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

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Abstract

Background—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.

Objectives—To evaluate genetic damage and immunological alterations in workers of both traditional and organic farming.

Methods—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.

Results—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).

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Competing financial interest declaration

The authors declare that they have no conflicts of interest.

Overall, organic farmers presented similar levels of genetic damage as controls, in some cases modulated by *GSTT1* and *GSTM1*, *GSTP1* 105Ile/Ile and *XRCC1* 399Gln/Gln genotypes.

Conclusions—Results confirmed the increased presence of DNA damage in farmers exposed to pesticides, and showed as exposure conditions and genetic background influence observed effects. Findings from this study indicate that no evident genetic or immunologic damage can be observed in organic farmers.

Keywords

biomarkers; chromosomal aberrations; comet assay; genetic polymorphisms; lymphocyte subpopulations; micronucleus test; pesticides; T-cell receptor mutation assay

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 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; Li, 2007; Corsini et al., 2008).

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.

Materials and Methods

Study population

The group of traditional agricultural system consisted of 85 farmers using pesticides (43 males and 42 females) from a main Portuguese agricultural 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. 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.

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 and occurrence of previous intoxications (those resulting of pesticide exposure that required medical treatment). 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).

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 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 3h 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.

Biomarkers of exposure

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.

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).

Urinary Thioethers (THIO) determination—The procedure was based on what was previously described by Vainio et al. (1978).

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's 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.

Biomarkers of effect

Micronuclei tests: Cytokinesis Block Micronuclei Assay (CBMN) and Micronuclei in reticulocytes (MN-RET)—CBMN was performed according to what

was previously described by Costa et al. (2007). 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 carried out according to 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.

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.

Chromosomal Aberrations—Slides for analysis of chromosomal aberrations were obtained following the protocol described by Costa 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, fifty 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.

Comet Assay—The alkaline comet assay was performed essentially as described by Costa et al. (2008). 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).

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.

Biomarkers of susceptibility

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.

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).

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).

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.

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).

Results

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).

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. Finally, MNL (p=0.009) and TCR-Mf (p=0.013) were significantly lower in individuals previously intoxicated.

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 NK 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 except for natural killer 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).

Biomarkers of susceptibility

The results of genotype analysis in pesticide workers, organic farmers and unexposed controls are presented in Table 7. There was no statistically significant difference in the frequency of studied polymorphisms among the different groups.

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).

The effects modification of different genetic polymorphisms on the parameters evaluated were contrasting. Different genotypes of *GSTM1*, *GSTT1*, *GSTP1* and *XRCC1* could modulate the frequencies of some biomarkers of genotoxicity and immunotoxicity, but only selected results have been presented and discussed.

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).

Discussion

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 are 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 (Riggs 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 what was previously described by Mikov et al. (2000). For this reason, we can say that although this is a non-specific biomarker it can be used to establish a risk trend 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).

Concerning BChE results, we found significant differences with age and gender. These findings are in accordance to what has been 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. In addition, also for men, it has been observed an increase after 45 years-old (Lepage et al. 1985). 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).

Biomarkers of effect

Exposure—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. 2006; 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. 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. 2006; 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 produce immune alterations sufficient to result in altered host resistance to infectious agents and neoplastic cells (Gos and Dean 1990).

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.

Host factors

Although gender is widely described as a demographic factor influencing observed cytogenetic damage assessed by means of MN frequency (Bonassi et al. 1995), 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.(2006;2008).

The lower values observed in the current work for %$CD3^+$ and %$CD4^+$ cells 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-Ballester 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. 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 lymphocytary 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 limits the value of the result and impairs any possible conclusion on this matter.

Exposure variables

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 pesticide preparation may be on the basis of this discrepancy; both MN-RET

and %T are able to detect very recent damage while 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.

