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REVIEW

Evidence for genotoxicity of pesticides in pesticide applicators: a review

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A systematic review of the literature has been conducted and studies reporting investigations of genotoxicity biomarkers in pesticide workers have been assessed with view to establishing whether there was evidence for any risk to those using pesticides approved in the United Kingdom. Each of the studies was evaluated using a set of criteria drawn up by members of the UK Committee of Mutagenicity based upon the guidelines proposed by the International Programme on Chemical Safety (IPCS) working group [R. J. Albertini, D. Anderson, G. R. Douglas, L. Hagmar, K. Hemminki, F. Merlo, A. T. Natarajan, H. Norppa, D. E. Shuker, R. Tice, M. D. Waters and A. Aitio (2000) *Mutat. Res*., 463, 111–172]; 24 out of 70 studies met the criteria for inclusion in the substantive evaluation. Positive findings were compared with occupational practices and evidence of exposure to specific pesticides with view to developing hypotheses for further consideration. Seventeen of the 24 studies reported positive findings, although in the majority of these the magnitude of increase was small. There was some limited evidence that the use of benzimidazoles was more consistently associated with positive findings. However, limitations in the data, particularly evidence of exposure, did not allow definitive conclusions to be drawn. Also, it was noted that the use (or not) of personal protective equipment (PPE) was not well documented and in the few studies in which its use was reported, the findings were more likely to be positive in the absence of PPE usage. An independent epidemiological review concluded that all studies were of limited design, particularly with regards to study size, the assessment of subject selection and potential recruitment bias. Variance in genotoxicity indices in the control population and a lack of understanding of the factors influencing this variability complicate attempts to characterize positive responses. More substantive data are needed in this respect so that the significance of relatively small increases in biomonitoring indices can be accurately assessed. Once these data are available, a study in workers using benzimidazoles would be appropriate.

Introduction

Pesticides undergo testing for genotoxicity and carcinogenicity prior to market authorization. Some prove positive for genotoxicity and/or carcinogenicity in tests systems. However, non-threshold genotoxic carcinogens are not approved for use in the United Kingdom and the conditions of approval are set to offset any potential risks to individuals who might be exposed to these compounds. Nevertheless, this does assume that the pesticides are used according to the stipulated conditions. Possible abuse or misuse could lead to significant levels of exposure, particularly among those exposed occupationally. There are also concerns that the risk of genotoxicity from some pesticides might be appreciably greater than that predicted from toxicity tests (1). Cytogenetic damage in circulating lymphocytes has been widely used as a biomarker of exposure (and perhaps of effect) in those exposed to pesticides. This has resulted in a number of reports in which pesticide exposure has been associated with increases in chromosome aberrations (CA), micronuclei (MN) and sister chromatid exchanges (SCE) in cultured lymphocytes isolated from peripheral blood taken from exposed individuals [reviewed in (1)]. It has been suggested that the assessment of cytogenetic effects in exposed subjects can serve as an early indicator of increased risk of cancer (2–4) although the evidence is somewhat contradictory (5).

There is much uncertainty surrounding studies of pesticide exposure and genotoxic damage, including the reliability of exposure assessment, the power of the studies, the suitability of control groups and the protocols used for determining genotoxicity. In addition, there is by no means agreement as to the significance of an increase in cytogenetic damage for cancer risk and therefore the health risks associated with pesticide exposure (6,7). However, it is noteworthy that 'pesticides' *per se* comprise a wide variety of chemical classes and structures, most of which are not considered to represent an *in vivo* genotoxic hazard. Indeed, registration policies are focused upon approval of pesticide products that should pose no risk to users. Nevertheless, a review conducted by Bolognesi (1) concluded that occupational exposure to pesticides was associated with an increase in genotoxic damage and that this was dose-related. Attention was drawn also to the potential for cumulative effects with continuous exposure, even though the chromosomal damage itself is regarded as transient. However, due to the fact that pesticide products generally comprise a mixture of different chemicals and that more than one product may be used simultaneously, it is largely unfeasible to tease out the potential effects of any specific pesticide of concern.

