978). Briefly, ascorbic acid was added to 10 mL of sample and an aliquot (2 mL) was then deproteinized ((H3PO4 2 M; EDTA 5 mM; NaCl 5 mM; 1:1:1). This mixture was centrifuged at 1000 rpm for 15 min and 1 mL of supernatant underwent alkaline hydrolysis (NaOH 5 M; 100 °C for 50 min). Samples were neutralized with HCl and thioethers were detected by reaction with DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)]. Sample absorbance was read at 412 nm using an UV/VIS spectrophotometer (UNICAM). Non-hydrolysed urine blanks were treated similarly to determine background absorbance. Mercaptoacetic acid was used to prepare the standard curve.

2.3.4. Plasma butyrylcholinesterase (BChE) activity (EC 3.1.1.8)

BD Vacutainer™ CPT™ cell preparation tubes (Becton Dickinson) with sodium heparin were centrifuged according to manufacturer' instructions. After centrifugation an aliquot of the upper layer (plasma) was used for immediate BChE determination with ChE Gen 2 kit (PN 04498577190, Roche Diagnostics) on COBAS INTEGRA 800 according to company insert. Enzymatic activity was obtained in U/L.

2.4. Biomarkers of effect

2.4.1. Micronuclei tests: Cytokinesis Block Micronuclei Assay (CBMN) and Micronuclei in reticulocytes (MN-RET)

CBMN was performed according to what was previously described by Teixeira et al. (2004). Microscopic analysis was carried out on a Nikon Eclipse E400 light microscope. For each subject, 1000 binucleated lymphocytes with well-preserved cytoplasm were scored blindly by the same reader and the total number of MN in binucleated cells (MNL) was considered for statistical analysis. MN were identified using a 500× magnification for detection and a 1250× magnification for confirmation following the criteria of Fenech (2000).

Determination of MN-RET was based on the method described by Dertinger et al. (2002)with modifications previously described in Costa et al. (2011). Flow cytometric analysis was performed on a Coulter Epics XL-MCL (Beckman Coulter, Inc.). Data obtained provided necessary information to determine MN-RET relative frequency for each sample.

2.4.2. T-cell receptor (TCR) mutation assay

TCR mutation assay was performed by a flow cytometric methodology following García-Lestón et al. (2011). Cell suspensions were analyzed by a FACScalibur flow cytometer with Cell Quest Pro software (Becton Dickinson). A minimum of 2.5×10^5 lymphocyte-gated events were acquired, and mutation frequencies of TCR (TCR-Mf) were calculated as the number of events in the mutant cell window ($CD3^-CD4^+$ cells) divided by the total number of events corresponding to $CD4^+$ cells.

2.4.3. Chromosomal aberrations

Slides for analysis of chromosomal aberrations were obtained following the protocol described by (Roma-Torres et al., 2006). Microscope analyses were performed on a Nikon Eclipse E400 light microscope and scored blind by the same reader. One hundred metaphases were analyzed for each individual, 50 from each duplicate culture, using a 1250× magnification for aneuploidies and different types of aberrations according to the criteria of Therman (1980).

Total CA frequency was defined as the number of aberrations, excluding gaps, per 100 cells. Chromosome-type aberrations (CSA) included chromosome-type breaks, ring chromosomes, and dicentrics, whereas chromatid type aberrations (CTA) included chromatid-type breaks.

2.4.4. Comet assay

The alkaline comet assay was performed essentially as described by Singh et al. (1988) with minor modifications. Each slide contained two replicates of each donor. The slides were coded and examined by a 'blind' scorer using a magnification of 400×. One hundred randomly selected cells (50 per replicate) were examined from each subject. Image capture and analysis were performed with Comet Assay IV software (Perceptive Instruments); percentage of tail DNA (%*T*) was the DNA damage parameter evaluated according to what has been recommended by Kumaravel et al. (2009).

2.4.5. Lymphocyte subpopulations

Lymphocyte subpopulations percentages were determined by means of a flow cytometry methodology as previously described by García-Lestón et al. (2012). At least 10^4 events in the lymphocytes window were acquired. The lymphocyte subsets determined were $CD3^+$ T-lymphocytes, $CD4^+$ T-helper lymphocytes, $CD8^+$ T-cytotoxic lymphocytes, $CD19^+$ B-lymphocytes, and $CD16^+56^+$ natural killer (NK) cells.

2.5. Biomarkers of susceptibility

2.5.1. Genotyping

Heparinized blood samples were stored at −20 °C until use. Genomic DNA was obtained from 200 μL of whole blood using a commercially available kit according to the manufacturer instructions (QIAamp DNA extraction kit, Qiagen). All genotype analyses were performed at least in duplicate.

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The *EPHX1* codons 113 and 139 polymorphisms were determined by polymerase chain reaction (PCR) and restriction fragment length polymorphisms (RFLP) according to the method of Salama et al. (2002), with minor modifications described in Teixeira et al. (2004).

GSTM1 and *GSTT1* genotyping for gene deletions were carried out by a multiplex PCR as described by Lin et al. (1998), with minor modifications described in Teixeira et al. (2004).

The *GSTP1* codon 105 polymorphism was determined by PCR and RFLP according to the method of Harries et al. (1997), with minor modifications in Teixeira et al. (2004).

