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REVIEW

Micronuclei and pesticide exposure

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Micronucleus (MN) is a biomarker widely used in biomonitoring studies carried out to determine the genetic risk associated to pesticide exposure. Many *in vitro* and *in vivo* studies, as well as epidemiological approaches, have demonstrated the ability of certain chemical pesticides to produce genetic effects including cancer and other chronic pathologies in humans; thus, biomonitoring studies have been carried out to characterise the genetic risk associated to pesticide exposure. It must be noted that 'pesticide exposure' is a broad term covering complex mixtures of chemicals and many variables that can reduce or potentiate their risk. In addition, there are large differences in pesticides used in the different parts of the world. Although pesticides constitute a wide group of environmental pollutants, the main focus on their risk has been addressed to people using pesticides in their working places, at the chemical industry or in the crop fields. Here, we present a brief review of biomonitoring studies carried out in people occupationally exposed to pesticides and that use MN in lymphocytes or buccal cells as a target to determine the induction of genotoxic damage. Thus, people working in the chemical industry producing pesticides, people spraying pesticides and people dedicated to floriculture or agricultural works in general are the subject of specific sections. MN is a valuable genotoxic end point when clear exposure conditions exist like in pesticide production workers; nevertheless, better study designs are needed to overcome the uncertainty in exposure, genetic susceptibility and statistical power in the studies of sprayers and floriculture or agricultural workers.

Introduction

A large number of synthetic pesticides have been introduced in the market since the mid-1940s. At present, the pesticide manual includes 900 main entries and lists over 2600 products (1). Pesticides, as a heterogeneous category of biologically active compounds, are characterised by various degrees of toxicity also to non-target species, including human beings. Most pesticides are acutely toxic to humans. Cases of acute pesticide poisonings account for significant morbidity and mortality worldwide, especially in developing countries, where the pattern of pesticide use is different (2,3).

Chronic health effects have been associated to pesticide exposure, including neurological effects, reproductive or development problems and cancer. Epidemiological studies on farmers, pesticide manufacturers, pesticide sprayers and on accidentally exposed industrial workers or residents have shown that exposure to pesticides may increase the risk of site-specific cancers. Increased risks have been detected for brain cancer, leukaemia and Ewing's bone sarcomas, kidney cancer, acute leukaemia, soft tissue sarcoma, non-Hodgkin's lymphoma, brain cancer, testicular, colorectal, endocrine glands and brain cancers in children exposed to pesticides in their home or whose parents were occupationally exposed to pesticides (4). Reproductive effects (5,6), developmental problems and very recently neurodegenerative disorders, such as Parkinson (7,8) and Alzheimer disease (9,10), have been also associated to occupational exposure to pesticides. Many pesticides involved in carcinogenic risk, and classified as probable or possible carcinogens by the International Agencies, were banned or their use was restricted in some countries; but, due to their bioaccumulation and persistence in the ecosystems, they are widespread environmental pollutants. Residues of these pesticides have been detected in the food chain and in different biological media in humans.

At present, the regulations concerning the introduction of plant protection products on the market in the developed countries (e.g. Dir. 91/414/EEC, EPA Regulations) involve the evaluation of all the active substances in a pesticide product. Pesticides containing substances that are carcinogenic (except for those with a threshold mode of action) and/or genotoxic are not allowed to be placed on the market and for already authorised compounds, if new data become available showing that the substances may have these potentials, they will be withdrawn from the market. Acute and chronic effects are determined by observing symptoms in test animals, resulting from lifetime exposure to the active substances. However, delayed adverse health effects can be often identified or confirmed only through epidemiological studies in occupationally exposed populations.

Genotoxicity risk of pesticide exposure

Genotoxic potential is a primary risk factor for long-term effects, such as carcinogenic and reproductive toxicology and degenerative diseases. Biomonitoring studies focusing on genomic modifications have been carried out in pesticide-exposed populations from different countries to elucidate the risk associated to the exposure to specific compounds or classes of compounds or to specific cultivation practices (11,12).

Among them, several studies employing the micronucleus (MN) test in peripheral lymphocytes or in exfoliated buccal mucosa cells are available in the last decades. Occupational exposure is the normal source of information on the risk associated to pesticide exposure. Nevertheless, this exposure usually involves complex mixtures of pesticides belonging to different chemical classes varying with the type of crop, the season and the geographical area

Taken into account the complexity of these exposures, in this review, we have structured the studies applying the MN test in peripheral blood lymphocytes (PBL) according to the following topics: (i) results obtained in people working in the chemical industry producing pesticides, (ii) studies on pesticide sprayers, (iii) studies in floriculturists and (iv) studies in agricultural workers not included in the previous sections. A further section (v) includes studies that have used the MN assay in buccal cells.

MN in PBL of workers from pesticide industries

The available studies on workers from pesticide industries showed a statistically significant increase of MN frequency in PBL (Table I). The MN frequency in 41 workers exposed to chlorinated compounds, including hexachlorobenzene (HCB) in Sao Paulo (Brazil), is significantly higher than in controls, showing also a correlation with working time and with serum concentration of HCB (13). Two studies carried out in Croatia in workers exposed to 2,4-dichlorophenoxyacetic acid (2,4-D), atrazine, alachlor, cyanazine and malathion, during the process of production, show significant increases in the MN frequency after 8 months of high exposure (14,15). In a recent study carried out in Pakistan with workers from an industry producing pesticides, belonging to the organophosphate and pyrethroid classes, significant increases in the MN frequency were observed in workers, showing a linear correlation with length of exposure (16).

MN in the PBL of pesticide sprayers

Pesticides sprayers are directly involved in treating specific pests by spraying/fumigating the crops and represent the most exposed group among the agricultural workers. Among sprayers, we can find workers applying specifically one or few pesticides, while others use mixtures of pesticides. The biomonitoring studies concerning the use of one or few pesticides are all related to professional applicators working under controlled conditions: no increase in chromosomal damage was observed (Table IIa). The frequency of MN in a group of 31 fumigators of commercial grain stores in Australia using phosphine was not significantly different to that observed in controls, indicating a lack of genotoxic risk keeping low levels (2.4 p.p.m./h) of exposure (17).