The current article is based upon a review prepared by the Department of Health Toxicology Unit, Imperial College London and the DH Secretariat for the UK's Department of Health's, Committee on Mutagenicity (COM). The aim was to use a structured approach to examine, as far as reasonably possible, all published investigations of DNA damage including clastogenicity in pesticide applicators, and workers exposed to pesticides during manufacture, formulation

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or during and after use (e.g. those handling cut flowers). The ultimate objective was to establish whether there was evidence for any risk of genotoxicity posed by the use of the pesticides approved in the United Kingdom.

After a detailed literature review, all the articles identified were evaluated for methodological robustness using exclusion criteria drawn up by COM members using the guidelines proposed by the International Programme on Chemical Safety (IPCS) working group (8) as a framework. A further assessment was undertaken by an independent epidemiologist with regard to the rigour of studies, which included evaluation of study design, selection of controls, bias, confounding and use of statistical methods.

Relationships were sought between any evidence of genotoxicity findings and specific occupations, the use (or not) of protective clothing and the exposure to specific pesticides. In this way, it was hoped to identify active ingredients or occupational practises that could then be submitted for regulatory review or targeted for focused research.

Recently, Guzelian *et al*. (9) highlighted the need for toxicologists to address the shortcomings of present methodologies and evaluations, encouraging the use of evidence-based toxicology based on answering questions about general and specific causations of effects. A stepwise approach is suggested, wherein data are collected, appropriate questions framed and the evidence reviewed with regards to causation. In this spirit, the current assessment is considered to be novel and to augment the review conducted by Bolognesi (1). It is hoped that this evidence-based evaluation of the current literature will provide a more realistic framework for a true risk assessment. Furthermore, by establishing the strengths and weaknesses of the published investigations, it has been possible to formulate recommendations for future studies that should be more informative than many of those published to date.

Identification of groups appropriate for study

Individuals working with pesticides were categorized into five main groups, depending on their place of work and/or activity with the active ingredients, e.g. spraying.

Pesticide sprayers and applicators. Those involved directly in the preparation and application of pesticides to crops and who potentially represent the most exposed group of workers (1). However, as the potential for direct contact with pesticides is anticipated during these activities, the use of personal protective equipment (PPE) is frequently a condition of use and is thought to be more commonplace than in those working with the sprayed crops, which may impact on outcomes (10,11). PPE usage is discussed below.

Floriculturists and greenhouse workers. Those involved in the production of flowers and ornamental plants which are commonly sprayed with pesticides in greenhouses. It has been suggested that these groups of workers may potentially have an increased risk of cytogenetic damage due to working in small confined areas, humid conditions and a potential continuous exposure through re-entry activities such as cutting and potting, all militating against the regular use of protective clothing (12,13). Additionally, compared to other classes of workers, floriculturists may be relatively highly exposed to pesticides during loading, mixing and application as well as during manual activities following regular contact with flowers and ornamental plants (14). Furthermore, the climatic conditions within greenhouses allow for the continuous production of fruits, vegetables and flowers, requiring a regular application of pesticides throughout the year, resulting in potentially continuous exposure (15,16).

Agricultural workers and farmers. Those involved in the production of crops, fruits and vegetables and hence indirectly exposed to pesticides. In most instances, workers were also involved in the mixing and loading of the pesticides. Exposure to pesticides may be lower than in other groups due to the seasonal application of pesticides and the working environment, i.e. outdoors (17).

Forestry workers. Those involved in the spraying of trees and shrubs. In general, fewer active ingredients were used by forestry workers compared with other occupational groups (18).

Production workers. Those involved in the manufacture of pesticides. The production of pesticides is undertaken throughout the year, as opposed to pesticide applications which, in general, occur seasonally. Workers are therefore potentially exposed to pesticides continuously, as well as to the raw materials such as formaldehyde, toluene and benzene, some of which also have genotoxic activity.

