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RESEARCH ARTICLE

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Assessment of DNA damages in lymphocytes of agricultural workers exposed to pesticides by comet assay in a cross-sectional study

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ABSTRACT

Purpose: To assess the predictive power of the comet assay in the context of occupational exposure to pesticides.

Materials and methods: The recruited subjects completed a structured questionnaire and gave a blood sample. Exposure to pesticides was measured by means of an algorithm based on Dosemeci's work (Agricultural Health Study). Approximately 50 images were analyzed for each sample via fluorescence microscopy. The extent of DNA damage was estimated by tail moment (TM) and is the product of tail DNA (%) and tail Length.

Results: Crude significant risks (odds ratios, ORs) for values higher than the 75th percentile of TM were observed among the exposed subjects (score > 1). The frequency of some confounding factors (sex, age and smoking) was significantly higher among the exposed workers. A significant dose–effect relationship was observed between TM and exposure score. Significant high-risk estimates (ORs), adjusted by the studied confounding factors, among exposure to pesticides and TM, % tail DNA and tail length were confirmed using unconditional logistic regression models.

Conclusions: The adjusted associations (ORs) between the comet parameters and exposure to pesticides were significant. The sensitivity of the comet test was low (41%), the specificity (89%) and the predictive positive value (0.77) were found acceptable.

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KEYWORDS
Comet assay; lymphocytes; pesticides; DNA damage; exposure score

Introduction

Evaluation of DNA damage by the comet assay

Direct DNA damage of single-stranded DNA or DNA strand breaks can be caused by chemical agents or their metabolites (Sies 1985, Barnett and Barnett 1998, Van Gent *et al.* 2001, Jackson 2002, Krejci *et al.* 2003), the processes of DNA excision, replication and recombination or by apoptosis processes (Eastman and Barry 1992) or the interaction of reactive oxygen species (ROS) with DNA (Møller and Wallin 1998). The comet assay, or single cell gel electrophoresis (SCGE), is a genotoxicity test that exploits the ability of DNA to migrate when immersed in an electric field (Lindahl and Andersson 1972), allowing it to cumulatively evaluate both DNA damage and alkaline-labile sites in any type of eukaryotic cell; alkaline labile sites exhibit alkylation of phosphate and are susceptible to filament rupture related to pH and alkaline exposure (Azqueta *et al.* 2014). This simple economical technique has been used for several years in various applications, such as for in vitro and in vivo tests of the potential genotoxicity of substances and preparations, biomonitoring of human exposure to mutagenic agents (Anderson *et al.* 2013), or evaluations of the efficacy of DNA repair systems (Rojas *et al.* 1999, Collins 2004). The combination of the standard comet assay with enzymes that recognize oxidized nucleotides and cut the DNA backbone have made this technique a valuable means to study oxidative damage of DNA (Collins 2014a).

Integral DNA filaments migrate rarely and tend to concentrate in a nucleoid, while fragments generated by filament or double-strand breaks migrate in inverse proportion to their length and molecular weight (Grandi *et al.* 2006). Therefore, after electrophoresis, when viewed under a fluorescence microscope, each cell yields a comet-shaped image is obtained, the head of which consists of the integral DNA and the tail of which consists of DNA fragments of varying length that have migrated away from the nucleus. The images are analyzed using a software that identifies and delimits the

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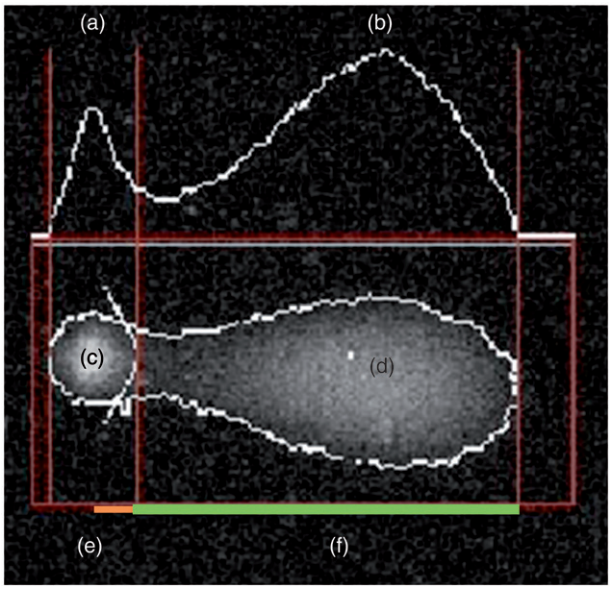

Figure 1. Analysis of the image of a comet. (a) Head DNA; (b) tail DNA; (c) head area; (d) tail area; (e) head radius; (f) tail length.

areas that constitutes the head and the one constituting the tail (Figure 1) (Collins *et al.* 1997, 2001).

Similar to other genotoxicity tests, and in light of the currently available studies, the comet assay is indicative of the potential genotoxicity of exposures for individuals but is not predictive of their cancer risk.

The comet assay has proven to be a valid predictor of DNA damage due to various types of pesticides in Colombia (Varona-Uribe *et al.* 2016), Brazil (Khayat *et al.* 2013), India (Kaur *et al.* 2011) and Argentina (Simoniello *et al.* 2010), and it has been used for various crops, such as soybean (Benedetti *et al.* 2013), orchards (Kasiotis *et al.* 2012), vineyards (Kvitko *et al.* 2012) and tobacco (Da Silva *et al.* 2012, 2014).

Diffusion of pesticides in the study area

The study area was located in Italy between two provinces (Bari and Taranto) of the Apulia region where the most relevant agricultural production is based on vegetables, grapes and olive trees. In this area, the most used insecticides are chlorpyrifos and deltamethrin. Chlorpyrifos is an organophosphate (phosphorothioate) that undergoes oxidative desulfuration to form Chlorpyrifos-oxon, which is generally able to phosphorylate acetylcholinesterase, with a reduction in the metabolism of acetylcholine to choline and acetate. The ensuing neurotoxicant effects are currently an area of interest in terms of the oxidative DNA damage response (Thakur *et al.* 2017). Deltamethrin (Type 2 Pyrethroid) has recently been shown to have a potential carcinogen effect in mouse skin, but the underlying mechanism remains elusive (George and Shukla 2013). The most used herbicides were glyphosate and dimethoate. Glyphosate (a nonselective nitro-aniline compound) has only a low irritative effect, and in many recent studies, no consistent association was found with multiple myeloma (De Roos *et al.* 2005, Burstyn and De Roos 2016). Dimethoate (an organophosphate) affects hemoglobin contents and hematocrit, but shows no effects on total blood cell counts. It has been demonstrated that the mouse gastric tissue has the potential to become cancerous (Wang *et al.* 2013). Finally, the most diffuse fungicides were mancozeb and fosetyl-aluminum. Mancozeb (a thiocarbamate) yields a minimal irritant reaction and is a sensitizer when combined with in thiophanate. This compound is responsible for DNA damage and may be involved in the pathogenesis of diseases including cancer (Calviello *et al.* 2006). Fosetyl-aluminum (a systemic fungicide based on an aluminum salt of an organic compound) is classified as a minimal irritant, and DNA adducts were reported for treated Pacific oysters (Geret *et al.* 2013).