XRCC1 codon 194 and 399 polymorphisms were genotyped by PCR followed by RFLP according to Silva et al. (2007).

The genotyping of *XRCC2* 188Arg/His was also determined by PCR-RFLP as described by Bastos et al. (2009).

2.6. Statistical analysis

The departure from normality for the analyzed continuous variables was evaluated with the graphical approach as well as with Kolmogorov–Smirnov test. Where the assumption of normality was not met, data transformations were applied to normalize the distribution or data were categorized (cigarettes/day, time since last exposure and age). Within age limits included in the study age distribution is normal, and therefore parametric measures of central tendency were used (differences arising from median use were analysed but not found and therefore only the mean values are presented).Considering biomarkers of exposure, PYR was dichotomized as present or non-present; OP/CRB and THIO were transformed on the log-scale and BChE was normally distributed. Considering biomarkers of effect measuring genotoxic damage, *%T* (comet assay) was transformed on the log-scale; no transformation was applied to other biomarkers. Considering immunological biomarkers, $\%CD3^+$, $\%CD4^+$, and $\%CD19^+$ were normally distributed, while $\%CD8^+$ and $\%CD16^+56^+$ needed log-transformation to be analyzed with parametric statistics. The relationship between biomarkers was performed by means of Spearman's correlation analysis.

One-way/two-way analysis of variance/covariance (ANOVA/ANCOVA) and Kruskal–Wallis test were used for all statistical comparison between the three groups in normally and not normally distributed variables, respectively. As regards qualitative variables, the presence of heterogeneity between groups was tested with the Chi-square test.

To take into account the presence of confounding, and to test for the presence of effect modification, genetic and immunological damage in the three groups was investigated with a regression modeling approach, after adjusting for age, gender and smoking habit. Furthermore, the role of variables describing occupational exposure to pesticides was tested in the group of pesticides workers. The presence of a significant difference in the proportion of smokers among the study groups may generate confounding that was taken into consideration including smoking habit in all regression models as a fixed covariate.

Comet assay data was analyzed applying the log-normal regression approach, while counts were studied with the Poisson regression model. In the presence of over-dispersion the binomial negative regression was applied instead of Poisson. Over-dispersion can be increased by an elevated number of zero counts. When this was the case in our data, the zero-inflated negative binomial regression was fitted (Ceppi et al., 2010).

Mann–Whitney and Kruskal–Wallis tests were employed to test the presence of difference between biomarkers of effect by genotype. Differences in genotype distributions were evaluated by the chi-square test.

Statistical significance was set at a *p*-value below 0.05. Analyses were carried out using the SPSS software package V.13.0 (SPSS, Inc) and STATA software package V. 10 (StataCorp LP).

3. Results

3.1. Biomarkers of exposure

No significant differences were found among study groups regarding age, gender, smoking habits and cigarettes/day. All results concerning biomarker of exposure are reported in Table 3. Concentration of OP/CRB compounds in urine was the only marker presenting significant differences among the three studied groups.

The analysis including possible confounders showed that host factors such as age and gender and a few variables related with exposure, i.e., last exposure (days) and season, significantly influenced the concentrations of these biomarkers. Females presented significantly higher concentrations of PYR (within controls; $p = 0.005$), OP/CRB and THIO (both among organic farmers; $p = 0.001$). Regarding BChE enzymatic activity, males consistently presented higher activity values than females (difference statistically significant in the control group; $p = 0.001$). THIO presented a significant increase with age (in both control and pesticide workers groups; $p = 0.002$ and $p = 0.015$, respectively). The same increase with age was also observed in BChE activity (among pesticide workers group; $p = 0.001$).

As regards exposure variables and their influence on the results of biomarkers of exposure, higher levels of OP/CRB ($p = 0.002$) and THIO ($p = 0.003$) were observed in individuals recently exposed to pesticides; individuals whose samples were collected during spring and summer presented significantly higher concentrations of OP/CRB ($p = 0.001$).

3.2. Biomarkers of effect

Univariate analysis showed significantly higher frequencies of MNL, MN-RET, total CA, CTA and *%T* among pesticide workers when compared with the remaining studied groups (data not shown). Age, gender and smoking habit were included in all regression models for genotoxicity biomarkers as reported in Table 4. Pesticide workers were compared with unexposed controls and presented significantly higher means of MN in lymphocytes (6.69 ± 0.47 vs. 2.33 ± 0.23) and reticulocytes (1.14 ± 0.09 vs. 0.51 ± 0.05), total CA (1.56 ± 0.15 vs. 0.92 ± 0.14) and *%T* (15.05 ± 0.85 vs. 8.03 ± 0.73). Results were not consistent for organic farmers when compared to controls, displaying a 48% increase of MNL frequency ($p = 0.016$) but significant decreases for TCR-Mf ($p = 0.001$) and *%T* ($p = 0.001$).