Biomarkers of susceptibility

Literature suggests that null variants of GST genotypes confer increased susceptibility to pesticide-induced damage. In opposition, we found that both for GSTM1 and GSTT1 significantly higher levels of damage were observed in 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. Due to these disparities, some authors cautioned the use of GST polymorphisms in predicting disease risk associated with pesticide exposure (Peck and Eaton 2010).

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.

DNA repair is a very important mechanism in protection against gene mutation and cancer initiation. Few studies were carried out on pesticide exposed populations to understand the influence of polymorphisms of repair enzymes on observed genetic damage. In this work,

we report an increase in genetic damage (evidenced by CSA and TCR-Mf) in XRCC1 codon 399Gln/Gln individuals. Although Wong et al.(2008) found an opposite result (variant homozygous individuals presented lower levels of damage) in a pesticide-exposed population, our finding is in accordance to what has been previously suggested by other authors (Duell et al. 2000). Indeed, XRCC1 399Gln allele has been associated with increased DNA adducts level (Lunn et al. 1999), increased p53 mutations (Hsieh et al. 2003), and prolonged cell cycle delay (Hu et al. 2002).

Pesticides are not only substrates to metabolizing enzymes, but also act as inhibitors or inducers, in either case often with selectivity for specific isoforms (Hogdson 2010). In the particular case studied herein of multiple exposures, these interactions and inhibitions carried out by pesticides may contribute to conflicting results.

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 and to assess the influence of individual variability on the genotoxic effects.

Results obtained show 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. In addition, it was also observed that in general organic farmers present similar levels of genetic damage that unexposed controls demonstrating that the health status of farm workers may be influenced by the type of agriculture they practice.

Regarding the effect of the genetic polymorphisms on the different biomarkers studied, results suggest that positive genotypes of *GSTT1* and *GSTM1*, *GSTP1* 105Ile/Ile and *XRCC1* 399Gln/Gln genotypes are associated with increased genetic damage, but possible interactions and inhibition processes contribute to the difficult interpretation of the results.

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List of relevant abbreviations used in the manuscript

PPE	personal protective equipment
PYR	pyrethroids
OP	organophosphates
CRB	carbamates
THIO	urinary thioethers
BChE	butyrylcholinesterase
CBMN	cytokinesis-block micronuclei assay

MN-RET	micronuclei in reticulocytes
CA	chromosomal aberrations
CSA	chromosome-type aberrations
CTA	chromatid-type aberrations
TCR	T-cell receptor

References

Au W, Cajas-Salazar N, Salama S. Factors contributing to discrepancies in population monitoring studies. Mutation Research. 1998; 400:467. [PubMed: 9685705]

Aw D, Silva AB, Palmer DB. Immunosenescence: emerging challenges for an ageing population. Immunology. 2007; 120:435–446. [PubMed: 17313487]

Bajpayee M, Dhawan A, Parmar D, Pandey AK, Mathur N, Seth PK. Gender-related differences in basal DNA damage in lymphocytes of a healthy Indian population using the alkaline Comet assay. Mutation Research. 2002; 520:83–91. [PubMed: 12297147]

Bastos H, Antão M, Silva S, Azevedo A, Manita I, Teixeira V, et al. Association of polymorphisms in genes of the homologous recombination DNA repair pathway and thyroid cancer risk. Thyroid. 2009; 19(10):1067–1075. [PubMed: 19772428]

Bhalli JA, Khan QM, Nasim A. DNA Damage in Pakistani Pesticide-Manufacturing Workers Assayed Using the Comet Assay. Environmental and Molecular Mutagenesis. 2006; 47:587–593. [PubMed: 16917935]

Bhalli JA, Khan QM, Khalid AM, Nasim A. Cytogenetic analysis of Pakistani individuals occupationally exposed to pesticides in a pesticide production industry. Mutagenesis. 2006; 21:143–148. [PubMed: 16540494]

Bolognesi C. Genotoxicity of pesticides: a review of human biomonitoring studies. Mutation Research. 2003; 543:251–272. [PubMed: 12787816]