The main purposes of this study were to assess the association between the chronic pesticide exposure of agricultural workers operating in the provinces of Bari and Taranto and the DNA damage of peripheral lymphocytes, to evaluate the predictive positive value (PPV) of the comet assay and to assess a dose–effect relationship between the increase of the pesticide exposure score and the most relevant comet parameter [tail moment (TM)]. This is a cross-sectional study based on a group of agricultural workers exposed to various pesticides (insecticides, herbicides and fungicides) and a group of unexposed subjects.

Clinical significance

- The study of oxidative stress induced in peripheral lymphocytes by aneuploidizing substances may be useful in the future to identify biological markers that predict malignancy to evaluate DNA repair. Many studies have been designed to assess the predictive power of the comet assay in the context of occupational exposure to pesticides.
- The comet assay is able to estimate risks (ORs) to observe lymphocytes DNA oxidative damages parameters (TM, % tail DNA and tail length) among subjects exposed to pesticides adjusted by the studied confounding factors.
- The specificity of the test was found to be acceptable (89%), and the PPV was also acceptable (77%).

Methods

The study of the comet assay, which is part of the biological studies related to a larger project, was approved by the Ethics Committee of the "Policlinico-Giovanni XXIII" University Hospital of Bari, Italy.

Study groups

The subjects involved in the study are 22 agricultural workers from the provinces of Bari and Taranto, and 24 nonexposed subjects living in the same areas. The first group was selected from agricultural enterprises in Bari and Taranto and the nonagricultural group from hematologic outpatients of the University Hospital as well as University volunteers. Overall, 2374 images were globally analyzed. The study size was of 1122 images for the exposed group and 1252 for the nonexposed group. After signing an informed consent form, each subject gave a venous blood sample. All recruited

subjects completed two questionnaires: one general, aimed at gathering identification and socio-demographic data, those about schooling, family history, pathological history, habits such as smoking and alcohol, and working history, and a second questionnaire related to specific aspects of exposures during farm work. For each participant, two fresh blood cells aliquots (OECD 2014) were immediately prepared prior to the start of the test by treating the samples under yellow light and paying particular attention to avoiding DNA damage via direct exposure to UV light (Nowsheen *et al.* 2012).

Comet assay

The technique was conducted on the basis of the standardized comet assay methodology described by Hartmann *et al.* (2003a, 2003b) and Burlinson *et al.* (2007), according to the recommendations reported by Araldi *et al.* (2015). The test is now recognized as a method for human biomonitoring in accordance with FDA and WHO guidelines (Singh 2016).

For the comet assay, we used FLUO Plus DNA stain®, a new, very sensitive nuclear fluorophore, whit high affinity to single-stranded DNA and double-stranded DNA (ssDNA and dsDNA), with virtually no background signal that proved to be very sensitive. Cell specimens were prepared using the following components: lysis solution (Lysis Sol); low melting point agarose (LMPA); disodium EDTA, 0.5 M; neutralizing buffer solution (Neutral Sol); FLUO Plus; slides; doubly distilled water; sodium hydroxide pellets; bovine serum solution (PBS), free of Ca^{2+} and Mg^{2+} ions (to avoid endonuclease activity); and dimethyl sulfoxide (DMSO).

Peripheral heparin blood was diluted 1:1 with PBS. The buffy coat was separated via centrifugation on a density gradient at 800 rotations per minute (rpm) for 20 min; the buffy coat is where lymphocytes are concentrated. Cells were counted in a Neubauer chamber, and vitality was verified through trypan blue, which allows the identification of nonviable cell membranes, for which the cell membrane had become permeable. The comet assay requires that at least 75% of the cells are vital. Vital cells were counted in the Neubauer chamber prior to addition to pre-pretreated slides (Singh sandwich technique) (Singh *et al.* 1988) and were coated with layered agarose gel. On a third layer was placed the cell suspension for analysis, followed by another gel layer of agarose, which was melted at the time. For the test to be valid, the number of cells should be between 1 and 20×10^4. Counting is followed by the cell lysis (1 h, dark, 4 °C), and unwinding with alkaline solution at pH 13, which tests damage to both the single and the double filaments by electrophoresis: DNA, negatively charged, migrates to the positive pole proportionally to its damage and at an inverse speed proportional to the size of its fragments. After neutralization, DNA was stained with FLUO Plus® (excitation wave length: 490 nm, emission wave length: 535 nm), which emits green fluorescence when bound to DNA. Finally, within 48 h, a fluorescence microscope (50 randomized comets, 20–40× magnifications) was read and linked to the "Comet Assay Laboratory Universal Computer Image Analysis" software for image analysis (Figure 1). For each sample, 20–25 cells or comets were randomly selected to give a total of 40–50 records per subject, for statistical analysis purposes. The interpretation of images is based on their classification into five classes: class 0 = tail-free comet, no DNA damage index; class 1 = slight damage to DNA due to the presence of a few fragments that create a single around the head of the comet; class 2 = moderate DNA damage; class 3 = extended damage; and class 4 = almost completely fragmented DNA (remarkable tail length) (Figure 2). The software allows the calculation of the following parameters: length of the comet head (head length); tail length (L); fluorescence intensity, expressed as the percentage of DNA in the head (% head DNA) and the percentage of DNA present in the tail (% tail DNA, I); TM equal to the product L × I and the TM mean; and the damage index (DI), that is the sum of (% of class 1 × 1 cells) + (% of class 2 × 2 cells) + (% of class 3 × 3 cells) + (% of class 4 × 4 cells). The DI therefore has a range from 0 (if all cells are of class 0) to 400 (if all cells are of class 4). The software returns a table of useful values as an Excel file to interpret the results of the comet assay.