Gender difference was significant only in *%T*, with males presenting higher DNA damage levels ($p = 0.022$). No significant effect of age was observed, excepting an increase of TCR-Mf ($p = 0.017$) in individuals aged 30–38 with regard to the youngest group. Smoking habits did not influence MNL, MN-RET and total CA, but a reduction of damage assessed by TCR-Mf ($p = 0.001$) and *%T* ($p = 0.008$) was found in smokers. A few exposure variables showed a significant influence on biomarkers frequencies as shown in Table 5. Applicators show significantly higher frequencies of TCR-Mf ($p = 0.002$); pesticide preparation and activity during spring-summer increased frequencies of MN-RET ($p = 0.041$ and $p = 0.013$) and *%T* ($p = 0.031$ and $p = 0.020$), but reduced damage evaluated by TCR-Mf ($p = 0.013$ and $p < 0.001$). Contrasting results were observed when looking at the influence of workplace: individuals working in greenhouses presented higher levels of mutations (TCR-Mf; $p = 0.009$) but lower levels of DNA damage (*%T*; $p = 0.014$). Inadequate usage of pesticides (against manufacturer's instructions) significantly influenced TCR-Mf ($p = 0.001$), with those reporting inadequate usage presenting much higher means than those using them properly.

Results of univariate analysis of lymphocytes subpopulations showed statistically significant differences only for B lymphocytes (higher frequencies in controls when compared with organic farmers; $p = 0.023$) and natural killer cells (higher frequencies in organic farmers when compared with controls; $p = 0.001$). In all studied lymphocyte subpopulations, cell frequency was similar in pesticide workers and unexposed controls. Multivariate analyses based on regression modeling (Table 6) showed that, after correction by *age*, *gender* and *smoking habit*, significantly lower percentages of B lymphocytes were found in both farmers groups than in unexposed controls ($p = 0.021$ for organic farmers and $p = 0.031$ for pesticide workers), and the results of univariate analysis were confirmed for natural killer cells.

Total T lymphocytes and T helper cells were higher in females than in males ($p = 0.012$ and $p < 0.001$, respectively), and the opposite was observed for T cytotoxic cells ($p = 0.006$). The proportion of T helper cells significantly increased with age ($p = 0.011$) while a decrease was perceived for B lymphocytes ($p = 0.009$). Levels of all studied subpopulations were similar in smokers and non-smokers

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Table 3
Mean concentrations of biomarkers of exposure in studied groups (data are reported as mean ± SE and range in brackets).

	Study group							
	n	Unexposed Controls	*n*	Organic Farmers	*n*	Pesticide workers	*p*-value	*p*-value adjusted for age and last exposure
PYR (μg/mmol creat)	60	0.13 ± 0.04 (nd–1.35)	36	0.06 ± 0.05 (nd–1.92)	85	0.08 ± 0.03 (nd–1.66)	0.137	0.185
OP/CRB (μg/mmol creat)	60	1.54 ± 0.23[a] (nd–11.73)	36	1.86 ± 0.30[a,b] (nd–9.61)	85	2.23 ± 0.19[b] (nd–8.22)	0.030	0.002
THIO (μmol/mmol creat)	60	51.83 ± 3.28 (20.95–187.46)	35	62.56 ± 5.60 (23.49–217.95)	85	54.33 ± 3.16 (20.08–229.41)	0.111	0.003
BChE[c] (U/L)								
Class 1	41	6425 ± 224 (3347–9083)	29	6246 ± 191 (4408–8647)	56	7064 ± 202 (4142–9707)	0.943	0.059
Class 2	7	5966 ± 642 (3902–8966)	6	5804 ± 662 (3964–7890)	21	6240 ± 289 (3539–9044)		
Class 3	13	6553 ± 444 (4944–10192)	1	7074 (7074–7074)	7	6951 ± 467 (5485–9050)		

nd–not detected; creat–creatinine. BChE classes: class 1 includes all men and women above 40 years-old; class 2 includes women between 16 and 39 years-old that do not take hormonal contraceptives; class 3 comprises women between 18 and 41 years old under hormonal contraceptive medication.
[a,b]Homogeneous groups according to multiple comparison Tukey' test.
[c]Classes established according to age and gender.

Table 4
Effect of exposure and host-factors on biomarkers of genotoxicity (with estimates of mean frequency ratio–FR and mean ratio - MR).