Two studies were conducted in California (USA) with workers involved in the Mediterranean Fruit Fly Eradication Program. In 38 intermittently malathion-exposed sprayers, no increase in the frequency of MN in PBL was detected (18). In a second study, a slight but significant increase in the MN frequency was observed in workers exposed to malathion for >50 h during the last 8 months or with levels of malathion diacid >100 p.p.b. (19).

Methyl bromide fumigators have also been the subject of a biomonitoring study testing the levels of MN in lymphocytes (20). This study was carried out in USA and no increases were observed in the MN frequency of fumigators. These negative findings contrast with those observed in the same group of workers, when the frequency of MN was measured in oropharyngeal cells and when hypoxanthine–guanine phosphoribosyl transferase gene (*HPRT*) mutations were measured in lymphocytes.

No genotoxic risk was associated to the herbicide 2,4-D exposure as evaluated in a group of sprayers from eastern Kansas (USA): no significant difference in MN frequency was observed between workers and controls and before and after the spraying period (21). A biomonitoring study carried out with 11 fumigators at the tobacco fields in western Greece, using metalaxil as fungicide and imidacloprid as insecticide, did not show any significant increase in the frequency of MN in PBL (22).

The studies carried out with sprayers applying complex mixtures of pesticide (Table IIb) include heterogeneous populations involved in cultivation of different crops, in sanitisation and indirectly exposed by aerial spraying. Four of five studies give positive results. Significant increases of MN associated to the duration of exposure were observed in a study carried out with sprayers from central Italy (23). A study conducted in vineyards workers from Serbia, applying mainly insecticides and fungicides, showed higher MN frequency compared to controls 1 month after the start of the spraying period, with a further increase at the end of the spraying season (24). No significant effects were observed in workers from Concepción (Chile), who sprayed a variety of pesticides, mainly the insecticides deltamethrin and dichlorvos (25).

Positive effects were also reported in a group of sanitation workers from Belo Horizonte (Brazil), using different pesticides including organophosphates and pyrethroid insecticides, as well as hydroxycoumarinic rodenticides. No time exposure association was found (26).

Table I. Biomonitoring studies using peripheral blood lymphocytes from human populations exposed to pesticides: MN in chemical plant workers

Study subjects/ controls	Exposure (chemicals)	Duration (years)	Result (fold difference versus controls)	PPE	Time dependence	Country	Reference
41/28	Chlorinated compounds, including HCB	9	Pos (+3.6)	NA	No evaluated	Brazil	da Silva Augusto *et al.* (13)
20/20	Pesticide production limited to 8 months/year (2,4-D, atrazine, alachlor, cyanazine, malathion)	4–30	Pos; after 8 months of high exposure (+3.63), after 8 months of non-exposure (+1.86)	NA	Yes	Croatia	Garaj-Vrhovac and Zeljezic (14)
10/20	Pesticide production limited to 8 months/year (2,4-D, atrazine, alachlor, cyanazine, malathion)	4–30	Pos (+7.9)	NA	No evaluated	Croatia	Garaj-Vrhovac and Zeljezic (15)
35/29	Complex mixtures, mainly organophosphates and pyrethroids	3–18	Pos (+2.06)	No	Yes	Pakistan	Bhalli *et al.* (16)

NA, not available; PPE, personal protective equipment.

Table II. Biomonitoring studies using peripheral blood lymphocytes from human populations exposed to pesticides: MN in pesticide sprayers

Study subjects/ controls	Exposure (chemicals)	Duration (years)	Result (fold difference versus controls)	PPE	Time dependence	Country	Reference
a) Exposure to single pesticide							
31/21	Fumigators: phosphine (2.4 p.p.m. in enclosed spaces)	1.5–32	Neg	NA	NA	Australia	Barbosa and Bonin (17)
38/16	Medfly eradication programme: malathion, exposure below the genotoxic dose	NA	Neg, after spraying season (no correlation with metabolites in urine)	NA	NA	USA	Titenko-Holland *et al.* (18)
1992 cohort, 13/4, 1993 cohort, 24/10	Medfly eradication programme: malathion fumigations	NA	Pos (+1.4), malathion diacid, >100 p.p.b. in urine (+1.58), Neg	NA	NA	USA	Windham *et al.* (19)
31/27	Fumigant appliers: methyl bromide	0.3–22	Neg	NA	No	USA	Calvert *et al.* (20)
12/9	Pesticide applicators: 2,4-D (240 + 100 p.p.b.), 12–1285 p.p.b.	Discontinuous use	Before and after Neg	Yes	No	USA	Figgs *et al.* (21)
11/11	Tobacco fields sprayers using metalaxyl and imidacloprid	23.64 ± 4.13	Neg	Yes (50%)	No	Greece	Vlastos *et al.* (22)
b) Exposure to mixture of pesticides							
48/50	Farmers (cereals, fruits, vegetables): pesticide mixture	4–50	Pos (+1.20)	Yes (29%)	Pos	Italy	Pasquini *et al.* (23)
27/20	Vineyard workers: pesticides most used: diazinon and dithiocarbamate	12.1	Pos (+7.67) end of spraying season	NA	Pos ($P = 0.016$)	Serbia	Joksic *et al.* (24)
22/16	Pesticides most used: bromadialone, captan, deltamethrin, diazinon dichlorvos, linuron, methamidophos	7	Neg	NA	NA	Chile	Venegas *et al.* (25)
29/30	Sanitation workers. Complex mixtures and types of application	23.64 ± 4.1 (1.5–18)	Pos (+3.35)	Yes	Neg	Brazil	Kehdy *et al.* (26)
62/60	Pesticide mixture	NA	Pos (+2.71)	NA	NA	Colombia	Bolognesi *et al.* (27)
60/60	Glyphosate aerial spraying for control of illicit crops		Pos (+2.53)				
64/60			Pos (+3.26)				
28/60	Glyphosate aerial spraying for sugar cane maturation		Pos (+4.72)				

Neg, negative; Pos, positive; PPE, personal protective equipment.

A recent study was carried out in Colombia to investigate the health effects associated with glyphosate exposure, in the aerial spraying programme for control of illicit crops and in the maturation of sugar cane in comparison with the exposure to pesticide mixture (27). In regions where glyphosate was being sprayed, blood samples were collected prior, during and 4 months after spraying. Results showed significant increases in MN frequency after glyphosate exposure, mainly when it is applied for maturation of sugar cane.