Bolognesi C, Carrasquilla G, Volpi S, Solomon KR, Marshall EJP. Biomonitoring of Genotoxic Risk in Agricultural Workers from Five Colombian Regions: Association to Occupational Exposure to Glyphosate. Journal of Toxicology and Environmental Health Part A. 2009; 72:986–997. [PubMed: 19672767]

Bolognesi C, Lando C, Forni A, Landini E, Scarpato R, Migliore L, et al. Chromossomal damage and ageing: effect on micronuclei frequency in peripheral blood lymphocytes. Age and ageing. 1999; 28:393–397. [PubMed: 10459794]

Bolognesi C, Perrone E, Landini E. Micronucleus monitoring of a floriculturist population from western Liguria, Italy. Mutagenesis. 2002; 17:391–397. [PubMed: 12202626]

Bonassi S, Bolognesi C, Abbondandolo A, Barale R, Bigatti P, Camurri L, et al. Influence of Sex on Cytogenetic End Points: Evidence from a Large Human Sample and Review of the Literature. Cancer Epidemiology Biomarkers & Prevention. 1995; 4:671–679.

Bonassi S, Ugolini D, Kirsch-Volders M, Strömberg U, Vermeulen R, Tucker JD. Human Population Studies With Cytogenetic Biomarkers: Review of the Literature and Future Prospectives. Environmental and Molecular Mutagenesis. 2005; 45:258–270. [PubMed: 15688363]

Bull S, Fletcher K, Boobis AR, Battershill JM. Evidence for genotoxicity of pesticides in pesticide applicators: a review. Mutagenesis. 2006; 21:93–103. [PubMed: 16567350]

Ceppi M, Biasotti B, Fenech M, Bonassi S. Human population studies with the exfoliated buccal micronucleus assay: Statistical and epidemiological issues. Mutation Research/Reviews in Mutation Research. 2010; 705:11–19.

Chng WJ, Tan GB, Kaperan P. Establishment of adult peripheral blood lymphocytes subset reference range for an asian population by single-platform flow cytometry: Influence of age, sex, and race and comparison with other published studies. Clinical and Diagnostic Laboratory Immunology. 2004; 11(1):168–173. [PubMed: 14715565]

Corsini E, Liesivuori J, Vergieva T, Loveren HV, Colosio C. Effects of pesticide exposure on the human immune system. Human & Experimental Toxicology. 2008; 27:671–680. [PubMed: 19042949]

Costa C, Silva S, Coelho P, Roma-Torres J, Teixeira J, Mayan O. Micronucleus analysis in a Portuguese population exposed to pesticides: Preliminary survey. international Journal of Environmental Health. 2007; 210(3–4):415–418.

Costa C, Silva S, Neves J, Coelho P, Costa S, Laffon B, et al. Micronucleus frequencies in lymphocytes and reticulocytes in a pesticide-exposed population in Portugal. Journal of Toxicology and Environmental Health, Part A. 2011; 74(15–16):960–970. [PubMed: 21707421]

Costa, C.; Teixeira, JP.; Mayan, O. Chapter III: Pesticides as Genetic Damage Inducers. In: Miura, S.; Nakano, S., editors. Progress in DNA Damage Research. Nova Science Publishers, Inc; 2008.

Costa C, Teixeira JP, Silva S, Roma-Torres J, Coelho P, Gaspar J, et al. Cytogenetic and molecular biomonitoring of a Portuguese population exposed to pesticides. Mutagenesis. 2006; 21(5):343–350. [PubMed: 16980312]

Costa S, Coelho P, Costa C, Silva S, Mayan Olga, Santos LS, et al. Genotoxic damage in pathology anatomy laboratory workers exposed to formaldehyde. Toxicology. 2008; 252:40–48. [PubMed: 18721846]

Dangour AD, Dodhia SK, Hayter A, Allen E, Lock K, Uauy R. Nutritional quality of organic foods: a systematic review. American Journal of Clinical Nutrition. 2009; 90:680–685. [PubMed: 19640946]