	MNL		MN-RET		TCR-Mf		Total CA		CTA (%)		%*T*	
Variables	*n*	FR and CI (95%)	*n*	FR and CI (95%)	*n*	FR and CI (95%)	*n*	FR and CI (95%)	*n*	FR and CI (95%)	*n*	MR and CI (95%)
Exposure												
Unexposed controls	61	1.00	61	1.00	61	1.00	59	1.00	59	1.00	59	1.00
Organic farmers	36	1.48 (1.08, 2.03)*	36	0.93 (0.70, 1.24)	36	0.20 (0.09, 0.44)**	25	0.95 (0.60, 1.52)	25	0.89 (0.59, 1.37)	35	0.52 (0.39, 0.69)**
Pesticide workers	84	2.80 (2.18, 3.59)**	83	1.89 (1.41, 2.54)**	77	0.48 (0.23, 0.99)*	80	2.19 (1.41, 3.40)**	80	1.77 (1.29, 2.62)**	79	1.71 (1.36, 2.15)**
Gender												
Females	95	1.00	95	1.00	92	1.00	88	1.00	88	1.00	92	1.00
Males	86	0.87 (0.70, 1.07)	85	0.97 (0.76, 1.23)	82	1.54 (0.81, 2.92)	76	0.84 (0.57, 1.23)	76	0.87 (0.61, 1.23)	81	1.27 (1.04, 1.56)*
Age												
18–29	46	1.00	45	1.00	43	1.00	42	1.00	42	1.00	43	1.00
30–38	45	1.10 (0.82, 1.46)	44	1.06 (0.76, 1.48)	42	3.12 (1.22, 7.93)*	41	1.24 (0.75, 2.06)	41	0.99 (0.66, 1.47)	44	0.77 (0.58, 1.02)
39–49	47	1.10 (0.83, 1.47)	48	1.07 (0.77, 1.50)	48	0.51 (0.23, 1.13)	42	1.34 (0.79, 2.28)	42	1.38 (0.87, 2.18)	48	0.94 (0.71, 1.23)
≥50	43	1.04 (0.77, 1.39)	43	0.98 (0.71, 1.36)	41	0.69 (0.29, 1.65)	39	0.83 (0.52, 1.31)	39	0.96 (0.63, 1.45)	38	1.01 (0.75, 1.35)
Smoking Habits												
Non-smokers	160	1.00	159	1.00	154	1.00	147	1.00	147	1.00	154	1.00
Smokers	21	0.70 (0.48, 1.03)	21	0.96 (0.67, 1.36)	20	0.24 (0.10, 0.58)**	17	1.19 (0.61, 2.32)	17	1.06 (0.62, 1.82)	19	0.64 (0.46, 0.89)**

* $p < 0.05$.
** $p < 0.01$; CI–confidence interval.

except for NK cells, which were significantly increased in smokers (MR = 1.44, $p = 0.029$).

Percentages of T lymphocytes and NK cells were significantly influenced by season; T lymphocytes were significantly decreased during spring-summer ($p = 0.011$) while NK cells were significantly elevated during this period ($p < 0.001$) when compared with autumn-winter. Individuals working in greenhouses presented significantly higher percentages of T helper cells than subjects working in open-field ($p = 0.039$).

Table 5
Effect of pesticides exposure variables on biomarkers of genotoxicity (with estimates of mean frequency ratio–FR and mean ratio–MR).

	MNL		MN-RET		TCR-Mf		%*T*	
Variables	*n*	FR and CI (95%)	*n*	FR and CI (95%)	*n*	FR and CI (95%)	*n*	MR and CI (95%)
Task								
Non-applicators	29	1.00	29	1.00	26	1.00	29	1.00
Applicators	55	0.93 (0.65, 1.32)	54	1.18 (0.65, 2.16)	51	5.70 (1.92, 16.91)**	50	1.03 (0.68, 1.57)
Pesticide preparation								
No	29	1.00	30	1.00	29	1.00	28	1.00
Yes	55	0.95 (0.67, 1.34)	53	1.74 (1.02, 2.97)*	48	0.25 (0.09, 0.75)*	51	1.52 (1.04, 2.23)*
Season								
Autumn–winter	45	1.00	46	1.00	44	1.00	41	1.00
Spring–summer	39	1.07 (0.79, 1.44)	37	1.86 (1.14, 3.05)*	33	0.07 (0.03, 0.17)**	38	1.45 (1.06, 1.99)*
Workplace								
Open-field	13	1.00	13	1.00	9	1.00	11	1.00
Greenhouses or both	71	0.83 (0.56, 1.25)	70	0.72 (0.35, 1.52)	68	4.50 (1.45, 13.97)**	68	0.56 (0.35, 0.89)*
Inadequate usage								
No	66	1.00	65	1.00	60	1.00	61	1.00
Yes	18	0.86 (0.59, 1.26)	18	0.64 (0.37, 1.09)	17	7.60 (2.19, 26.34)**	18	0.92 (0.61, 1.39)

* $p < 0.05$.
** $p < 0.01$; CI–confidence interval.

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Table 6
Effect of exposure and host-factors on lymphocyte subpopulations (with estimates of mean ratio - MR).