MN in PBL of floriculturists

Floriculturists are involved in the production of flowers and ornamental plants, which are commonly treated with high quantities of agrochemical formulations in greenhouses.

Several studies have been carried out with this collective (Table III), mainly in Italy, where in 1993, one study was performed in the region of Liguria (Northwest of Italy). This study carried out with 71 workers showed significant increases in the frequency of MN in people occupationally exposed to pesticides. The MN frequency showed a dose–response relationship with duration of exposure, with a maximum increment of 71% in the MN frequency in subjects exposed for over 30 years (28,29). Further studies in this population indicated that the conditions of exposure influenced the MN frequency. Thus, increased relative risks (RR) in greenhouse workers (RR = 1.31) and in people working alternately in the greenhouse and in the open field (RR = 1.46) were observed with respect to the reference population (30).

A further study in the same area and by the same group was carried out in workers producing ornamental plants and vegetables. A statistically significant increase in the MN of 107 floriculturists was detected with respect to the control population, and a positive correlation between years of farming and MN frequency was observed. The conditions of exposure were also associated with an increase in cytogenetic damage, with a 28% higher MN frequency in greenhouse workers compared with subjects working only in open fields. Finally, workers not using protective measures during high exposure activities showed an increase in the MN frequency (34).

To determine the mechanisms producing MN, 52 floriculturists and 24 controls were evaluated by using the cytokinesis-block methodology associated with fluorescence *in situ* hybridisation with a pan-centromeric probe that allowed distinguishing centromere-positive (C+) and centromere-negative (C−) MN. The percentage of C+ MN was not related to the duration of exposure or to the number of genotoxic pesticides used, but a higher percentage (66.52 versus 63.78%) was observed in a subgroup of subjects using benzimidazolic compounds compared with the floriculturist population exposed to a complex pesticide mixture not including benzimidazolics (35).

Two other studies including floriculturists were carried in Tuscany (Central Italy). In this area, floriculturists used many different formulations and performed two types of

Table III. Biomonitoring studies using peripheral blood lymphocytes from human populations exposed to pesticides: MN in floriculturists

Study subjects/ controls	Exposure (chemicals)	Duration (years)	Result (fold difference versus controls)	PPE	Time dependence	Country	Reference
71/75	Complex pesticide mixtures	2–55	Pos (1.29)	Yes	Yes	Italy	Bolognesi *et al.* (28–30)
43/41	Greenhouse workers: >100 agrochemical formulations	NA	Neg	NA	NA	Italy	Scarpato *et al.* (31)
23/22	Greenhouses using: benzimidazoles, carbamates, diphenylethanoles, dithiocarbamates, organophosphates, thiophthalimides	NA	Neg	Yes	NA	Italy	Scarpato *et al.* (32)
34/33, 17/— highly exposed sprayers	Greenhouse workers: complex mixture of pesticides	7–41	Neg, Pos (+1.22)	Yes	NA	Italy	Falck *et al.* (33)
107/61	Greenhouse and open field workers	2–70	Pos (+1.45), greehouses/open field (+1.22), No PPE/PPE (+1.17)	Yes (15%)	Yes	Italy	Bolognesi *et al.* (34)
51/24	Greenhouses (80%) and open field (20%) using >50 different pesticides	26.3 ± 14.5	Neg	NA	Yes	Italy	Bolognesi *et al.* (35)
31/30	Women field workers, complex mixtures	10.97 (2–22)	Pos	Yes (49.2%)	No	Colombia	Varona *et al.* (36)

Neg, negative; Pos, positive; PPE, personal protective equipment.

work: culture treatment (mixing and spraying of pesticides) or re-entry activities (cutting and harvesting flowers several hours after the end of pesticide spraying). MN frequency in PBL from the floriculturists did not show differences compared with controls (31). Blood samples obtained during and 1 month after the end of intensive pesticide treatments were analysed to cover a period of high and low exposure, respectively, but no effect of pesticide exposure was detected. Each donor was genotyped for polymorphisms in the *GSTM1*, *GSTT1* and *NAT2* genes, involved in xenobiotic metabolism, but no association was observed between MN frequency and the genetic polymorphisms analysed (32). Nevertheless, a subsequent study showed that *GSTM1* positive and *NAT2* fast appear associated to MN increases (33). Finally, a study carried out in Colombia with women working in open fields observed significant increases in MN associated to pesticide exposure (36).

MN in PBL of agricultural workers

A survey of studies carried out in agricultural workers is shown in Table IV. A first study was carried out in Italy with open field and greenhouse workers exposed to complex pesticide mixtures, but no effects were detected (37). Negative results were also obtained in seasonal farm workers from British Columbia (Canada) harvesting berry crops. Subjects were 39 females of South Asian descent, 18 farm workers and 21 age-matched controls. Interestingly, the highest frequency of MN cells was found in the group with the longest history of employment as a farm worker. In addition, farm workers had a lower frequency of kinetochore-positive MN than controls (38).

Two studies were carried out in the south-eastern of Spain. PBL samples from 64 workers exposed to complex mixtures of pesticides did not show any increase in the frequency of MN. This lack of genotoxic effects did not change when agricultural workers were classified according their genotypes for *GSTM1* and *GSTT1* (39). A follow-up study, carried out with 39 greenhouse workers from the same group, compared the effects of high exposure (spring–summer) and lower exposure (autumn–winter). Results indicated that no statistically significant differences in the MN frequencies were found neither between the two sampling periods nor between the exposed and controls (44).

The same research group carried out three different studies with three other European populations in Poland, Greece and Hungary. Neither the Poland group (49 subjects) nor the Greece (50 workers) and the Hungarian group (84 workers) presented significant increases in MN frequency in their PBL (41–43). In spite of this lack of genotoxic effects, decreases in the cell proliferation index were observed, indicating some type of effect related to pesticide exposure. A summing up study was carried out with the above-cited populations, including 239 agricultural workers and 231 unexposed controls. The results indicated that, for the overall population, there were no increases in MN frequencies in the agricultural workers when compared with the controls (45).