Doorn RV, Leijdekkers C, Bos R, Brouns R, Henderson P. Detection of human exposure to electrophilic compounds by assay of thioether detoxication products in urine. Annals of Occupational Hygiene. 1981; 24(1):77–92. [PubMed: 7015964]

Duell EJ, Wiencke JK, Cheng T-J, Varkoni A, Zuo ZF, Ashok TDS, et al. Polymorphisms in the DNA repair genes *XRCC1* and *ERCC2* and biomarkers of DNA damage in human blood mononuclear cells. Carcinogenesis. 2000; 21(5):965–971. [PubMed: 10783319]

Ergene S, Çelik A, Çavas T, Kaya F. Genotoxic biomonitoring study of population residing in pesticide contaminated regions in Göksu Delta: Micronucleus, chromosomal aberrations and sister chromatid exchanges. Environment International. 2007; 33:877–885. [PubMed: 17493680]

Falck G, Hirvonen A, Scarpato R, Saarikoski S, Migliore L, Norppa H. Micronuclei in blood lymphocytes and genetic polymorphism for GSTM1, GSTT1 and NAT2 in pesticide-exposed greenhouse workers. Mutation Research. 1999; 441:225–237. [PubMed: 10333536]

Fenech M. The in vitro micronucleus technique. Mutation Research. 2000; 455:81–95. [PubMed: 11113469]

Franceschi C, Monti D, Sansoni P, Cossarizza A. The immunology of exceptional individuals: the lesson of centenarians. Immunology Today. 1995; 16(1):12–16. [PubMed: 7880382]

Garaj-Vrhovac V, Zeljezic D. Assessment of Genome Damage in a Population of Croatian Workers Employed in Pesticide Production by Chromosomal Aberration Analysis, Micronucleus Assay and Comet Assay. Journal of Applied Toxicology. 2002; 22:249–255. [PubMed: 12210542]

García-Lestón J, Roma-Torres J, Mayan O, Schroecksnadel S, Fuchs D, Moreira AO, et al. Assessment of Immunotoxicity Parameters in Individuals Occupationally Exposed to Lead. Journal of Toxicology and Environmental Health, Part A. 2012; 75(13–15):807–818. [PubMed: 22788368]

García-Lestón J, Roma-Torres J, Vilares M, Pinto R, Cunha LM, Prista J, et al. Biomonitoring of a population of Portuguese workers exposed to lead. Mutation Research/Genetic Toxicology and Environmental Mutagenesis. 2011; 721:81–88.

Garry VF, Tarone RE, Kirsch IR, Abdallah JM, Lombardi DP, Long LK, et al. Biomarker Correlations of Urinary 2,4-D Levels in Foresters: Genomic Instability and Endocrine Disruption. Environmental Health Perspectives. 2001; 109(5):495–500. [PubMed: 11401761]

Garte S, Gaspari L. Metabolic gene polymorphism frequencies in control populations. Cancer Epidemiology, Biomarkers & Prevention. 2001; 10:1239–1248.

Gaspar J, Rodrigues S, Gil OM, Manita I, Ferreira TC, Limbert E, et al. Combined effects of glutathione S-transferase polymorphisms and thyroid cancer risk. Cancer Genetics and Cytogenetics. 2004; 151:60–67. [PubMed: 15120911]

Globerson A, Effros RB. Ageing of lymphocytes and lymphocytes in the aged. Immunology Today. 2000; 21(10):515–521. [PubMed: 11071531]

Gos, J.; Dean, J. Methods for assessing the effects of chemicals on the immune system. In: Bourdeau, P., editor. Short-term toxicity tests for non-genotoxic effects. Jon Wiley & Sons, Ltd; 1990. p. 239-262.