Literature search strategy

Relevant study reports were retrieved by systematically searching MEDLINE and TOXBASE using the following search terms: each of the pesticide worker definitions listed above plus genotoxic(ity), mutagen(icity), DNA damage, micronucleus, CA, cytogenetic, COMET assay or biomonitoring. These were cross-checked against a similar review conducted by Bolognesi (1) and literature searches conducted by the UK Pesticides Safety Directorate (PSD).

A total of 70 studies were retrieved (2–4,10–76). Reports were obtained from diverse parts of the world, including the United States, Europe and South America. However, no studies conducted in the United Kingdom were identified. The majority of studies were analyses of cytogenetic damage (MN and CAs) in circulating lymphocytes, a few investigated cytogenetic endpoints in buccal mucosa cells and some measured damage using the COMET assay or DNA adduct technology.

Overview of exclusion/inclusion criteria

All the articles retrieved were analysed in detail, and their suitability for inclusion in the substantive assessment was evaluated according to predetermined criteria. These criteria had been set by the COM in consultation with an independent epidemiologist, based on guidelines proposed by the IPCS working group for monitoring genotoxic effects. Blood sampling procedures, storage and quality control parameters as well as details of statistical analyses were addressed; the reader is referred to the article from the IPCS for details of these criteria (8). The approach adopted was designed to avoid bias and provide clear guidance for the reviewer. Studies were excluded largely due to the use of inappropriate or unsubstantiated experimental procedures or inadequate presentation of data (lack of control data, duplicated data).

The exclusion and inclusion criteria employed were as follows:

Sister chromatid exchange. Studies measuring only SCE as the endpoint were excluded. The Committee had concluded that the true biological relevance of the SCE endpoint to

mutagenicity or carcinogenic risk was questionable and therefore not useful to the current assessment (77). A total of nine studies were excluded; five reported positive data and four negative.

Micronuclei: incubation period. Studies which utilized a 72 h incubation period to assess MN were excluded. This decision was based on the understanding that by this time many cells will have passed through three cell divisions post-sampling and the extent of DNA repair and cell loss may influence the results, possibly leading to false negative data. However, if data on the proliferation of cells had been provided and it was shown that less than three rounds of cell division had occurred, these studies were included. A total of nine studies were excluded, one reported positive and eight negative data.

Chromosome aberrations. Chromosome/chromatid gaps are not considered to be a true representation of a cytogenetic effect (78). Therefore, if the percentage of aberrant cells without gaps could not be extracted from the data provided, these studies were excluded from further evaluation. A total of nine studies were excluded based on this criterion, two of which were negative.

Sampling time. Studies in which samples were collected more than 48 h after cessation of pesticide exposure were excluded. The IPCS guidelines recommend that sampling for all endpoints be carried out during chronic exposure when damage and repair mechanisms are at steady state, or within 2 days after acute or cessation of chronic exposure (8). This is necessary as persistence of genetic damage is generally transient following chemical exposure (79), which could lead to false negative results. Two studies were rejected on this basis, one positive and one negative.

Inappropriate protocols. Advice was sought from members of the COM with particular expertise in different fields of genotoxicity testing, on the appropriateness or robustness of protocols identified as unusual. Elements of protocol design, such as the number of cells scored, were taken from Albertini *et al*. (8). A study which omitted a butanol extraction step during a ^{32}P-post-labelling assay (14) and studies in which lymphocytes were isolated prior to culturing rather than culturing whole blood were excluded as being inappropriate. Furthermore, some studies in which samples had been shipped were excluded; this criterion was based on the probability of a long interval between sampling and analysis, inadequately controlled temperatures or the possibility that samples had passed through X-ray machines, thus resulting in additional cell damage. However, several of these studies were rejected based on other criteria in the flow diagram depicted in Figure 1 and therefore a single positive study by Nehez *et al*. (54) was excluded on the basis that only 10 metaphase spreads were scored and therefore at variance with guidelines.