	%CD3$^+$ (T Lymphocytes)		%CD4$^+$ (T helper cells)		%CD8$^+$ (T cytotoxic cells)		%CD19$^+$ (B Lymphocytes)		%CD56$^+$CD16$^+$ (natural killer cells)	
Variables	*n*	MR and CI (95%)	*n*	MR and CI (95%)	*n*	MR and CI (95%)	*n*	MR and CI (95%)	*n*	MR and CI (95%)
Exposure										
Unexposed controls	61	1.00	61	1.00	61	1.00	61	1.00	61	1.00
Organic farmers	36	0.98 (0.94, 1.03)	36	1.06 (0.98, 1.14)	36	1.01 (0.80, 1.27)	36	0.85 (0.73, 0.97)*	36	1.65 (1.24, 2.19)**
Pesticide workers	85	0.99 (0.95, 1.02)	85	1.01 (0.95, 1.07)	85	0.97 (0.80, 1.17)	85	0.88 (0.78, 0.99)*	85	1.09 (0.87, 1.38)
Gender										
Females	96	1.00	96	1.00	96	1.00	96	1.00	96	1.00
Males	86	0.96 (0.93, 0.99)*	86	0.90 (0.85, 0.95)**	86	1.27 (1.07, 1.50)**	86	1.1 (1.00, 1.23)	86	0.96 (0.78, 1.18)
Age										
18–29	46	1.00	46	1.00	46	1.00	46	1.00	46	1.00
30–38	45	1.02 (0.97, 1.06)	45	1.06 (0.98, 1.14)	45	0.98 (0.77, 1.23)	45	1.00 (0.87, 1.15)	45	1.03 (0.78, 1.37)
39–49	48	1.02 (0.98, 1.07)	48	1.13 (1.05, 1.22)**	48	0.93 (0.74, 1.16)	48	0.86 (0.75, 0.99)*	48	0.89 (0.67, 1.17)
≥50	43	1.01 (0.97, 1.06)	43	1.11 (1.02, 1.20)*	43	0.80 (0.64, 1.02)	43	0.82 (0.71, 0.95)**	43	1.26 (0.94, 1.67)
Smoking habits										
Non-smokers	162	1.00	162	1.00	162	1.00	162	1.00	162	1.00
Smokers	21	0.98 (0.93, 1.03)	21	1.06 (0.97, 1.16)	21	0.93 (0.71, 1.22)	21	0.91 (0.71, 1.07)	21	1.44 (1.04, 1.99)*

* $p < 0.05$.
** $p < 0.01$; CI–confidence interval.

3.3. Biomarkers of susceptibility

The results of genotype analysis in pesticide workers, organic farmers and unexposed controls are presented in Table 7 (the distribution of all studied polymorphisms is in Hardy–Weinberg equilibrium, except the GSTM1 and GSTT1 deletion polymorphisms, for which no information on allele distribution was available). There was no statistically significant difference in the frequency of studied polymorphisms among the different groups.

Although the main aim of genotype analysis was to exclude possible differences in the genetic background of individuals in the three different groups, the effects modification of different genetic polymorphisms on the parameters evaluated were also analysed but contrasting results were obtained. As regards *GSTM1*, in the group of unexposed controls higher levels of damage were found among individuals with the positive genotype. In the pesticide workers group, significantly increased %*T* and decreased percentage of NK cells were found among individuals with the positive genotype. A significant effect of *GSTT1* was evident only for TCR-Mf, with increased frequencies among positive individuals of the control group. For *GSTP1* genotype, significantly lower CA frequencies were found in organic farmers homozygous for the variant GSTP 105Val allele, while pesticide workers with the same genotype presented significantly higher percentages of NK cells. *XRCC1* polymorphism in codon 399 significantly influenced the levels of TCR-Mf in unexposed control individuals and CSA among pesticide workers (variant XRCC1 399Gln/Gln presented higher levels of damage).

4. Discussion

4.1. Biomarkers of exposure

A major limitation in most epidemiological studies conducted on the adverse health effects of pesticides has been the poor characterization of exposure. In humans, although a minor portion of pesticide compounds are excreted unchanged (as they are rapidly metabolized by mammalian species to their inactive acids and alcohol components), original molecules of PYR (including deltamethrin, fenvalerate and permethrin) have been quantified in urine in different studies (He et al., 1988; Zhang et al., 1991) showing that these are detectable in urine after PYR exposure. Furthermore, in a context of multiple exposure, it may be useful to obtain information using a non-specific biomarker (such as thioethers determination) as this provides information on the level of exposure to total electrophilic compounds (Doorn et al., 1981).

There is no published literature using the same methods here performed to assess exposure to PYR and OP/CRB. Results obtained for PYR were not reliable as they seem to be affected by factors other than occupational exposure (that could not be identified here) and therefore do not offer helpful information as a biomarker of exposure to pesticides. In opposition, OP/CRB determination based on a very simple method provided meaningful information and was related to recent exposure (self-reported by the subjects). Further studies using this method will be necessary to fully understand the potential of this indicator as a biomarker of exposure to pesticides and its reliability.

THIO excretion is known to be highly affected by diet as some foodstuffs that contain thioethers (e.g., horseradish, cabbage, onions) or electrophilic substances (e.g., charcoal-grilled meats, red wine) are metabolized to free radicals (Rosen et al., 1999). Similarly to what was observed for OP/CRB determinations, THIO concentrations were found to be significantly higher among those recently exposed to pesticides when compared with those reporting past exposure, what is in accordance with what was previously described by Mikov et al. (2000). Although this is a non-specific biomarker since it is affected by age and diet, it can be used to roughly estimate the exposure risk within the exposed populations.

Previous studies on thioethers excretion report different results regarding the influence of age and gender; Aringer and Lidums 1988 did not find association either with age or gender while Hagmar et al. (1988) reported an increase of THIO excretion with age and Vainio et al. (1978) and Kilpikari (1981) observed increased THIO excretion in females. Our observed increase in THIO excretion with age may be related to an increase of the endogenous levels of free radicals that are normally conjugated with glutathione and are thus detected as thioethers in urine (Sohal, 2002). Regarding gender, increased excretion of THIO in females may be attributed to estrogen conjugates (Raftogianis et al., 2000).