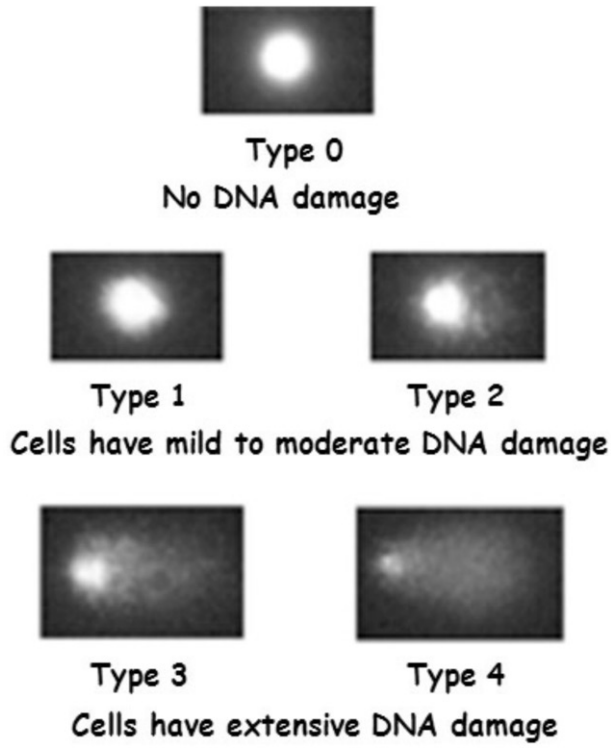

Figure 2. Classes of DNA damage. Source: Waters DJ *et al.* (2007).

Retrospective evaluation of pesticide exposure

An algorithm based on the experience of Dosemeci *et al.* (2002) within the Agricultural Research Study conducted by the National Cancer Institute in the United States was developed for the definition of an exposure intensity score and it was applied to the recruited subjects. Specifically, information was collected using dedicated questionnaire related to the use of mixing systems, pesticide application methods, the presence or absence of wet clothing, personal hygiene,

the use of gloves and personal protective equipment, the repair of tanks and the use of tractors with cabins.

Using the following *items* is possible to calculate an intensity level score (IL):

$$IL = [(m \times e)) + (a \times c) + r1 + W] \times p \times r2 \times h \times s$$

m = MIX (conditions for product mixing) [0 = no mix; 9 = mix]; e = ENCLOSED (use of an included mixing system) [0.5 = present; 1 = nonpresent]; a = APPL (application method) [0 = none system; 1 = air application system; 2 = furrow application system; 3 = spraying bar system; 8 = shoulder pump system; 9 = motorpump system; 9 = shoulder atomizer system; 9 = towing atomizer system]; c = CAB (tractor equipped with a cabin and/or with activated carbon filter) [0.1 = cabin with filter; 0.5 = cabin without filter; 1 = no cabin no filter]; r_1 = REPAR (use of PPE) [0 = Yes; 2 = No); W = WASH (equipment washing after application) [0 = no wash; 0.5 = sprinkler rinsing; 0.5 = tractor rinsing; 3 = cleaning of nozzless; 1 = tank cleaning]; p = PPE (personal protective equipment types used) [1 = non PPE use or hat; 0.8 = dust mask/facial screen/goggles/gloves/overall; 0.7 = cartridge respirators/antigas mask/resistant boots/disposable clothing; 0.6 = chemical resistant gloves]; r_2 = REPL* (frequency of replacement of worn gloves) [1 = after single use; 1.1 = one time/month; 1.2 = when they are consumed]; h = HYG (types of personal hygiene, e.g., clothes-change habits, washing of arms and hands, and/or shower or bath habits) [0.2 = changes clothes immediately after/wears disposable clothing/washes arms and hands immediately after/bathes or showers immediately after/bathes or showers for lunch; 0.4 = change clothes immediately after/wears disposable clothing and/or washes arms and hands for lunch or at the end of the day; 0.8 = changes clothes the day after or bathes or showers at the end of the day; 1 = changes clothes on the weekend or washes arms and hands at the end of the day]; s = SPILL (behavior in the event of spills of products on work clothes) [1 = changes clothes immediately after; 1 = wears disposable clothing; 1.1 = changes clothes for lunch; 1.2 = changes clothes at the end of the next day; 1.4 = changes clothes at the end of the day after]. Every recruited exposed subject was assigned a variable score for IL. Every unexposed recruited subject was assigned a score of IL equal to 0 (Dosemeci *et al.* 2002).

Statistical analysis

Two slides per recruited subject and an average of 25 images for every slide were analyzed (25 images per slide [total images = 50]) based on previous experience (Grandi *et al.* 2006). The images were finalized to the increase of the power of the comet parameters measurements. Samples were taken from patients to prepare slides at a single time point, and the choice of two slides per sample was finalized to the increase certainty and give at least 25 measurements also in the case of technical mistakes. Slides were prepared just after the blood draw.

The difference in the two groups was due to difference between agricultural workers and nonagricultural workers. Unexposed people may be present among the agricultural worker group, and exposed people may be present among the nonagricultural workers.

Therefore, power was not assessed for a case-control study with outcomes such as disease or death but for a (transitional) cross-sectional study, primarily based on multiple image measurements for every participant with an overall number of 1122 images for the exposed group and 1252 for the nonexposed group. Supposing a $Z\ \alpha/2 = 1.96$, $Z\ \beta = 0.84$, an expected probability of exposure among controls ($\pi 1$) = 0.47 and an expected probability of exposed among Comet positive subjects ($\pi 2$) = 0.57 with a difference of 0.10, this would require approximately 366 observations for the group for a power of 0.84. Thus, with 1122 observations for the exposed group and 1252 for the controls, the power is very strong.

Statistical analysis was performed using STATA 12 software. Preliminary statistical evaluation of the measured parameters was performed. All the measured parameters are quantitative variables and the exposure score is a quantitative estimate. All the measured parameters were dichotomized using a cutoff value corresponding to the 75th percentile of their cumulative distributions, because the distributions were not-normal. Then, for all the measured parameters, the risks (ORs) were calculated to determine values above the 75th percentile that were associated with pesticide exposure. The risks associated with some important individual variables were also studied, and a multivariate analysis was used to adjust the estimates for the confounding variables using an unconditional logistic regression model. Natural logarithmic transformation of all the parameters was performed, and a linear regression model was used to verify the presence of a dose–effect relationship between an increase in exposure score and the individual log transformed parameters. Finally, an evaluation of the PPV and a sensitivity and specificity assessment of the comet assay related to the pesticides exposure were also performed.

The test used for Table 1 is the Pearson's chi-square test, which has two degrees of freedom for age classes and one degree of freedom for sex and smoking habits.

Multicollinearity was not evaluated for the linear regression model, because it was not a "multiple" regression model but only an instrument to study the linear dose–effect relationship between the TM and the exposure score.

In the multiple unconditional logistic regression, there were assessed nine different multiple models with only one measured parameter and tree different confounding variables. For specificity and sensitivity, no statistical test was used. The positive and negative likelihood ratios were calculated, but were not reported due to their irrelevance. The accuracy was also calculated. The unconditional logistic method was used due to the nonparametric distribution of the measured parameters. This model was more congruent then the conditional one because the logistic model for outcome probabilities and the related assessment of the PORs must be adapted to our cross sectional (transitional study) (Breslow and Day 1980). For parameter distribution testing, the skewness and kurtosis values were calculated.

Table 1. General characteristics of the two groups.