Inadequate control data. Reports not presenting control subject data variability were excluded from further evaluation as this is regarded as important for assessing the significance of any positive findings [e.g. a series of studies by Rupa *et al*. (65–67)]. In addition, studies in which no attempt had been made to match control subjects with exposed subjects with regards to age, sex and smoking habits were excluded (3,11,41,61). In control subjects, the effects of age, sex and smoking on cytogenetic endpoints such as MN and CA were also considered to serve as a positive internal control for the study. Studies that could not demonstrate an age-, sex-, or smoking-related increase in MN or CA were noted and less weight was placed on data obtained from such studies (41).

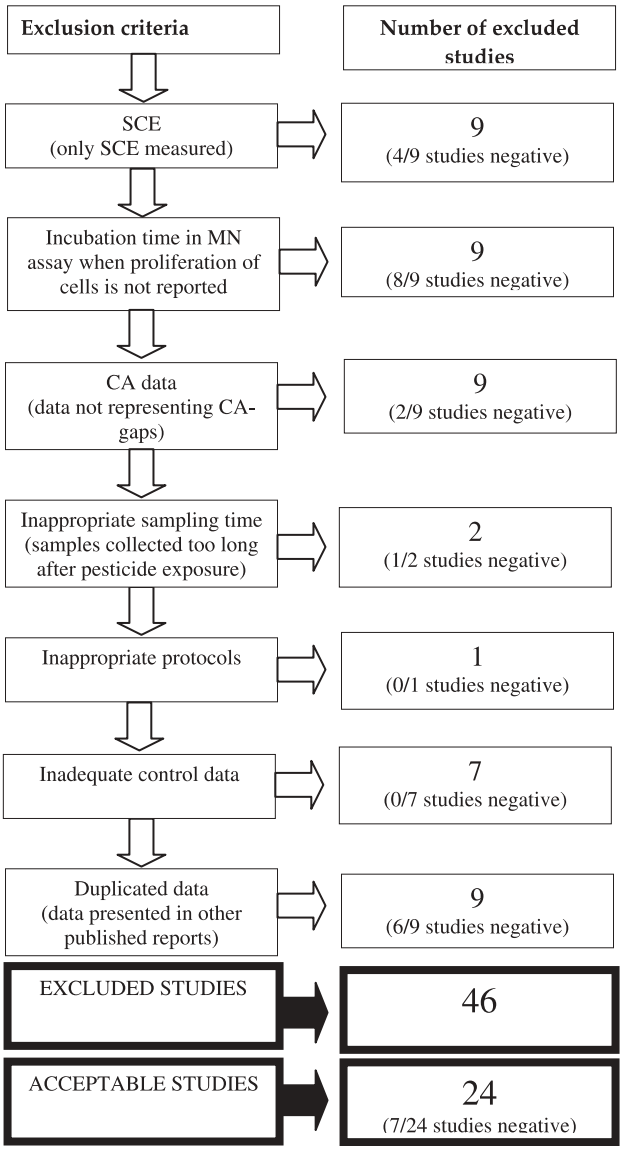

Fig. 1. Flow chart showing the exclusion criteria and number of studies excluded at each step (*Note*: some studies were excluded on the basis of more than one criterion).

Duplicated data. Those reports found to contain duplicated data from previous publications were excluded from further evaluation. Most notably, in a series of articles reporting on studies conducted in Croatia by Zeljezic and Garaj-Vrhovac (32,33,74–76), there was substantial evidence that the subjects used in each study were the same and that some parameters (e.g. frequency of binucleated lymphocytes) are inaccurately reported. Similarly, two reports from Bolognesi *et al*. (23,25) appear to reproduce datasets.

Confounding factors. No studies were excluded based on a lack of age or sex concurrence between controls and exposed subjects, but this was noted and taken into consideration when placing weight of evidence on data from these studies.