It may be important to highlight that PYR, OP/CRB and UTIO concentrations were not corrected for creatinine concentration, possibly generating biased results (Barr et al., 2005). Urinary creatinine is lower in women, condition that may lead to higher values in corrected concentrations. Nevertheless, being impractical the collection of 24 h urine samples, the use of creatinine to correct variable dilutions among spot samples is the best available option. Concerning BChE results, we found significant differences with age and gender. These findings are in accordance to what has been

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Table 7
Frequency of genotypes in the study population.

	Study group								
	n	All	*n*	Unexposed controls	*n*	Organic farmers	*n*	Pesticide workers	*p*-value
EPHX1 codon 113									
Tyr/Tyr	116	64.4%	41	67.2%	21	60.0%	54	64.3%	0.913
Tyr/His	45	25.0%	15	24.6%	9	25.7%	21	25.0%	
His/His	19	10.6%	5	8.2%	5	14.3%	9	10.7%	
EPHX1 codon 139									
His/His	100	54.9%	37	60.7%	21	58.3%	42	49.4%	0.673
His/Arg	71	39.0%	20	32.8%	13	36.1%	38	44.7%	
Arg/Arg	11	6.0%	4	6.6%	2	5.6%	5	5.9%	
GSTM1 deletion									
Positive	90	49.5%	31	50.8%	15	41.7%	44	51.8%	0.577
Null	92	50.5%	30	49.2%	21	58.3%	41	48.2%	
GSTT1 deletion									
Positive	155	85.2%	53	86.9%	28	77.8%	74	87.1%	0.379
Null	27	14.8%	8	13.1%	8	22.2%	11	12.9%	
GSTP1 codon 105									
Ile/Ile	74	40.7%	21	34.4%	13	36.1%	40	47.1%	0.354
Ile/Val	84	46.2%	31	50.8%	20	55.6%	33	38.8%	
Val/Val	24	13.2%	9	14.8%	3	8.3%	12	14.1%	
XRCC1 codon 194									
Arg/Arg	167	91.8%	60	98.4%	32	88.9%	75	88.2%	0.202
Arg/Trp	14	7.7%	1	1.6%	4	11.1%	9	10.6%	
Trp/Trp	1	0.5%	0	0.0%	0	0.0%	1	1.2%	
XRCC1 codon 399									
Arg/Arg	62	34.1%	19	31.1%	14	38.9%	29	34.1%	0.843
Arg/Gln	83	45.6%	28	45.9%	17	47.2%	38	44.7%	
Gln/Gln	37	20.3%	14	23.0%	5	13.9%	18	21.2%	
XRCC2 codon 188									
Arg/Arg	129	70.9%	49	80.3%	27	75.0%	53	62.4%	0.068
Arg/His	50	27.5%	10	16.4%	9	25.0%	31	36.5%	
His/His	3	1.6%	2	3.3%	0	0.0%	1	1.2%	

previously stated in the literature and are related to hormonal status. Cholinesterase activity decreases after menarche and increases again in postmenopausal women, reaching the values for men. This increase in BChE activity may be due to a decrease in sexual hormones, which could result in reduced hepatic synthesis or in the release of enzymes. In addition, also for men, it has been observed an increase after 45 years-old (Lepage et al., 1985). This age-associated increase observed in men, associated to the concurrent increase of body mass and the biological role of cholinesterase in lipid and lipoprotein metabolism (Kutty et al., 1983).

The interpretation of BChE values is complicated by the larger inter- and intra-variability of this enzyme making the distinction between physiologically low levels and inhibited enzymes impossible (inter-variability is estimated from 12 to 46%). In addition, and because BChE has no attributed physiological function, this indicator may only reflect the degree of exposure but has no significance in terms of health (Lotti, 2010).

4.2. Biomarkers of effect

Cytogenetic markers are very frequently used in studies due to their sensitivity when measuring exposure to genotoxic agents (Bonassi et al., 2005). In this study, we found a significant increase of MNL, MN-RET, total CA, CTA frequencies and %*T* in pesticide exposed individuals compared with unexposed controls.

MNL increase is in accordance to the results reported in other investigations (Bhalli et al., 2006b; Bolognesi et al., 2009; Costa et al., 2006; Ergene et al., 2007; Sailaja et al., 2006). Results also show an increase in MN-RET frequencies in pesticide workers suggesting that pesticide exposure can be responsible for injury in hematopoietic cells. In the more recent years, this biomarker has been found to be a suitable technique to access genetic damage in humans due to chemical or physical exposure (Abramsson-Zetterberg et al., 2006; Offer et al., 2005; Sun et al., 2005).

Most studies assessing genetic damage in pesticide-exposed population, by means of CA, also report significant increases in this frequency (Ergene et al., 2007; Garaj-Vrhovac and Zeljezic, 2002; Garry et al., 2001; Sailaja et al., 2006; Varona et al., 2003). Herein, we also observed a significant increase in total CA mostly due to CTA. This increase observed for CTA, but not for CSA (data not shown), agrees with the model proposing that mainly CTA are to be expected by the action of chemical mutagens (Schleiermach, 1971).

Percentage DNA in the comet tail (%*T*) was also significantly increased among pesticide workers when compared with controls. Still, the percentages obtained in the pesticide workers group are very close to established reference values (range from 4.4 to 14.5%) (Moller, 2006). This increase agrees with the majority of results found in the literature on this matter (Bhalli et al., 2006a; Liu et al., 2006; Remor et al., 2009; Sailaja et al., 2006; Shadnia et al., 2005) although some studies also report negative findings (Piperakis et al., 2006; Piperakis et al., 2003).