	Exposure groups							
	1 ($n = 1122$)		0 ($n = 1252$)		Total ($n = 2374$)			
	n	%	*n*	%	*n*	%	Pearson's chi-square	*p*
Age (years)								
≤38	96	8.6	475	37.9	571	24.1	–	–
38–51	533	47.5	196	15.7	729	30.7	–	–
>51	493	43.9	581	46.4	1074	45.2	409	0.000
Sex								
Females	49	4.4	126	10.1	175	7.4	–	–
Males	1073	95.6	1126	89.9	2199	92.6	28	0.000
Smoking habits								
No smokers	727	64.8	1108	88.5	1835	77.3	–	–
Smokers	395	35.2	144	11.5	539	22.7	189	0.000

Results

The studied pesticides were herbicides, fungicides and insecticides. The most important crops were vegetables (80%), grapes (10%) and olives (10%). The used insecticides were chlorpyrifos and deltamethrin. The used herbicides were glyphosate and dimetoate. The used fungicides were mancozeb and fosetyl. All the recruited subjects used individual protective devices (mask, gloves and suits), and there were reportedly at least three treatments per year.

An individual exposure score (semi-quantitative evaluation) was assigned using the Dosemeci algorithm. All the subjects exposed to pesticides had scores above 1. Images of nuclei with tails, indicating greater DNA damage, were commonly observed among the pesticides-exposed workers (Figure 3). In unexposed subjects, lymphocytes showed minor DNA damage or quiescent nuclei (Figure 4).

Overall, 2374 images were globally analyzed. The study size was of 1122 slides for the exposed group and 1252 for the unexposed group. Automatic reading of the recruited subjects' slides returned comet parameters with a non-Gaussian distribution, and, thus, a logarithmic transformation that normalized the distributions was performed (Figure 5). A significant dose–effect relationship between pesticide exposure (score) and the natural logarithm of the TM was also found in a significant linear regression [$F = 152.29$; $p(F) \leq .000$; Adj $R^2 = 0.1307$; $t = 12.34$; $p(t) \leq 0.00$; $\beta = 0.20$] (Figure 6). A correlation matrix of log transformed parameters showed significant correlations among the different measures (Figure 7).

Statistical evaluation was performed based on the number of images analyzed that was 1122 in the exposed group and 1252 in the unexposed group. The frequency of subjects in older age groups (≥38 years) was significantly higher in the exposed group than in the unexposed one. Both groups were predominantly male; the frequency of smokers among the exposed group was significantly higher than among the unexposed group (Table 1). We can therefore consider these three characteristics of the groups as potential confounding variables.

To compare the two groups in terms of the different parameters of the comet assay, dichotomic variables were created using a cutoff value corresponding to the 75th percentile. The ORs of the observed parameters values

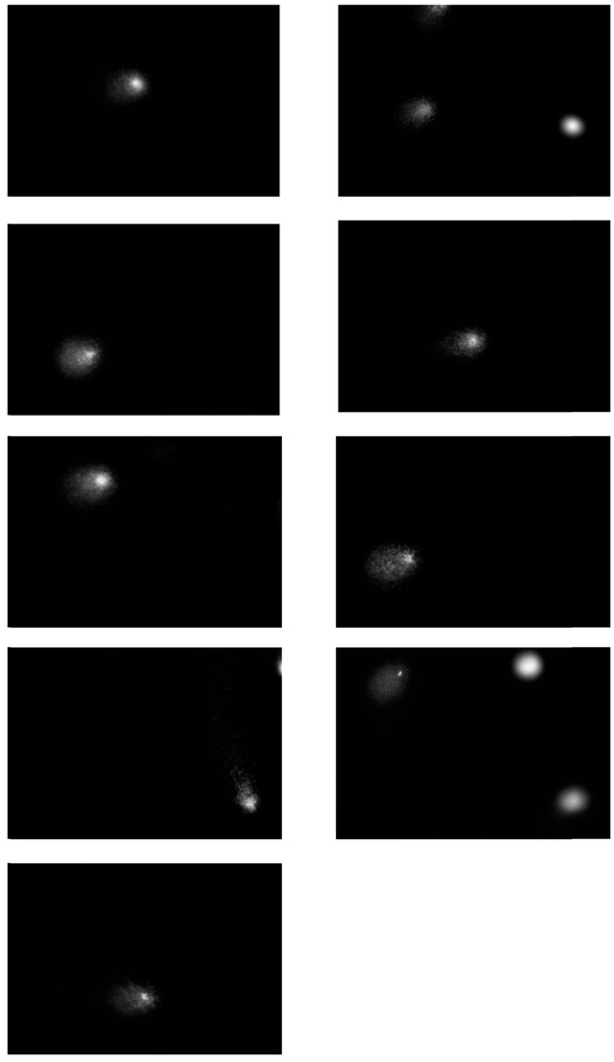

Figure 3. Fluorescence microscopy images of cells corresponding to some pesticide exposed subjects.

categorized above the 75th percentile were significantly higher for tail length (OR = 6.36 [5.1–7.95]) and TM (OR = 5.77 [4.63–7.21]) (Table 2).

Multivariate analysis was applied to each variable that characterize the comet measures.

For the TM, the risk of observing values above the 75th percentile (0.42), adjusted for age and smoking was 4.61