Gruver A, Hudson L, Sempowski G. Immunosenescence of ageing. Journal of Pathology. 2007; 211:144–156. [PubMed: 17200946]

Hagmar L, Bellander T, Persson L, Holmén A, Attewell R, Hogstedt B, et al. Biological effects in a chemical factory with mutagenic exposure. III. Urinary mutagenicity and thioether excretion. International Archives of Occupational and Environmental Health. 1988; 60:453–456. [PubMed: 3410555]

Harries LW, Stubbins MJ, Forman D, Howard GC, Wolf CR. Identification of genetic polymorphisms at the glutathione S-transferase Pi locus and association with susceptibility to bladder, testicular and prostate cancer. Carcinogenesis. 1997; 18 (4):641–644. [PubMed: 9111193]

He F, Sun J, Han K, Wu Y, Yao P, Wang S, et al. Effects of pyrethroid insecticides on subjects engaged in packaging pyrethroids. British Journal of Industrial Medicine. 1988; 45:548–551. [PubMed: 3415921]

Hogdson, E. Metabolism of Pesticides. In: Krieger, R., editor. Hayes' Handbook of Pesticide Toxicology. Elsevier Inc; 2010. p. 893-921.

Hsieh L, Chien H, Chen I, Liao C, Wang H, Jung S, et al. The XRCC1 399Gln polymorphism and the frequency of p53 mutations in Taiwanese oral squamous cell carcinomas. Cancer Epidemiology, Biomarkers & Prevention. 2003; 12:439–443.

Hu J, Smith T, Miller M, Lohman K, Case L. Genetic regulation of ionizing radiation sensitivity and breast cancer risk. Environmental and Molecular Mutagenesis. 2002; 39:208–215. [PubMed: 11921191]

Huppert FA, Solomou W, O'Connor S, Morgan K, Sussams P, Brayne C. Aging and lymphocyte subpopulations:whole-blood analysis of immune markers in a large population sample of healthy elderly individuals. Experimental Gerontology. 1998; 33(6):593–600. [PubMed: 9789736]

Jensen T, Giwercman A, Carlsen E, Scheike T, Skakkebaek N. Semen quality among members of organic food associations in Zeland Denmark. Lancet. 1996; 347:1844. [PubMed: 8667966]

Jentsch-Ulrich K, Koenigsmann M, Mohren M, Franke A. Lymphocyte subsets' reference ranges in an age- and gender- balanced population of 100 healthy adults - A monocentric German study. Clinical Immunology. 2005; 116:192–197. [PubMed: 15993366]

Juhler R, Larsen S, Meyer O, Jensen N, Spano M, Giwercman A, et al. Human semen quality in relation to dietary pesticide exposure and organic diet. Archives of Environmental Contamination and Toxicology. 1999; 37:415–423. [PubMed: 10473800]

Kilpikari I. Correlation of urinary thioethers with chemical exposure in a rubber plant. British Journal of Industrial Medicine. 1981; 38:98–100. [PubMed: 7470409]

Kumaravel T, Vilhar B, Faux SP, Jha AN. Comet Assay measurements: a perspective. Cell Biology and Toxicology. 2009; 25:53–64. [PubMed: 18040874]

Laffon B, Fraga-Iriso R, Perez-Cadahía B, Mendez J. Genotoxicity associated to exposure to prestige oil during autopsies and cleaning of oil-contaminated birds. Food and Chemical Toxicology. 2006; 44:1714–1723. [PubMed: 16814914]

Lepage L, Schiele F, Gueguen R, Siest G. Total cholinesterase in plasma: biological variations and reference limits. Clinical Chemistry. 1985; 31(4):546–550. [PubMed: 3978785]

Lin D-X, Tang Y-M, Peng Q, Lu S-X, Ambrosone CB, Kadlubar FF. Susceptibility to Esophageal Cancer and Genetic Polymorphisms in Glutathione S-Transferases T1, P1, and M1 and Cytochrome P450 2E1. Cancer Epidemiology, Biomarkers & Prevention. 1998; 7:1013–1018.

Liu Y-J, Huang P-L, Chang Y-F, Chen Y-H, Chio Y-H, Xu Z-L, et al. *GSTP1* genetic polymorphism is associated with a higher risk of DNA damage in pesticide-exposed fruit growers. Cancer Epidemiology Biomarkers & Prevention. 2006; 15:659–666.