Results obtained for TCR-Mf contradict the information obtained in the remaining biomarkers of genotoxic effect. In contrast with other genotoxicity assays, that detect chromosomal structure alterations, TCR-Mf is an indication of overall genotoxicity of a chemical resulting in subtle, selective mutations (Zhijian et al., 2006). This increased sensitivity may lead to detection of mutations originated by environmental factors, a possibly significant factor for the unexposed control population.

In addition to genotoxicity biomarkers, lymphocytes subpopulations were also studied as a marker of alterations on the immune system. These alterations constitute a sensitive parameter for detecting subclinical toxic injury; exposure to certain chemicals at doses that do not cause overt toxicity can

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produce immune alterations sufficient to result in altered host resistance to infectious agents and neoplastic cells (Gos and Dean, 1990). Some pesticide chemicals and groups of chemicals were already recognized as immunotoxic. They include organophosphate compounds, organochlorine insecticides, carbamates, phenoxy herbicides, pentachlorophenol, dithiocarbamates and organotin compounds (Colosio, 1999). In the scientific literature the decrease of %CD26$^+$, %CD4$^+$, neutrophil function, the decrease of antibody production by B lymphocytes, and the decrease of natural killer cells activity among other alterations in pesticide-exposed populations,are extensively reported (Colosio, 1999; Li, 2007; Corsini, 2008). As regards autoimmunity, lupus-like syndrome was also reported after exposure to chlorpyrifos, and other studies describe findings of autoantibodies in pesticide exposed individuals (Holsapple, 2002).

In this study, significant alterations were found on %CD16$^+$56$^+$ cells between controls and organic farmers (higher in organic farmers) and %CD19$^+$ between unexposed controls and the two other considered groups (lower in farmers). NK cells are involved in immunosurveillance and therefore an increase in its presence would correspond to an improvement in the immune response (Corsini et al., 2008). However, NK increase in number (or percentage) may not be associated with a better response as the activity of these cells is more important than the number (Panda et al., 2009), and in this study the functional capacity of NK cells was not addressed. A decrease of %CD19$^+$ in organic farmers and pesticide workers when compared with unexposed controls suggests immunosuppression, as a decreased presence of B lymphocytes will lead to a decrease in the production of antibodies and therefore a weakened immune function (Weiskopf et al., 2009).

A novelty in this study concerns the inclusion of an organic farmers group in the study population. These constitute a group of individuals not exposed to synthetic pesticides and generally with healthier lifestyles. Although there were some conflicting results, overall it seems that organic farmers present a level of genetic damage similar to unexposed controls.

Although gender is widely described as a demographic factor influencing observed cytogenetic damage assessed by means of MN frequency (Bonassi et al., 1995; Nefic and Handzic, 2013), in this study only a non-significant increase was observed among females.

Although the influence of gender on Comet assay is still not fully clarified, it is more usually observed an increase in the levels of DNA damage among males (Moller et al., 2000). In our study, this difference was actually significant as the one found by Bajpayee et al. (2002), Laffon et al. (2006) and Pérez-Cadahía et al. (2008).

The lower values observed in the current work for %CD3$^+$ and %CD4$^+$ cells in males were profusely documented in European populations (García-Dabrio et al., 2012; Laffon et al., 2013; Santagostino et al., 1999). On the contrary, percentage of CD8$^+$ lymphocytes usually does not differ between males and females (Andreu-Ballister et al., 2012; García-Dabrio et al., 2012; Jentsch-Ulrich et al., 2005) but herein we found a significant increase of this subset in males. These differences in T cells between genders may be due to sex hormones; two different mechanisms have been suggested: either androgens accelerate thymocyte apoptosis and therefore shape the peripheral T cell repertoire (Jentsch-Ulrich et al., 2005) or there is an effect of binding to cell receptors for the sex steroid present on T cells (Chng et al., 2004).

Increasing chromosome instability with age is a phenomenon well described in literature (Bolognesi et al., 1999) that relates to diminished DNA repair capacity and increase of oxygen free radicals. In the current study, we could only find a significant increase of TCR-Mf when comparing individuals of 18–29 years-old with those of 30–38 years-old.

Obtained data confirmed the effect of age on lymphocytes subpopulations. In the overall population studied in this project, we found a significant decrease of %CD19$^+$ cells and a significant increase in %CD4$^+$ lymphocytes with age, supporting previous reports (García-Dabrio et al., 2012). Alterations in the immune system with age are often designated by immunosenescence that is characterized by a decrease in cell-mediated immune function as well as by reduced humoral immune responses. Concerning B lymphocytes (CD19$^+$) and ageing, results are conflicting as some authors state that the number of these cells are stable throughout the life of individuals (Weiskopf et al., 2009), while others report a decrease in the number of these cells with age (Globerson and Effros, 2000; Huppert et al., 1998). Decrease of CD19$^+$ cells is explained by an increased number of B cells in organs other than peripheral blood and/or an increased lifespan of B lymphocytes in germinal centers. This would predict age-related alterations in B-cell homing and the propensity to undergo apoptosis (Franceschi et al., 1995). In respect to T lymphocytes, again different trends have been described with age (Aw et al., 2007; Huppert et al., 1998). Although it is certain that the thymus involutes with age leading to a significant decrease in the T cell output, it is expected to observe constancy in the peripheral T cell number (Gruver et al., 2007). In regard to smoking habits, our population has a quite unbalanced number of smokers and non-smokers what can be a bias to outcome analysis. This discrepancy may affect final estimates of effect, although the potential confounding due to this parameter was taken into account adjusting all regression models for this covariate.