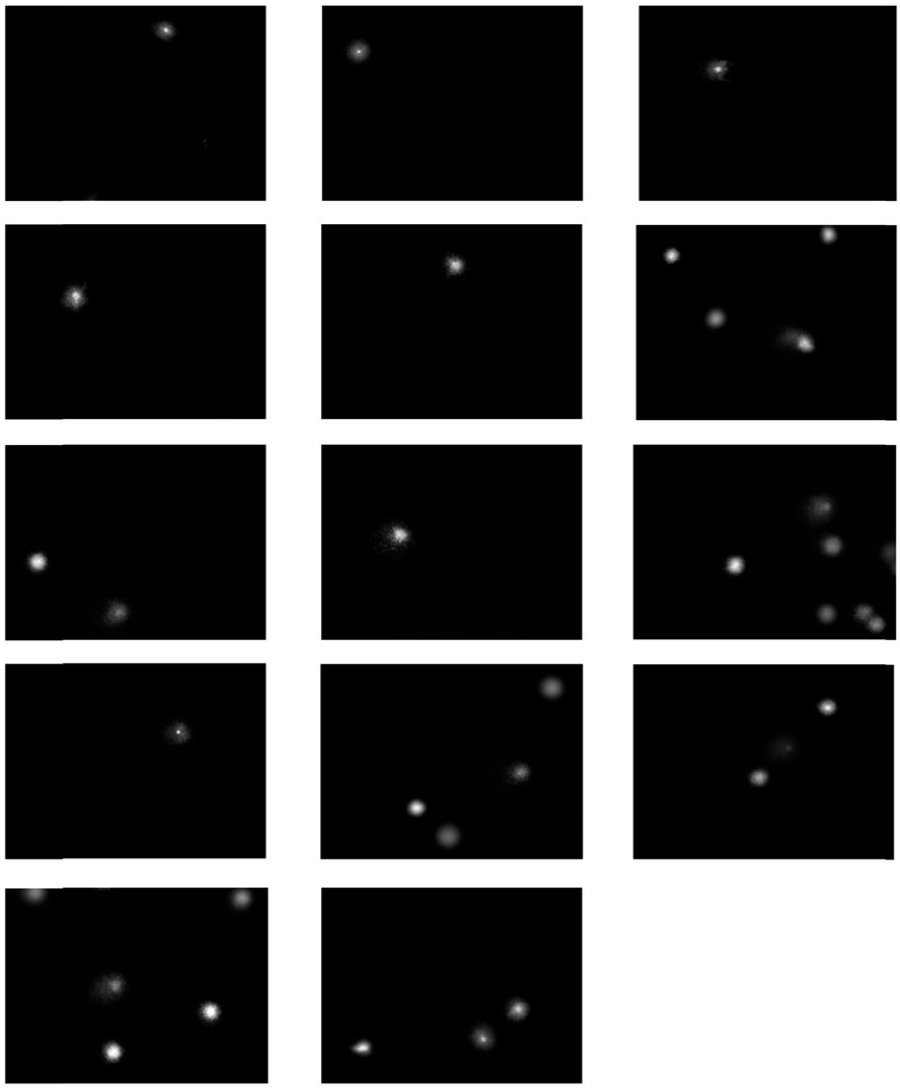

Figure 4. Fluorescence microscopy images of cells of some controls.

[3.84–5.54]; for tail length, it was 4.61 (3.83–5.54); for tail DNA, it was 3.61 (3.02–4.30); for olive moment, it was 3.30 (2.76–3.94); and for tail area, it was 2.46 (2.06–2.94). The integral intensity was 1.35 (1.14–1.60). All of these risks are independent of the confounding factors considered while these factors have shown a differentiated influence among the various parameters. Age had an influence on TM, tail DNA, tail length and olive moment but did not affect tail area and integral intensity. Sex affected TM, integral intensity, tail length and olive moment. Smoke only affected the head DNA (Table 3).

The specificity and the PPV of the comet test were related to the exposure to pesticides (and not to disease). The comet assay's sensitivity to pesticides exposure (the ratio between the number of positive in the comet test for the exposure to pesticides [true positive] and all exposed to pesticides) was low (41%), and there were many false negatives among the exposed workers). The specificity was 89% with was very few false positives among unexposed subjects. The PPV of the test associated with pesticides exposure was 77% (Table 4).

Discussion

Our study used the comet assay as a biomarker for exposure to genotoxic agents. To our knowledge, this is the first study that calculated the OR for comet assay parameters. One possible limitation of the study is the small numbers of subjects. However, many studies are performed with fewer subjects, e.g. 19 females and 12 males (Bausinger and Speit 2016) and 21 welders and 21 nonwelders (Villarini *et al.* 2015).

The results, in addition to confirming the genotoxicity of pesticides used by the study participants, suggest the utility of the comet assay in the biomonitoring of occupational exposure to genotoxic agents. Specifically, the TM and other parameters related to the comet "tail" (% tail DNA, tail length and tail area) that quantitatively express DNA damage showed higher values and a direct correlation to the exposure of subjects to pesticides.

Our findings agree with other studies on pesticides. Using the comet assay, Muhammad *et al.* (2016) showed a strong correlation ($R^2 = 0.91$) between DNA damage in terms of tail

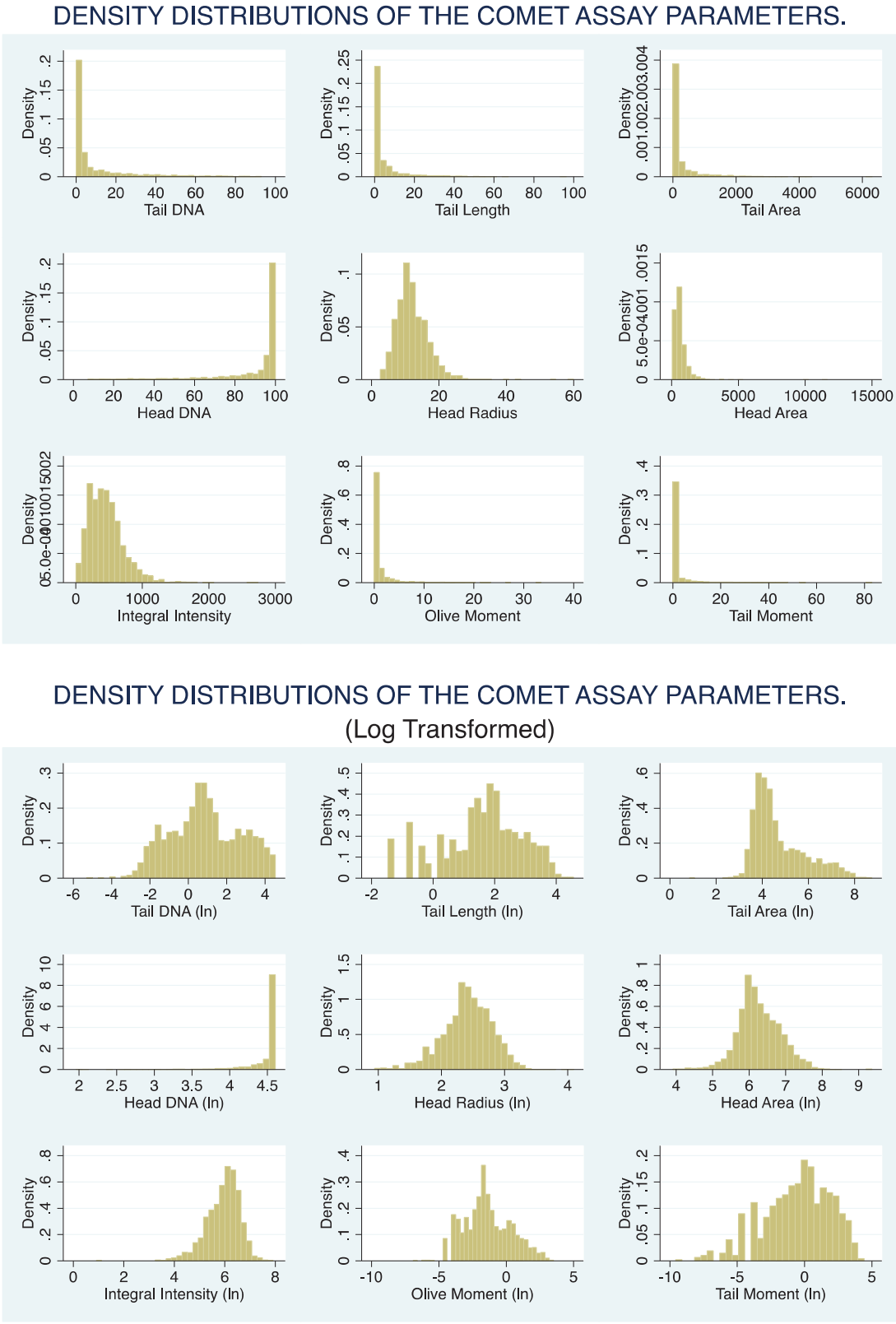

Figure 5. Untransformed and log-transformed distributions of comet assay parameters.