In a study carried out in Costa Rica in banana farms, no increases in MN frequency were observed in women, exposed for at least 4 months to the commonly applied compounds imazalile, thiabendazole and chlorpyriphos. Nevertheless, women with a high frequency of abortions showed increased frequencies of MN (40).

The Bío-Bío Region is a major fruit-growing area of Chile that makes intensive use of agricultural pesticides. In a group of 64 females harvesting and packing different significant increases in MN frequency were found without correlation with the duration of exposure (46). A statistically significant increase in MN frequencies was observed in a small group of 11 agricultural workers growing vineyards and olive trees in Crete (Greece) and exposed to complex mixtures of pesticides (47).

A study with 15 agricultural workers from Kentucky (USA), exposed for 6 months to several pesticides, showed a 76% increase in the average MN frequency in lymphocytes. In addition, MN frequency peaked during the period of highest exposure (48). In a biomonitoring study with 28 agricultural workers from the region of the Atoyac River (Mexico), increase in the MN frequency was observed, with higher values

Table IV. Biomonitoring studies using peripheral blood lymphocytes from human populations exposed to pesticides: MN in agricultural workers

Study subjects/ controls	Exposure (chemicals)	Duration (years)	Result (fold difference versus controls)	PPE	Time dependence	Country	Reference
62/29	Open field and greenhouse workers. Complex pesticide mixtures	2–52	Neg	NA	Yes	Italy	Bolognesi *et al.* (37)
18/21	Berry pickers exposed mainly to simizine, paraquat, napropamide, glyphosphate captan, triforine, diazinon, malathion, carbofuran, endosulfan	1–24	Neg	NA	Yes	Canada	Davies *et al.* (38)
64/50	Greenhouse workers. Complex pesticide mixture	9.82 ± 1.0	Neg	Yes (80%)	No	Spain	Lucero *et al.* (39)
32/37	Banana farms. Imalzalile and thiabenzadole (fungicides) and chlorpyriphos (insecticide)	>4 consecutive months	Neg	NA	No	Costa Rica	Ramírez and Cuenca (40)
49/50	Greenhouse and open field: vegetables and ornamental plants	16.28 ± 1.1	Neg	Yes (78%)	NA	Poland	Pastor *et al.* (41)
50/66	Open field: vegetables and ornamental plants	8.62 ± 1.13	Neg	Yes (62%)	NA	Greece	Pastor *et al.* (42)
84/65	Open field/greenhouse workers: pesticide mixture	18.75 ± 0.89	Neg	Yes (85%)	NA	Hungary	Pastor *et al.* (43)
39/22	Greenhouse workers	8.31 ± 1.12	Neg	Yes (93%)	No	Spain	Pastor *et al.* (44)
239/231	Open field/greenhouses. Complex pesticide mixtures	13.92 ± 0.58	Neg	Yes	No	Spain, Greece, Hungary, Poland	Pastor *et al.* (45)
64/30	Thinning and pruning fruit trees, harvesting and packaging fruits	8 ± 4.8	Pos (+3.72)	No	NO	Chile	Márquez *et al.* (46)
11/11	Vineyards and olive tree cultures. Organophosphates and pyrethroids, the most used	26.45 ± 3.38 (25–60)	Pos (+1.40)	NA	NA	Greece	Vlastos *et al.* (47)
15/10	Complex mixtures including endosulfan, chlorpyriphos, dimethoate, diazinon and maleic hydrazide	18.2 ± 1.3	Pos (+1.76)	NA	NA	USA	Tope *et al.* (48)
28/21	Polluted areas including pesticide-polluted areas	NA	Pos (+1.92)	NA	NA	Mexico	Montero *et al.* (49)
33/33	Open field and greenhouses	15.0 ± 13.0 (0.5–48)	Pos (+2.76), greenhouses/open field, Pos (+1.86)	33% (gloves)	No	Portugal	Costa *et al.* (50, 51)
69/69	Cotton pickers (carbamates, organophosphates, pyrethroids)	10.3 ± 6.1	Pos (+2.92)	NA	Yes	Pakistan	Ali *et al.* (52)
108/65	Open fields: grapes growers	NA	Pos (+1.69)	NA	NA	Brazil	da Silva *et al.* (53)

Neg, negative; Pos, positive; PPE, personal protective equipment.

in people with the *GSTT1* null allele (49). In the area of Oporto (Portugal), a biomonitoring study was conducted in a group of 33 farmers exposed to pesticides. MN frequency was significantly higher in the exposed group and it was possible to relate a specific working environment (greenhouses) with higher levels of genetic damage and the use of personal protective equipments with lower frequencies of MN. No association was found between MN frequency and duration of pesticide exposure and, when the effect of polymorphic genes of xenobiotic-metabolising enzymes (*GSTM1*, *GSTT1*, *GSTP1*, *CYP2E1* and *EPHX1*) was evaluated, results suggest that low microsomal epoxide hydrolase activity as well as *GSTT1*-positive genotype are associated with increased cytogenetic damage (50,51). An increase of MN frequency was also shown in a biomonitoring study with 69 females involved in cotton-picking activity in the Bahawalpur area (Pakistan) (52).

In Caxias do Sul (Brazil), 108 vineyard workers showed high rates of MN than controls. When the subjects were genotyped for *GSTT1*, *GSTM1*, *GSTP1*, *CYP1A1*, *CYP2E1* and *PON*, it was shown that genetic polymorphisms in *PON* modulated the frequency of MN in the exposed group. In addition, some associations between *GSTM1*, *GSTT1* and *CYP2E1* polymorphisms were suggested (53).

A study was performed in the umbilical cord blood of 16 newborns, in an agricultural area in Delicias, Chihuahua, in the North of Mexico characterised by the use of pesticide mixtures (mainly organophosphates) during the summer and autumn spraying cycles. No significant increases in MN were observed in this group compared to 35 controls (not exposed to pesticides), although more babies with a higher MN frequencies were within the pesticide-exposed group (54).

MN in buccal cells of pesticide-exposed workers

Table V summarises the studies on MN in buccal cells. The first study reporting effects in buccal cells was carried out in workers exposed to methyl bromide, where higher but not significant MN frequency was observed (20).