Lotti, M. Clinical Toxicology of Anticholinesterase Agents in Humans. In: Krieger, R., editor. Hayes' Handbook of Pesticide Toxicology. Elsevier Inc; 2010. p. 1543-1589.

Lunn R, Langlois R, Hsieh L, Thompson C, Bell D. XRCC1 polymorphisms: effects on aflatoxin B1-DNA adducts and glycophorin A variant frequency. CAncer Research. 1999; 59:2557–2661. [PubMed: 10363972]

Mage DT, Alavanja MCR, Sandler DP, McDonnell CJ, Kross B, Rowland A, et al. AModel for Predicting the Frequency of High Pesticide Exposure Events in the Agricultural Health Study. Environmental Research Section A. 2000; 83:67–71.

McClure G, Helm R, Stine K, Burks A, Jones S, Gandy J. Evaluation of Immune Parameters in Propanil-Exposed Farm Families. Archives of Environmental Contamination and Toxicology. 2001; 41:104–111. [PubMed: 11385596]

Mikov I, Milosevic M, Mikov A, Mikov M. Increased urinary excretion of thioethers as a marker for detecting exposure to herbicide containing 2,4-dichlorophenoxyacetic acid dimethylamine - experimental study on mice. Annals of Agricultural and Environmental Medicine. 2000; 7:61–63. [PubMed: 10865247]

Moller P. Assessment of reference values for DNA damage detected by the comet assay in human blood cell DNA. Mutation Research. 2006; 612:84–104. [PubMed: 16314140]

Moller P, Knudsen LE, Loft S, Wallin H. The comet assay as a rapid test in biomonitoring occupational exposure to DNA-damaging agents and effect of counfounding factors. Cancer Epidemiology, Biomarkers & Prevention. 2000:1005–1015.

PAN. List of Lists:Pesticide Action Network. 2009

Panda A, Arjona A, Sapey E, Bai F, Fikrig E, Montgomery RR, et al. Human innate immunosenescence: causes and consequences for immunity in old age. Trends in Immunology. 2009; 30(7):325–333. [PubMed: 19541535]

Pastor S, Lucero L, Gutiérrez S, Durban R, Gomez C, Parron T, et al. A follow-up study on micronucleus frequency in Spanish agricultural workers exposed to pesticides. Mutagenesis. 2002; 17(1):79–82. [PubMed: 11752238]

Peck, EC.; Eaton, DL. Genetic Polymorphisms and Susceotibility to Pesticides. In: Krieger, R., editor. Hayes' Handbook of Pesticide Toxicology. Elsevier Inc; 2010. p. 525-549.

Pérez-Cadahía B, Méndez J, Pásaro E, Lafuente A, Cabaleiro T, Laffon B. Biomonitoring of human exposure to prestige oil: Effects on DNA and endocrine parameters. Environmental Health Insights. 2008; 2:83–92. [PubMed: 21572833]

Piperakis SM, Kontogianni K, Siffel C, Piperakis MM. Measuring the Effects of Pesticides on Occupationally Exposed Humans with the Comet Assay. Environmental Toxicology. 2006; 21(4): 355–359. [PubMed: 16841319]

Piperakis SM, Petrakou E, Tsilimigaki S, Sagnou M, Monogiudis E, Haniotakis G, et al. Biomonitoring With the Comet Assay of Greek Greenhouse Workers Exposed to Pesticides. Environmental and Molecular Mutagenesis. 2003; 41:104–110. [PubMed: 12605379]

Raftogianis R, Creveling C, Weinshilboum R, Weisz J. Chapter 6: Estrogen Metabolism by Conjugation. Journal of the National Cancer Institute. 2000; 27:113–124. [PubMed: 10963623]

Remor AP, Totti CC, Moreira DA, Dutra GP, Heuser VD, Boeira JM. Occupational exposure of farm workers to pesticides: Biochemical parameters and evaluation of genotoxicity. Environmental International. 2009; 35:273–278.