length and the blood concentration of malathion in workers involved in pesticide manufacturing industries. Agricultural workers involved in tobacco collection are regularly exposed to large quantities of pesticides; Alves *et al.* (2016) demonstrated, through the comet assay and micronucleus test, that genetic damage and changes in oxidative balance were induced by workers' exposure to complex mixtures of pesticides in the presence of inorganic compounds. DNA damage was investigated in the exfoliated buccal cells of workers exposed to pesticides in Guerrero, Mexico, using the comet assay and the micronucleus test: the results of the study revealed that DNA migration in the tail and the frequency of

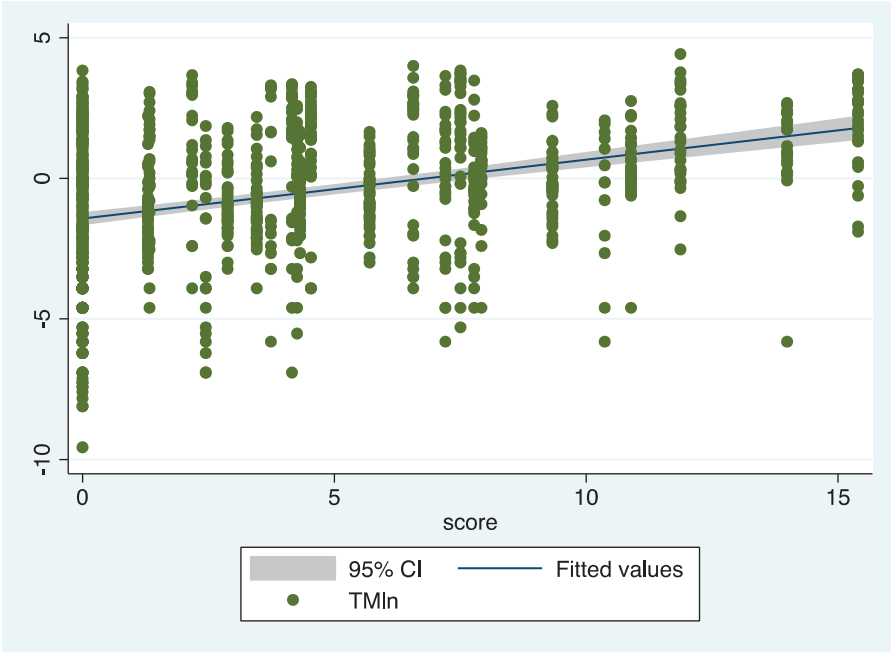

Figure 6. Regression line between Exposure increasing values of pesticides exposure and Tail Moment (Ln). ($F = 152.29$; $p(F) = .000$; Adj $R^2 = 0.1307$; $t = 12.34$; $p(t) = .000$; $\beta = 0.20$).

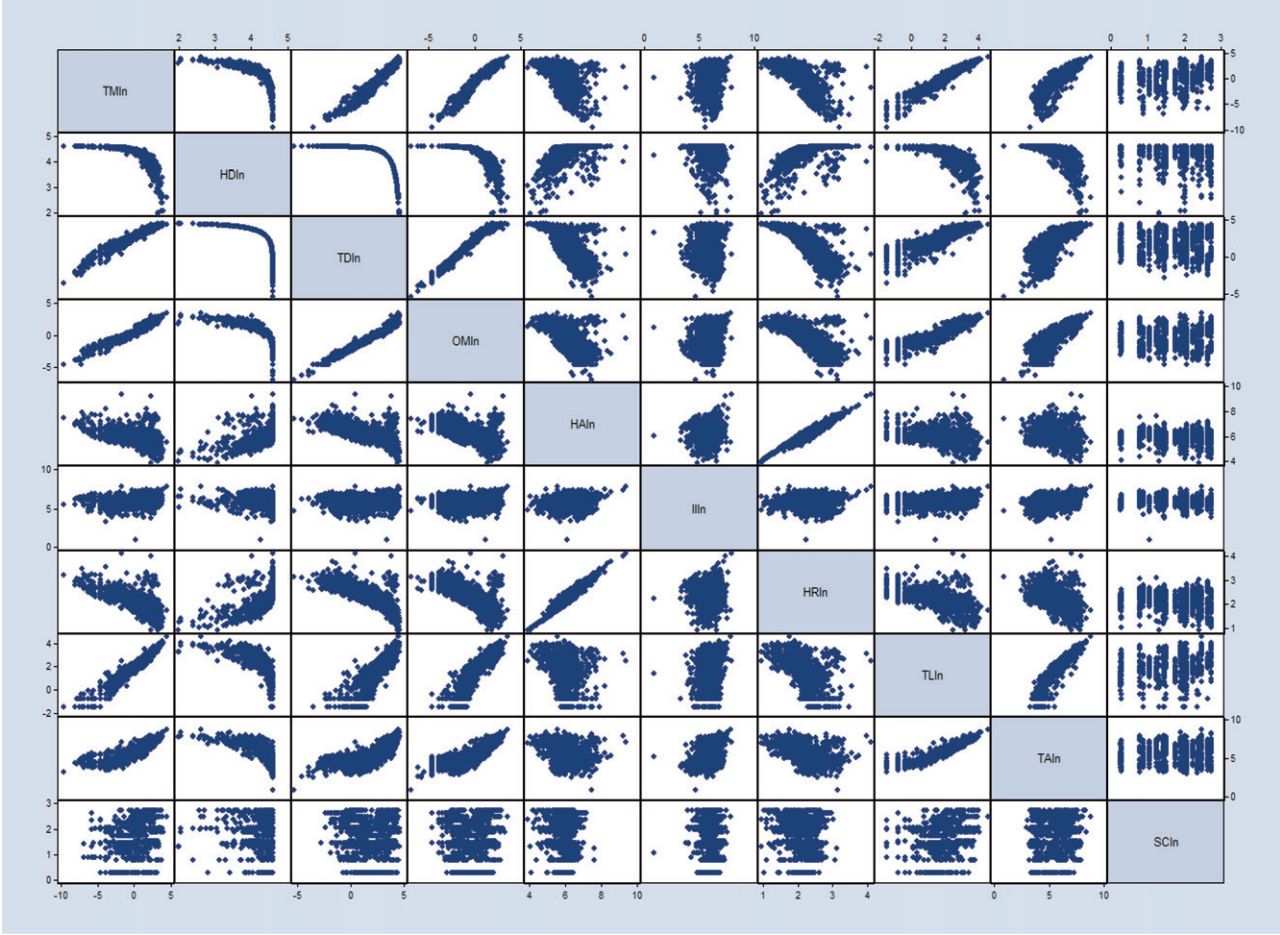

Figure 7. Correlation matrix of log transformed parameters measured by means of comet assay. TMln: tail moment[ln]; HDln: head DNA[ln]; TDln: tail DNA[ln]; OMln: olive moment[ln]; HAln: head area[ln]; IIln: Integral Intensity[ln]; HRln: head radius[ln]; TLln: tail length[ln]; TAln: tail area[ln]; SCln: score[ln].