Final listing of studies

A total of 24 studies were selected for detailed assessment ('the selected studies'). A breakdown of the numbers of studies which were excluded, including the proportions of which

were reported as negative or positive, is shown in Figure 1. This represents a sequential process and some studies may have been excluded on the basis of more than one criterion.

Results of analyses

Table I shows the occupations, endpoints measured, fold-increases in genetic damage in the pesticide-exposed group compared to controls and the authors' conclusion on whether the results in workers were significantly different to those in the controls, according to their statistical analyses. Overall, 17 of the 24 studies reported positive differences in exposed subjects, based on statistical significance (8/10 floriculturists, 4/8 agriculture-farmers, 3/4 sprayers, 1/1 foresters and 1/1 manufacturing).

The Committee commented on the relatively small increases (<1.5-fold) in genetic damage in a number of studies reported as positive by the authors (7/17 positive studies). For example, in a group of greenhouse workers Lander *et al*. (46) reported a genotoxic effect, but this appears negligible when gaps are excluded from the lymphocyte CA analyses (without gaps: 1.31% in controls and 1.37% in exposed workers; with gaps: 1.62% in controls and 2.34% in exposed workers). Pasquini *et al*. (57) reported a 1.2-fold, statistically significant increase in lymphocyte MN in agricultural workers (15.98/1000 and 13.30/1000 in exposed and controls, respectively), and a concurrent analysis of SCE's yielded negative results.

In light of these observations, an attempt was made to put into perspective the biological relevance of these small changes. The following points were addressed:

(i) the variation in values in the unexposed control subjects and
(ii) the magnitude of responses observed in subjects exposed to known mutagens.

Table I. Overall assessment of genotoxic endpoints measured in pesticide workers in the selected studies

Author	No. of subjects/controls and occupation	Endpoint	Results*	Highest magnitude of increase observed
Bolognesi *et al*. (24)	56 male and 15 female floriculturists 75 controls	MN	Positive	1.3-fold increase
Bolognesi *et al*. (12)	51 floriculturists 24 controls	MN	Negative	1.3-fold increase
De Ferrari *et al*. (29)	32 floriculturists 32 floriculturists with bladder cancer 31 controls	CA	Positive	1.9-fold increase
Dulout *et al*. (30)	36 floriculturists 15 controls	CA	Positive	4.3-fold increase
Falck *et al*. (4)	20 male and 14 female floriculturists 17 male and 16 female controls	MN	Positive	1.1-fold increase
Gomez-Arroyo *et al*. (35)	8 male and 22 female floriculturists 30 controls	MN	Positive	2.7-fold increase
Lander *et al*. (46)	116 male greenhouse workers 29 male controls	CA	Positive	1.0-fold increase
Munnia *et al*. (14)	40 male and 17 female greenhouse workers 22 male and 11 female controls	DNA adducts	Positive	3.9-fold increase
[a]Peluso *et al*. (62)	19 male and 7 female floriculturists 13 male and 9 females controls	DNA adducts	Positive	4.7-fold increase
Piperakis *et al*. (63)	30 male and 20 female greenhouse workers 41 male and 25 female controls	Comet	Negative	1.0-fold increase
Carbonell *et al*. (2)	29 male agricultural workers 2 matched control groups; Gp1, 29 males; Gp2, 24 males	CA	Positive	1.4-fold increase
Hogstedt *et al*. (38)	10 farmers 7 controls	CA	Negative	0.9-fold increase
Lebailly *et al*. (48)	29 male farmers 1 male control	Comet	Positive	1.4-fold increase
Lebailly *et al*. (49)	29 male farmers	Comet	Positive	1.3-fold increase
Lebailly *et al*. (47)	19 fruit growers	Comet	Negative	1.3-fold increase
Pasquini *et al*. (57)	48 male farmers 50 male controls	MN	Positive	1.2-fold increase
Pastor *et al*. (17)	30 male and 20 female agricultural workers 41 male and 25 female controls	MN	Negative	0.7-fold increase
Pastor *et al*. (16)	58 male and 26 female agricultural workers 53 male and 12 female controls	MN	Negative	1.0-fold increase
Garry *et al*. (18)	51 sprayers 33 controls	CA	Positive	3.5-fold increase
[a]Kourakis *et al*. (45)	29 sprayers 14 controls	CA	Positive	5.0-fold increase
Mustonen *et al*. (53)	19 male pesticide sprayers 15 male controls	CA	Negative	1.0-fold increase
Paldy *et al*. (56)	80 male mixers and sprayers 24 male controls	CA	Positive	3.9-fold increase
Garry *et al*. (34)	24 foresters 15 controls	CA	Positive	3.4-fold increase
Grover *et al*. (37)	54 pesticide manufacturers 54 controls	Comet	Positive	2.6-fold increase