Riggs M, Rosen PB, Snodgrass W. Dietary effect on urinary thioethers. Archives of Environmental Health. 1999

Sailaja N, Chandrasekhar M, Rekhadevi PV, Mahboob M, Rahman MF, Vuyyuri SB, et al. Genotoxic evaluation of workers employed in pesticide production. Mutation Research. 2006; 609:74–80. [PubMed: 16887377]

Salama SA, Au WW, Hunter GC, Sheahan RG, Badary OA, Abdel-Naim AB, et al. Polymorphic metabolizing genes and susceptibility to atherosclerosis among cigarette smokers. Environmental and Molecular Mutagenesis. 2002; 40(3):153–160. [PubMed: 12355548]

Schleiermach E. Chromosome Aberrations in Mitoses and Meioses in vivo. Archives of Toxicology. 1971; 28:105–114. [PubMed: 5123870]

Shadnia S, Azizi E, Hosseini R, Khoei S, Fouladdel S, Pajoumand A, et al. Evaluation of oxidative stress and genotoxicity in organophosphorus insecticide formulators. Human & Experimental Toxicology. 2005; 24:439–445. [PubMed: 16235732]

Shaham J, Kaufman Z, Gurvich R, Levi Z. Frequency of sister-chromatid exchange among greenhouse farmers exposed to pesticides. Mutation Research. 2001; 491:71–80. [PubMed: 11287300]

Shahghasempour S, Gerami M, Entezami Z. Enumeration of peripheral blood lymphocyte subsets in a healthy iranian population. Archives of Iranian Mdicine. 2001; 4(2):80–83.

Silva SN, Moita R, Azevedo AP, Gouveia R, Manita I, Pina JE, et al. Menopausal age and XRCC1 gene polymorphisms: Role in breast cancer risk. Cancer Detection and Prevention. 2007; 31(4): 303–309. [PubMed: 17935911]

Singh S, Kumar V, Singh P, Thakur S, Banerjee BD, Rautela RS, et al. Genetic polymorphisms of GSTM1, GSTT1 and GSTP1 and susceptibility to DNA damge in workers occupationally exposed to organophosphate pesticides. Mutation Research. 2011; 725:36–42. [PubMed: 21736951]

Sohal RS. Role of oxidative stress and protein oxidation in the aging process. Free Radical Biology & Medicine. 2002; 33(1):37–44. [PubMed: 12086680]

Teixeira J, Gaspar J, Silva S, Torres J, Silva S, Azevedo MC, et al. Occupational exposure to styrene: modulation of cytogenetic damage and levels of urinary metabolites of styrene by polymorphisms in genes CYP2E1, EPHX1, GSTM1, GSTT1 and GSTP1. Toxicology. 2004; 195:231–242. [PubMed: 14751678]

Therman, E., editor. Human Chromosomes: Structure, Behavior, Effects. New York: Springer-Verlag; 1980.

Vainio H, Savolainen H, Kilpikari I. Urinary thioether of employees of a chemical plant. British Journal of Industrial Medicine. 1978; 35:232–234. [PubMed: 698138]

Varona M, Cárdenas O, Crane C, Rocha S, Cuervo G, Vargas J. Alteraciones citogenéticas en trabajadoras con riesgo ocupacional de exposición a plaguicidas en cultivos de flores en Bogotá. Biomedica. 2003; 23:141–152. [PubMed: 12872553]

Vershenya S, Biko J, Drozd V, Lorenz R, Reiners C, Hempel K. Dose response for T-cell receptor (TCR) mutants in patients repeatedly treated with ^{131}I for thyroid cancer. Mutation Research. 2004; 548:27–33. [PubMed: 15063133]

Weiskopf D, Weinberger B, Grubek-Loebenstein B. The aging of the immune system. Transplantation International. 2009; 22:1041–1050.