A series of studies were carried out with agricultural workers from four European countries (Spain, Poland, Greece and Hungary). The overall results of this study, including 247 agricultural workers and 231 controls, did not indicate any increase in MN frequency in buccal cells related to pesticide exposure. In the Spanish population, an additional analysis determined that *GSTM1* and *GSTT1* polymorphisms did not modify the MN induction (39,41–43).

Table V. Biomonitoring studies using buccal mucosa cells from human populations exposed to pesticides

Study subjects/ controls	Exposure (chemicals)	Duration (years)	Result (fold difference versus controls)	PPE	Time dependence	Country	Reference
32/28	Methyl bromide (from fumigation)	NA	Neg	NA	No	USA	Calvert *et al.* (20)
64/50	Agricultural workers in greenhouses: tralomethrin	9.82 ± 1.0	Neg	Yes (80%)	No	Spain	Lucero *et al.* (39)
30/30	Floriculturists	1.5–10	Pos (+2.7)	No	NA	México	Gómez-Arroyo *et al.* (56)
49/50	Agricultural workers: open field/greenhouse	16.28 ± 1.1	Neg	Yes (78%)	NA	Poland	Pastor *et al.* (41)
50/66	Agricultural workers: open field—vegetables and ornamental plants	8.62 ± 1.13	Neg	Yes (62%)	No	Greece	Pastor *et al.* (42)
84/65	Agricultural workers open field/greenhouses, pesticide mixtures	18.75 ± 0.89	Neg	Yes (85%)	NA	Hungary	Pastor *et al.* (43)
239/231	Open field/greenhouses. Complex pesticide mixtures	13.92 ± 0.58	Neg	Yes	No	Spain, Greece, Hungary, Poland	Pastor *et al.* (45)
40/44	Women working as banana packing exposed to thiabenzadole and chlorpyrifos	6.4	Neg	NA	No	Costa Rica	Castro *et al.* (61)
54/54	Pesticide manufacturing unit: pyrethroids, organophosphates, carbamates	8.57 (3–13)	Pos (+3.9)	No	Yes	India	Sailaja *et al.* (59)
32/32	People living in a pesticide-contaminated area	34.6 ± 10.5	Pos	NA	NA	Turkey	Ergene *et al.* (57)
70/70	Agricultural workers	7.00 ± 3.95	Pos (+7.64)	No	NA	México	Martínez-Valenzuela *et al.* (58)
29/37	Agricultural workers: soybean growers	16.3 ± 10 (2–35)	Pos (+1.99)	Yes (31%)	No	Brazil	Bortoli *et al.* (60)
37/20	Agricultural workers	25.7 ± 10.1	Neg	67.6	No	Brazil	Remor *et al.* (55)

PPE, personal protective equipment; Neg, negative; Pos, positive.

No increase of MN frequency was detected in a group of 40 women working in banana packing facilities in Costa Rica (56). Negative results were also reported in sprayers from the region of Rio Grande do Sul (Brazil) exposed to a wide number of pesticides, although significant variations in the plasmatic levels of butyrylcholinesterase and δ-aminolevulinic acid dehydratase enzymes indicate that exposure did occur (61). In spite of the negative results above indicated, several studies reported significant MN increases in the buccal cells of workers exposed to pesticides.

In Mexico, a study with 30 subjects working as floriculturists in greenhouses shows an increase in MN frequency in buccal cells (55). A further study in Mexico (Sinaloa State) reported a clear increase in MN frequency in agricultural workers using mainly organophosphates and carbamates without any correlation with age, gender or exposure length to pesticides (59).

A study carried out in Hyderabad (India) in a chemical industry producing organophosphates, carbamates and pyrethroids showed significant increases in the MN frequency in subjects working for >10 years (57). Slight but significant increases in the frequency of MN were also reported in the Göksu Delta region (Turkey), a wetland area with intensive agriculture, where rice, cotton and peanuts are grown all over the year (58).

Significant increases in the frequency of MN were observed in the workers involved in soybean culture in the State of Rio Grande do Sul (Brazil); nevertheless, these increases were not related with the use of protective measures or the time of exposure (60).

Knowledge gaps and road map for future research and improvements

The general pattern in pesticide exposure is the simultaneous use of complex mixtures of chemical compounds that makes difficult to determine the possible synergic/antagonist effects among them. In this context, the appearance of the cytokinesis-block micronucleus assay in 1985 (62), as an easy alternative to the chromosome aberration test, opened the possibility to go further in the knowledge of the genotoxic risk associated to pesticide exposure. Nevertheless, the first biomonitoring study of a human population exposed to pesticides using the MN assay was published in 1993. Since then, an exponential use was not observed since 15 studies were reported between 1993 and 1999, 16 between 2000 and 2004 and 16 between 2005 and 2009. This means that, in spite of its advantages, the MN was not been widely used in the biomonitoring of human populations exposed to pesticides.

Actually, even if a number of studies in subjects exposed to single pesticides, or just to a few compounds, allowed to estimate a genotoxic risk associated to defined chemicals, the large majority of the available studies had not generated the reliable information needed for a risk assessment.

Some studies have an inadequate study design or a low statistical power. However, the main limitations of them are the lack of exposure assessment, information on the pesticide use pattern and the characterisation of the relevant factors modulating the exposure.

Surrogate factors for the exposure, such as pesticide consumption, number of genotoxic pesticides applied and duration of exposure were considered in some studies, where a relationship was observed between increased MN frequency and specific agricultural practices or inadequate working conditions. However, the lack of adequate evaluation of individual exposures severely limited any conclusions in regard to the identification of an active ingredient or occupational task, which are clearly identified as responsible for a genetic risk.

The MN test in its comprehensive application (Cytome) and for its role in predicting cancer risk is a useful tool to estimate the genetic risk from the integrated exposure to complex mixture of chemicals associated to the use of pesticides.

One advantage of the MN is that it makes easy to determine mechanism of action of the compounds through the detection

of the presence of kinetochore or centromere in the MN, as a way to distinguish between clastogenicity and aneugenicity, with relevant implications in risk assessment. These approaches were applied only in few studies (18,20,35), revealing an increase in kinetochore-negative or -positive MN related to the mechanism of action of the pesticides.