Percentage of time dedicated to different working activities such as mixing, loading, application, re-entry and maintenance can also influence exposure. A highly significant increase was found in TCR-Mf among applicators confirming results obtained by other authors (Mage et al., 2000; Shaham et al., 2001).

Regarding mixing/loading activities (pesticide preparation), genotoxic damage levels were found to be either similar or increased in individuals that perform this task, except for TCR-Mf. Time since last exposure may be on the basis of this discrepancy since few months are necessary for the expression of a TCR mutant phenotype (Vershenya et al., 2004). A variable that has not been assessed yet in any other study concerns the adequate usage of pesticides; a significant increase of TCR-Mf was observed in subjects not using pesticides adequately, highlighting the need for workers training.

Considering pesticide-exposed populations, seasonal variation can be determinant in the observed effects. As observed for time since last exposure, biomarkers of recent exposure (MN-RET and Comet assay) present significantly elevated frequencies in Spring-Summer period and TCR-Mf was significantly decreased. Damage due to pesticide exposure during Spring-Summer can only be assessed by TCR-Mf after a time gap and therefore increased frequencies are obtained when sampling is performed in Autumn-Winter.

The significant decrease of total T lymphocytes (%CD3$^+$) found in Spring-Summer period is possibly related to the fact that these period include the months of maximum usage of pesticides in agriculture. The increase found in the %NK cells in Spring-Summer was also reported by McClure et al. (2001). Again, the study of activity of these cells would be necessary to appreciate the significance of this result.

Working environment was found to be an extremely important factor on exposure risk. Two independent studies (Bolognesi et al., 2002; Costa et al., 2006) found increased genetic damage on farmers working mainly in greenhouses. The increased risk of greenhouse work was confirmed in the current study by a significant increase in TCR-Mf, which is considered to be one of the most sensitive biomarkers. Nevertheless, decreases of CSA and % were not consistent with the increased risk.

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4.3. Biomarkers of susceptibility

In this study, taking into consideration that individuals were exposed to several different chemical classes, it was decided to assess polymorphisms of genes coding for enzymes involved in main pathways of detoxification and DNA repair rather than pesticide-specific ones. In addition, as the strength of association between individual genetic variant and observed outcome (genotoxic damage or immune response) was likely to be low and difficult to identify, the relevance of data obtained with genotype analysis is related to the possible bias that the genetic background of individuals included in each group could introduce in the results of the different biomarkers of effect. Results showed no significant differences in the frequencies of studied polymorphisms among the different groups and therefore one can assume that results of comparison of biomarkers of effect among the three exposure groups were not influenced by the genetic background of the individuals.

The frequency of polymorphisms in xenobiotic metabolizing enzymes found in our study group are in accordance to what one should expect for Caucasian individuals (Garte and Gaspari, 2001) and are similar to those already described in the Portuguese populations (Costa et al., 2008; Costa et al., 2006; Gaspar et al., 2004; Teixeira et al., 2004).

Concerning the observed modulation of genetic damage, we found significantly higher levels of damage in GSTM1 and GSTT1 positive individuals; in addition, GSTM1 positive individuals also presented immunosupression as the percentage of NK cells found in these individuals was significantly lower. Falck et al. (1999) also reported an increase in genetic damage (MNL) in pesticide-exposed GSTM1 positive greenhouse workers. Our finding of lower damage in GSTP1 105Val/Val individuals confirms previous reports (Liu et al., 2006; Singh et al., 2011; Wong et al., 2008). It has been suggested that under the stress of high-dose pesticide, GSTP1 Val-containing enzyme is associated with increased levels of apoptosis, and therefore decreased levels of DNA damaged cells are observed (Liu et al., 2006). We also found significantly higher percentages of NK cells in these individuals, indicating an enhanced immune response.

Regarding genes of enzymes involved in DNA repair, we report an increase in genetic damage (evidenced by CSA and TCR-Mf) in XRCC1 codon 399Gln/Gln individuals in accordance to what has been previously suggested by other authors (Duell et al., 2000).

5. Conclusions

In this study, a set of biomarkers was used to evaluate whether work in different types of agricultural systems can cause genotoxic effects and immunological alterations.

Results obtained confirm that pesticides are able to induce genotoxicity evidenced by the results in different biomarkers and also to cause significant alterations in the percentage of B lymphocytes. An increased level of genetic damage was observed in conventional farmers when compared to organic farmers indicating that the health status of farm workers may be influenced by the type of agriculture they practice. Due to overall number of individuals and the unbalanced number of individuals in each group, this finding needs to be interpreted with caution and further investigation on this matter is required to confirm this conclusion.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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