Table 2. Risk (OR) to observe values of comet parameters above the 75th percentile.

		Exposure (score > 1)				
		1 ($n = 1122$)	0 ($n = 1252$)	OR	LIC (95%)	LSC (95%)
Tail moment (>0.42)	1	459	134	5.77	4.63	7.21
	0	663	1118	–	–	–
Tail area (>212.67)	1	547	136	5.63	4.53	7.03
	0	665	1116	–	–	–
Head DNA (>99.37)	1	105	490	0.16	0.12	0.2
	0	1017	762	–	–	–
Tail DNA (>9.87)	1	458	135	5.07	4.58	7.12
	0	664	1117	–	–	–
Integral intensity (>593)	1	312	282	1.32	1.09	1.6
	0	810	970	–	–	–
Head radius (>14.76)	1	49	547	0.05	0.04	0.08
	0	1073	705	–	–	–
Tail length (>4.42)	1	481	132	6.36	5.1	7.95
	0	641	1120	–	–	–
Olive moment (>0.88)	1	446	145	5.03	4.06	6.25
	0	676	1107	–	–	–
Head area (>779)	1	48	546	0.05	0.04	0.07
	0	1074	706	–	–	–

Table 3. Adjusted risk estimates (ORs) by confounding variables (age, sex and smoking habits) by means of unconditional logistic regression models.

Dependent variables	Independent variables	Odds ratio	SE	Z	$p > z$	95% CI		LR chi^2 (4)	$p >$ chi^2	Log likelihood	Pseudo R^2
Tail moment	Exposure score	4.61	0.43	16.32	0.000	3.84	5.54	327.67	0.00	−1454.29	0.10
	Age	1.22	0.07	3.24	0.001	1.08	1.39	–	–	–	–
	Sex	1.59	0.29	2.53	0.012	1.11	2.29	–	–	–	–
	Smoking habits	0.70	0.85	−2.87	0.004	0.55	0.89	–	–	–	–
Tail area	Exposure score	2.46	0.22	10.01	0.000	2.06	2.94	125.35	0.00	−1582.86	0.03
	Age	0.80	0.05	−3.63	0.000	0.71	0.9	–	–	–	–
	Sex	1.02	0.17	0.13	0.894	0.73	1.43	–	–	–	–
	Smoking habits	0.62	0.07	−4.04	0.000	0.49	0.78	–	–	–	–
Head DNA	Exposure score	0.27	0.02	−14.17	0.000	0.64	0.82	270.05	0.00	−1510.50	0.08
	Age	0.72	0.04	−5.17	0.000	0.64	0.82	–	–	–	–
	Sex	0.79	0.13	−1.33	0.182	0.56	1.11	–	–	–	–
	Smoking habits	1.27	0.15	2.02	0.043	1.01	1.61	–	–	–	–
Tail DNA	Exposure score	3.61	0.32	14.12	0.000	3.02	4.30	269.68	0.00	−1510.68	0.08
	Age	1.37	0.08	5.21	0.000	1.22	1.55	–	–	–	–
	Sex	1.23	0.21	1.24	0.215	0.88	1.72	–	–	–	–
	Smoking habits	0.79	0.09	−1.94	0.053	0.62	1.01	–	–	–	–
Integral intensity	Exposure score	1.35	0.11	3.47	0.001	1.14	1.60	55.74	0.00	−1617.66	0.01
	Age	0.74	0.04	−4.91	0.000	0.66	0.84	–	–	–	–
	Sex	1.65	0.28	2.88	0.004	1.17	2.33	–	–	–	–
	Smoking habits	1.19	0.13	1.56	0.118	0.95	1.49	–	–	–	–
Head radius	Exposure score	0.17	0.01	−18.22	0.000	0.14	0.21	441.36	0.00	−1424.82	0.13
	Age	0.82	0.05	−3.02	0.003	0.72	0.93	–	–	–	–
	Sex	1.28	0.22	1.42	0.154	0.9	1.81	–	–	–	–
	Smoking habits	0.87	0.10	−1.10	0.273	0.68	1.11	–	–	–	–
Tail length	Exposure score	4.61	0.43	16.32	0.000	3.83	5.54	327.67	0.00	−1454.29	0.10
	Age	1.22	0.07	3.24	0.001	1.08	1.39	–	–	–	–
	Sex	1.59	0.29	2.53	0.012	1.11	2.29	–	–	–	–
	Smoking habits	0.70	0.08	−2.87	0.004	0.55	0.89	–	–	–	–
Olive moment	Exposure score	3.30	0.29	13.18	0.000	2.76	3.94	243.48	0.00	−1523.13	0.07
	Age	1.44	0.08	6.01	0.000	1.28	1.63	–	–	–	–
	Sex	1.47	0.25	2.26	0.024	1.05	2.05	–	–	–	–
	Smoking habits	0.68	0.08	−3.20	0.001	0.54	0.86	–	–	–	–
Head area	Exposure score	0.22	0.02	−16.12	0.000	0.18	0.27	416.68	0.00	−1437.17	0.12
	Age	0.77	0.04	−4.03	0.000	0.68	0.87	–	–	–	–
	Sex	1.30	0.22	1.55	0.121	0.93	1.83	–	–	–	–
	Smoking habits	0.60	0.07	−3.96	0.000	0.47	0.77	–	–	–	–

micronuclei increased significantly in the exposed group (Carbajal-Lòpez *et al.* 2016). Paddy farm workers chronically exposed to mixtures of organophosphates reported a significant increase in DNA damage, as assessed by measuring comet tail length in the exfoliated buccal mucosa (How *et al.* 2015). The comet assay technique has been used to highlight genotoxic effects in the lymphocytes of farmers exposed to pesticides, suggesting in particular the possible role of fungicides (Lebailly *et al.* 2015). The comet assay showed that both the frequency and index of DNA damaging and the index of damage were significantly higher in growers from Southern Brazil's exposed to pesticide mixtures with genotoxic potential (Wilhelm *et al.* 2015). The comet assay showed that higher concentrations of individual pesticides (0.5–4.0 μM), but very low concentrations of pesticide mixtures caused significant DNA damage (Sultana Shaik *et al.*

Table 4. Sensitivity, specificity and predictive positive value (PPV) of the comet assay toward the pesticide exposure.