Further studies should be done in groups of subjects adequately characterised for the exposure in order to define the role of the MN test in pesticide risk assessment. Alternative methods have to be considered to estimate the exposure: the evaluation of dermal absorption and/or of the main urinary metabolites allows taking into account all the factors modulating the extent of exposure, such as the kind of crops, the type of application equipment and the use of protective devices. Other parameters can also be considered, as an example, inhibition of acetylcholinesterase activity could be a biomarker of exposure for widely used organophosphate pesticides with very short half-life (54).

In addition, the complex interaction of host defence mechanisms involved after a genotoxic exposure still need to be understand: interindividual differences in the ability to activate or detoxify genotoxic substances and to repair DNA damage could explain differential susceptibility to pesticides exposure.

The biomonitoring studies including the characterisation of allelic variants for genes involved in the metabolism of xenobiotics (32,33,39,50,53) reported contrasting results. Genetic polymorphisms in paraoxonase genes (*PON*s) were shown to modulate the frequency of MN in subjects exposed to complex mixture of pesticides (53). A recent *in vitro* study (63) showed that paraoxon caused a significant induction of MN only in subjects carrying the *PON1* QQ genotype with a lower PON1 activity, which was not able to hydrolyse the paraoxon.

A final aspect to be pointed out is the use of epithelial cells to evaluate the genetic risk associated to pesticide exposure. It must be emphasised that the MN assay can be applied in interphase to any proliferating cell population and allows the use of epithelial cells. The application of MN assay in buccal or nasal epithelial cells need to be further explored in groups of subjects exposed to pesticides considering the availability of a standardised protocol and of criteria of scoring for MN and other nuclear abnormalities.

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References

1. British Crop Protection Council (2009–2010). *The e-Pesticide Manual*. British Crop Protection Council, Hampshire, UK updated version 5.0.
2. Racke, K. D., Skidmore, M. W., Hamilton, D. J., Unsworth, J. B., Miyamoto, J. and Cohen, S. Z. (1997) Pesticide fate in tropical soils: technical report. *Pure Appl. Chem.*, **69**, 1349–1371.
3. Gonzalez-Arias, C. A. (2010) Patrón de uso y venta de plaguicidas en Nayarit, México. *Rev. Intern. Contam. Amb.*, **3**, 26(in press).
4. Blair, A. and Freeman, L. B. (2009) Epidemiologic studies of cancer in agricultural populations: observations and future directions. *J. Agromedicine*, **14**, 125–131.
5. Hanke, W. and Jurewicz, J. (2004) The risk of adverse reproductive and developmental disorders due to occupational pesticide exposure: an overview of current epidemiological evidence. *Int. J. Occup. Med. Environ. Health*, **17**, 223–243.
6. Lauria, L., Settimi, L., Spinelli, A. and Figà-Talamanca, I. (2006) Exposure to pesticides and time to pregnancy among female greenhouse workers. *Reprod. Toxicol.*, **22**, 425–430.
7. Dhillon, A. S., Tarbutton, G. L., Levin, J. L., Plotkin, G. M., Lowry, L. K., Nalbone, J. T. and Shepherd, S. (2008) Pesticide/environmental exposures and Parkinson's disease in East Texas. *J. Agromedicine*, **13**, 37–48.
8. Costello, S., Cockburn, M., Bronstein, J., Zhang, X. and Ritz, B. (2009) Parkinson's disease and residential exposure to maneb and paraquat from agricultural applications in the central valley of California. *Am. J. Epidemiol.*, **169**, 919–926.
9. Dosunmu, R., Wu, J., Basha, M. R. and Zawia, N. H. (2007) Environmental and dietary risk factors in Alzheimer's disease. *Expert Rev. Neurother.*, **7**, 887–900.
10. Santibáñez, M., Bolumar, F. and García, A. M. (2007) Occupational risk factors in Alzheimer's disease: a review assessing the quality of published epidemiological studies. *Occup. Environ. Med.*, **64**, 723–732.
11. Bolognesi, C. (2003) Genotoxicity of pesticides: a review of human biomonitoring studies. *Mutat. Res.*, **543**, 251–272.
12. Bull, S., Fletcher, K., Boobis, A. R. and Battershill, J. M. (2006) Evidence for genotoxicity of pesticides in pesticide applicators: a review. *Mutagenesis*, **21**, 93–103.
13. da Silva Augusto, L. G., Lieber, S. R., Ruiz, M. A. and de Souza, C. A. (1997) Micronucleus monitoring to assess human occupational exposure to organochlorides. *Environ. Mol. Mutagen.*, **29**, 46–52.
14. Garaj-Vrhovac, V. and Zeljezic, D. (2001) Cytogenetic monitoring of Croatian population occupationally exposed to a complex mixture of pesticides. *Toxicology*, **165**, 153–162.
15. Garaj-Vrhovac, V. and Zeljezic, D. (2002) Assessment of genome damage in a population of Croatian workers employed in pesticide production by chromosomal aberration analysis, micronucleus assay and comet assay. *J. Appl. Toxicol.*, **22**, 249–255.
16. Bhalli, J. A., Khan, Q. M., Haq, M. A., Khalid, A. M. and Nasim, A. (2006) Cytogenetic analysis of Pakistani individuals occupationally exposed to pesticides in a pesticide production industry. *Mutagenesis*, **21**, 143–148.
17. Barbosa, A. and Bonin, M. (1994) Evaluation of phosphine genotoxicity at occupational levels of exposure in New South Wales, Australia. *Occup. Environ. Med.*, **51**, 700–705.
18. Titenko-Holland, N., Windham, G., Kolachana, P., Reinisch, F., Parvatham, S., Osorio, A. M. and Smith, M. T. (1997) Genotoxicity of malathion in human lymphocytes assessed using the micronucleus assay *in vitro* and *in vivo*: a study of malathion-exposed workers. *Mutat. Res.*, **388**, 85–95.
19. Windham, G. C., Titenko-Holland, N., Osorio, A. M., Gettner, S., Reinisch, F., Haas, R. and Smith, M. (1998) Genetic monitoring of malathion-exposed agricultural workers. *Am. J. Ind. Med.*, **33**, 164–174.
20. Calvert, G. M., Talaska, G., Mueller, C. A., Ammenheuser, M. M., Au, W. W., Fajem, J. M., Fleming, L. E., Briggle, T. and Ward, E. (1998) Genotoxicity in workers exposed to methyl bromide. *Mutat. Res.*, **417**, 115–128.
21. Figgs, L. W., Titenko-Holland, N., Rothman, N. *et al.* (2000) Increased lymphocyte replicative index following 2,4-dichlorophenoxyacetic acid herbicide exposure. *Cancer Causes Control*, **11**, 373–380.
22. Vlastos, D., Demsia, G. and Matthopoulos, D. P. (2004) Evaluation of genetic damage in tobacco-growing farmers occupationally exposed to mixtures of metalaxyl and imidacloprid. *Int. J. Env. Anal. Chem.*, **84**, 183–191.
23. Pasquini, R., Scassellati-Sforzolini, G., Angeli, G., Fatigoni, C., Monarca, S., Beneventi, L., DiGiulio, A. M. and Bauleo, F. A. (1996) Cytogenetic biomonitoring of pesticide-exposed farmers in central Italy. *J. Environ. Pathol. Toxicol. Oncol.*, **15**, 29–39.
24. Joksic, G., Vidakovic, A. and Spasojevic-Tisma, V. (1997) Cytogenetic monitoring of pesticide sprayers. *Environ. Res.*, **75**, 113–118.
25. Venegas, W., Zapata, I., Carbonell, E. and Marcos, R. (1998) Micronuclei analysis in lymphocytes of pesticide sprayers from Concepcion, Chile. *Teratog. Carcinog. Mutagen.*, **18**, 123–129.
26. Kehdy, F. S. G., Cerqueira, E. M. M., Bonjardim, M. B., Camelo, R. M. and Castro, M. C. L. (2007) Study of the cytogenetic effects of occupational exposure to pesticides on sanitation workers in Belo Horizonte, Brazil. *Genet. Mol. Res.*, **6**, 581–593.
27. Bolognesi, C., Carrasquilla, G., Volpi, S., Solomon, K. R. and Marshall, E. J. (2009) Biomonitoring of genotoxic risk in agricultural workers from five Colombian regions: association to occupational exposure to glyphosate. *J. Toxicol. Environ. Health A*, **72**, 986–997.
28. Bolognesi, C., Parrini, M., Bonassi, S., Ianello, G. and Salanitto, A. (1993) Cytogenetic analysis of a human population occupationally exposed to pesticides. *Mutat. Res.*, **285**, 239–249.
29. Bolognesi, C., Parrini, M., Reggiardo, G., Merlo, F. and Bonassi, S. (1993) Biomonitoring of workers exposed to pesticides. *Int. Arch. Occup. Environ. Health*, **65** (Suppl. 1), S185–S187.