Werf H. Assessing the impact of pesticides on the environment. Agriculture, Ecosystems and Environment. 1996; 60:81–96.

WHO. Organization WH. Public health impact of pesticides used in agriculture. Geneva: 1990. p. 140

Wong R-H, Chang S-Y, Ho S-W, Huang P-L, Liu Y-J, Chen Y-C, et al. Polymorphisms in metabolic GSTP1 and DNA-repair XRCC1 genes with an increased risk of DNA damage in pesticide-exposed fruit growers. Mutation Research/Genetic Toxicology and Environmental Mutagenesis. 2008; 654:168–175.

Zhang Z, Sun J, Chen S, Wu Y, He F. Levels of exposure and biological monitoring of pyrethroids in spraymen. British Journal of Industrial Medicine. 1991; 48:82–86. [PubMed: 1998612]

Zhijian C, Jianlin L, Shijie C, Wei Z, Weib Wu, Lifen J, et al. Evaluating the genotoxic effects of workers exposed to lead using micronucleus assay, comet assay and TCR gene mutation test. Toxicology. 2006; 223:219–226. [PubMed: 16713056]

Table 1

Characteristics of study group

		Study group		
		Unexposed Controls (n=61)	**Organic Farmers (n=36)**	**Pesticide workers (n=85)**
Age[a]				
	(years)	39.5 ± 12.3	39.6 ± 14.5	40.0 ± 12.2
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)[1]				
	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				

	Study group		
	Unexposed Controls (n=61)	**Organic Farmers (n=36)**	**Pesticide workers (n=85)**
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%)
Previous intoxications			
No			79 (92.9%)
Yes			6 (7.1%)

[a] Mean ± SD; PPE: personal protective equipment

[1] Ten of the exposed individuals were not able to report the chemical concerning their last exposure

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
Fungicides	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
Insecticides	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
Herbicides	Paraquat dichloride	Bipyridylium	E	II
Aphicides	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.

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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	6425.44±224.15 (3347 – 9083)	29	6245.62±191.41 (4408 – 8647)	56	7063.66±202.31 (4142 – 9707)		
Class 2	7	5965.57±642.23 (3902 – 8966)	6	5803.83±661.51 (3964 – 7890)	21	6240.43±289.27 (3539 – 9044)	0.943	0.059
Class 3	13	6553.31±443.78 (4944 – 10192)	1	7074 (7074 – 7074)	7	6950.86±466.79 (5485 – 9050)		

[a, b] Homogeneous groups according to multiple comparison Tukey's test;

[c] classes established according to age and gender; nd – not detected; creat – creatinine.

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.

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)
Previous intoxications								
No	78	1.00	78	1.00	73	1.00	73	1.00
Yes	6	0.43 (0.22, 0.81)**	5	1.39 (0.47, 4.17)	4	0.17 (0.04, 0.68)*	6	1.15 (0.63, 2.12)

*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.

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%	
Tyr/His	45	25.0%	15	24.6%	9	25.7%	21	25.0%	0.913
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%	
His/Arg	71	39.0%	20	32.8%	13	36.1%	38	44.7%	0.673
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%	
Ile/Val	84	46.2%	31	50.8%	20	55.6%	33	38.8%	0.354
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%	
Arg/Trp	14	7.7%	1	1.6%	4	11.1%	9	10.6%	0.202
Trp/Trp	1	0.5%	0	0.0%	0	0.0%	1	1.2%	
***XRCC1* codon 399**									

	Study group								
	n	All	n	Unexposed Controls	n	Organic Farmers	n	Pesticide workers	p-value
Arg/Arg	62	34.1%	19	31.1%	14	38.9%	29	34.1%	
Arg/Gln	83	45.6%	28	45.9%	17	47.2%	38	44.7%	0.843
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%	
Arg/His	50	27.5%	10	16.4%	9	25.0%	31	36.5%	0.068
His/His	3	1.6%	2	3.3%	0	0.0%	1	1.2%	