		Exposed to pesticides (score ≥ 1)		
		+	−	Total
Comet assay	+	459	134	593
	−	663	1118	1781
	Total	1122	1252	2374

Sensitivity (41%); specificity (89%); PPV (77%).

2016). The comet assay was also used to confirm DNA damage in human peripheral blood lymphocytes after exposure to diazinon, an organophosphate pesticide, in agreement with the results of micronucleus (MN) and fluorescence in situ hybridization (FISH) assays (Gökalp Muranli *et al.* 2015). The comet assay is a valid test for human biomonitoring to assess the genotoxicity and carcinogenesis of various agents. In particular, the comet assay estimated that pesticide-exposed workers with the GSTP1 Ile-Ile and XRCC1399 Arg-Arg genotypes showed increased DNA damage (Saad-Hussein *et al*. 2017). The comet assay was also used to indicate that low level use of some conventional and green insecticides does not increase DNA damage (Zeljezic *et al.* 2017), while glyphosate, an important herbicide, may induce DNA damage in leucocytes (Kwiatkowska *et al*. 2017). Organophosphate and pyrethroid pesticides, in particular malondialdehyde (MDA), showed a positive correlation with DNA damage, via the comet assay, because they decrease the activity of antioxidant enzymes (Zepeda-Arce *et al*. 2017). DNA damage, as determined by the mean comet tail length, was high in women exposed to pesticides while picking cotton with bare hands, and a positive correlation of DNA damage with age and exposure time was observed (Ali *et al*. 2017). The comet assay showed that occupational exposure to pesticides was more dangerous than consumption of contaminated water, which was greater than controls; this difference between exposed and unexposed groups was not influenced by other factors, such as age or smoking or alcohol habits (Vazquez Boucard *et al*. 2017). The comet assay can also be useful in monitoring DNA damage in single cells due to exposure to chemotherapy and radiotherapy in cancer patients or to assess the cancer chemoprevention of some molecules (Santoro *et al*. 2016).

The comet assay appears to satisfy the need for a rapid, economical, noninvasive test to screen of genotoxic exposure in the workplace. The test also has many advantages in terms of ease of installation and rapid execution; it is generally very sensitive, but in our experience, it shows a low sensitivity (41%), with a high number of false negatives, and a high specificity (89%) with a low number of false positives. It can be applied to virtually any type of cell or tissue (Sasaky *et al.* 2000, Tice *et al.* 2000, Hartmann *et al.* 2003a, 2003b). For these reasons, numerous studies have been conducted (Neri *et al.* 2015) using the comet assay to monitor exposure to various genotoxic agents, predominantly in the workplaces (Gunasekarana *et al.* 2015), and to assess exposure to atmospheric pollution (Møller and Loft 2010). Jasso-Pineda *et al.* (2015) used the comet assay to analyze DNA damage in children living in highly contaminated areas in Mexico: children exposed to high levels of polycyclic aromatic hydrocarbons and DDTs showed the highest levels of DNA damage in their blood cells.

However, age (Chen *et al.* 2007, Heuser *et al.* 2008), tobacco smoke (Al-Amrah *et al.* 2014, Rajmohan *et al.* 2015) and other factors, such as diet (Duthie *et al.* 1996, Astley *et al.* 1999), physical activity (Møller *et al.* 1996), infections (Tice *et al.* 1990), gender (Betti *et al.* 1994, Bonassi *et al.* 1995) and exposure to ultraviolet radiation (Frenzilli *et al.* 1998, Møller *et al.* 2002), may affect the test and may sometimes lead to discrepancies among the various studies. The comet assay also has limitations that must be considered for the correct interpretation of results: for example, it is not possible to detect, except at very high exposure levels, the action of genotoxic agents such as aneugenes, which are compounds capable of inducing aneuploidy through interactions with the mitotic melt; it is not possible to detect agents that reduce the availability of nucleotides for synthesis or the shelter of nucleic acids using the comet assay, nor can it detect so-called cross-linkers, which form cross-linked links between DNA and proteins or within DNA at the single strand level (intra-strand) or between the two filaments inter-strand (Grandi *et al.* 2006).

Another limitation of this test concerns cytotoxicity-related influences that result in DNA fragmentation in cells during necrosis or apoptosis, leading to the formation of comet-headed comets, called "ghost cells" that are routinely excluded from the analysis (Grandi *et al.* 2006). Finally, in the choice of biological substrates, monocytes exhibit a faster DNA damage kinetics than lymphocytes (Knudsen *et al.* 1992). The International Program on Chemical Safety (IPCS) has proposed guidelines for the use of the comet assay in the biomonitoring of populations exposed to occupational or environmental genotoxicity (Albertini *et al.* 2000). Reaffirming that the test cannot be considered predictive of the individual risk of manifesting neoplastic disease, the multiplicity of mechanisms that can induce genotoxicity makes fundamental definitions of exposure through environmental and/or biological monitoring and the possible confounding factors. In particular, for DNA repair mechanisms, special attention should be paid to the effects on Comet Assay results of DNA repair mechanisms inhibitors and to the analysis of genetic polymorphisms (Albertini *et al.* 2000). Other technical aspects that could modulate the results of the comet assay include the uptake times for biological samples and the test run in relation to the work shift (start of turn, at regular intervals during the turn or at the end of the turn) and the timing during the work week (beginning or end of the week) (Grandi *et al.* 2006). It was also noted that the inter-laboratory variation in DNA damage measurements via the comet assay was greater than intra-laboratory variation (Frenzilli *et al.* 1998). Analysis of the effect of all these variables on the results of the comet assay has allowed standardization of the protocol and its further validation (Hartmann *et al.* 2003a, 2003b; Burlinson *et al.* 2007; Valverde and Rojas 2009; Collins *et al.* 2014b; OECD 2014; Araldi *et al.* 2015; Neri *et al.* 2015).

Conclusion

In conclusion, in our experience, the comet assay is a quick, reliable, easy-to-use test of genotoxicity, with high specificity and a good PPV. In our experience, the test is not predictive of individual cancer risk, but is predictive of chronic exposure to pesticides. Validation of comet assay in the biomonitoring of cancer diseases could be interesting for future studies and should be considered in the design of large multi-centric studies with large sample size, various routes of exposure and analytical characterization of confounding factors.

Disclosure assessment

No potential conflict of interest was reported by the authors.

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