30. Bolognesi, C., Parrini, M., Merlo, F. and Bonassi, S. (1993) Frequency of micronuclei in lymphocytes from a group of floriculturists exposed to pesticides. *J. Toxicol. Environ. Health*, **40**, 405–411.
31. Scarpato, R., Migliore, L., Angotzi, G., Fedi, A., Miligi, L. and Loprieno, N. (1996) Cytogenetic monitoring of a group of Italian floriculturists: no evidence of DNA damage related to pesticide exposure. *Mutat. Res.*, **367**, 73–82.
32. Scarpato, R., Migliore, L., Hirvonen, A., Falck, G. and Norppa, H. (1996) Cytogenetic monitoring of occupational exposure to pesticides: characterization of GSTM1, GSTT1, and NAT2 genotypes. *Environ. Mol. Mutagen.*, **27**, 263–269.
33. Falck, G. C., Hirvonen, A., Scarpato, R., Saarikoski, S. T., Migliore, L. and Norppa, H. (1999) Micronuclei in blood lymphocytes and genetic polymorphism for *GSTM1, GSTT1* and *NAT2* in pesticide-exposed greenhouse workers. *Mutat. Res.*, **441**, 225–237.
34. Bolognesi, C., Perrone, E. and Landini, E. (2002) Micronucleus monitoring of a floriculturist population from Western Liguria, Italy. *Mutagenesis*, **17**, 391–397.
35. Bolognesi, C., Landini, E., Perrone, E. and Roggieri, P. (2004) Cytogenetic biomonitoring of a floriculturist population in Italy: micronucleus analysis by fluorescence in situ hybridization (FISH) with an all-chromosome centromeric probe. *Mutat. Res.*, **557**, 109–117.
36. Varona, M., Cárdenas, O., Crane, C., Rocha, S., Cuervo, G. and Vargas, J. (2003) Cytogenetic alterations in field workers routinely exposed to pesticides in Bogota flowers farms. *Biomedica*, **23**, 141–152.
37. Bolognesi, C., Merlo, F., Rabboni, R., Roggieri, P., Reggiardo, G. and Abbondandolo, A. (1995) Genotoxic risk from occupational exposure to pesticides in floriculture. *Clin. Chem*, **41**, 1919–1922.
38. Davies, H. W., Kennedy, S. M., Teschke, K., Jenny, P. and Quintana, E. (1998) Cytogenetic analysis of South Asian berry pickers in British Columbia using the micronucleus assay in peripheral lymphocytes. *Mutat. Res.*, **416**, 101–113.
39. Lucero, L., Pastor, S., Suárez, S., Durbán, R., Gómez, C., Parrón, T., Creus, A. and Marcos, R. (2000) Cytogenetic biomonitoring of Spanish greenhouse workers exposed to pesticides: micronuclei analysis in peripheral blood lymphocytes and buccal epithelial cells. *Mutat. Res.*, **464**, 255–262.
40. Ramírez, V. and Cuenca, P. (2001) Micronuclei frequency in lymphocytes of individuals occupationally exposed to pesticides. *Rev. Biol. Trop.*, **49**, 1–8.
41. Pastor, S., Gutiérrez, S., Creus, A., Cebulska-Wasilewska, A. and Marcos, R. (2001) Micronuclei in peripheral blood lymphocytes and buccal epithelial cells of Polish farmers exposed to pesticides. *Mutat. Res.*, **495**, 147–156.
42. Pastor, S., Gutiérrez, S., Creus, A., Xamena, N., Piperakis, S. and Marcos, R. (2001) Cytogenetic analysis of Greek farmers using the micronucleus assay in peripheral lymphocytes and buccal cells. *Mutagenesis*, **16**, 539–545.
43. Pastor, S., Creus, A., Xamena, N., Siffel, C. and Marcos, R. (2002) Occupational exposure to pesticides and cytogenetic damage: results of a Hungarian population study using the micronucleus assay in lymphocytes and buccal cells. *Environ. Mol. Mutagen.*, **40**, 101–109.
44. Pastor, S., Lucero, L., Gutiérrez, S., Durbán, R., Gómez, C., Parrón, T., Creus, A. and Marcos, R. (2002) A follow-up study on micronucleus frequency in Spanish agricultural workers exposed to pesticides. *Mutagenesis*, **17**, 79–82.
45. Pastor, S., Creus, A., Parrón, T., Cebulska-Wasilewska, A., Siffel, C., Piperakis, S. and Marcos, R. (2003) Biomonitoring of four European populations occupationally exposed to pesticides: use of micronuclei as biomarkers. *Mutagenesis*, **18**, 249–258.
46. Márquez, C., Villalobos, C., Poblete, S., Villalobos, E., García, M. A. and Duk, S. (2005) Cytogenetic damage in female Chilean agricultural workers exposed to mixtures of pesticides. *Environ. Mol. Mutagen.*, **45**, 1–7.
47. Vlastos, D., Stivaktakis, P. and Matthopoulos, D. P. (2006) Pesticide exposure and genotoxicity correlations within a Greek farmers' group. *Int. J. Env. Anal. Chem.*, **86**, 215–223.
48. Tope, A., Bebe, F. N. and Panemangalore, M. (2006) Micronuclei frequency in lymphocytes and antioxidants in the blood of traditional limited-resource farm workers exposed to pesticides. *J. Environ. Sci. Health B*, **41**, 843–853.
49. Montero, R., Serrano, L., Araujo, A., Dávila, V., Ponce, J., Camacho, R., Morales, E. and Méndez, A. (2006) Increased cytogenetic damage in a zone in transition from agricultural to industrial use: comprehensive analysis of the micronucleus test in peripheral blood lymphocytes. *Mutagenesis*, **21**, 335–342.
50. Costa, C., Teixeira, J. P., Silva, S. *et al.* (2006) Cytogenetic and molecular biomonitoring of a Portuguese population exposed to pesticides. *Mutagenesis*, **21**, 343–350.
51. Costa, C., Silva, S., Coelho, P., Roma-Torres, J., Teixeira, J. P. and Mayan, O. (2007) Micronucleus analysis in a Portuguese population exposed to pesticides. Preliminary survey. *Int. J. Hyg. Environ. Health*, **210**, 415–418.
52. Ali, T., Bhalli, J. A., Rana, S. M. and Khan, Q. M. (2008) Cytogenetic damage in female Pakistani agricultural workers exposed to pesticides. *Environ. Mol. Mutagen.*, **49**, 374–380.
53. da Silva, J., Moraes, C. R., Vanina, D., Heuser, V., Andrade, M. and Silva, F. R. (2008) Evaluation of genetic damage in a Brazilian population occupationally exposed to pesticides and its correlation with polymorphisms in metabolizing genes. *Mutagenesis*, **23**, 415–422.
54. Levario-Carillo, M., Sordo, M., Rocha, F., González-Horta, C., Amato, D. and Ostrosky-Wegman, P. (2005) Micronucleus frequency in human umbilical cord lymphocytes. *Mutat. Res.*, **586**, 68–75.
55. Remor, A. P., Totti, A. C., Moreira, D. A., Dutra, G. P., Heuser, V. D. and Boeira, J. M. (2009) Occupational exposure of farm workers to pesticides: biochemical parameters and evaluation of genotoxicity. *Environ. Int.*, **35**, 273–278.
56. Gómez-Arroyo, S., Díaz-Sánchez, Y., Meneses-Pérez, M. A., Villalobos-Pietrini, R. and De León-Rodríguez, J. (2000) Cytogenetic biomonitoring in a Mexican floriculture worker group exposed to pesticides. *Mutat. Res.*, **466**, 117–124.
57. Ergene, S., Celik, A., Cavas, T. and Kaya, F. (2007) Genotoxic biomonitoring study of population residing in pesticide contaminated regions in Goksu delta: micronucleus, chromosomal aberrations and sister chromatid exchanges. *Environ. Int.*, **33**, 877–885.
58. Martínez-Valenzuela, C., Gómez-Arroyo, S., Villalobos-Pietrini, R., Waliszewski, S., Calderón-Segura, M. E., Félix-Gastélum, R. and Alvarez-Torres, A. (2009) Genotoxic biomonitoring of agricultural workers exposed to pesticides in the north of Sinaloa State, Mexico. *Environ. Int.*, **35**, 1155–1159.
59. Sailaja, N., Chandrasekhar, M., Rekhadevi, P. V., Mahboob, M., Rahman, M. F., Vuyyuri, S. B., Danadevi, K., Hussain, S. A. and Grover, P. (2006) Genotoxic evaluation of workers employed in pesticide production. *Mutat. Res.*, **609**, 74–80.
60. Bortoli, G. M., Azevedo, M. B. and Silva, L. B. (2009) Cytogenetic biomonitoring of Brazilian workers exposed to pesticides: micronucleus analysis in buccal epithelial cells of soybean growers. *Mutat. Res.*, **675**, 1–4.
61. Castro, R., Ramírez, V. and Cuenca, P. (2004) Micronuclei and other nuclear abnormalities in the oral epithelium of female workers exposed to pesticides. *Rev. Biol. Trop.*, **52**, 611–621.
62. Fenech, M. and Morley, A. (1985) Solutions to the kinetic problem in the micronucleus assay. *Cytobios*, **43**, 233–246.
63. Rojas-Garcia, A. E., Sordo, M., Vega, L., Quintanilla-Vega, B., Solis-Heredia, M. and Ostrosky-Wegman, P. (2009) The role of paraoxonase polymorphisms in the induction of micronucleus in paraoxon-treated human lymphocytes. *Environ. Mol. Mutag.*, **50**, 823–829.