*Positive = statistically significant differences between exposed and control groups as determined by author.

Table II. Statistical description of data from control subjects for micronucleus and chromosome aberration assays in selected studies

	MN (*n*/1000)	CA (%)
Mean ± SD	9.91 ± 6.15	2.73 ± 1.54
SE	1.38	0.36
Median	9.97	1.50
95% CI	7.34–12.86	1.38–3.32
Range	1.32–21.76	0.36–6.52
25% percentile	5.94	0.67
75% percentile	12.50	4.11
Coefficient of variation (%)	49	88
Fold increase between lowest and highest data	16.5	16.3

A statistical summary of the data from control subjects from the selected studies utilizing MN and CAs as their endpoints is shown in Table II. Analysis of the data revealed that the frequency of MN was consistent with a normal distribution, whilst the distribution of CAs was skewed. However, there was a large overall variation in all the biomonitoring indices evaluated in the control populations (~16-fold range for both MN and CA). Furthermore, it is suggested that the factors which influence the variability in control subjects are not well understood, further hindering attempts to qualify a positive response. Therefore, it was not possible to define historical control ranges from which it would be possible to define more precisely a clear positive response.

A series of studies examining increases in MN or CA, in patients treated with cytostatic medicines such as cyclophosphamide, or nurses exposed occupationally to the cytostatic drugs, were assessed and the reported fold increases determined. Tates *et al*. (80) reported MN increases in patients treated with a variety of drug regimens including cyclophosphamide, bleomycin and ifosfamide. The increases were not large, ~2.0- to 2.6-fold, and the authors communicate their surprise at the ineffectiveness of the drug regimes at inducing chromosomal damage. Similar responses were demonstrated in another study examining patients exposed to cyclophosphamide [2.7-fold increase in MN (81)]. Nurses occupationally exposed to cytostatic medicines had slightly lower fold increases in MN than patients [2.2- and 1.6-fold in (82) and (83), respectively]. These comparisons indicate that the magnitude of induced cytogenetic damage by known genotoxins was not as substantial as one might have expected on the basis of data from animal studies (e.g. compared to >10-fold increases as observed in rodent assays with cyclophosphamide), but it did provide information useful in helping to interpret the biomonitoring studies for genotoxicity. However, it was not possible to draw a definite conclusion on a minimum fold increase in genotoxicity indices associated with exposure to genotoxic compounds that might be considered of biological relevance.

Personal protective equipment

An attempt was made to relate the findings in these studies to the use (or lack of use) of protective clothing. Of the 24 studies assessed, only 12 stated whether protective clothing was used. Prominent differences in the use of protective clothing were noted between studies, although there were no studies in which the effects of wearing PPE on the extent of genetic damage had been specifically investigated. The usage of PPE, as noted in the individual articles, is summarized in Table III.

The extent of PPE usage varied considerably; some studies reporting that pesticides were mixed using bare hands and sprayed using no protection (44,45) and others describing conditions in which most workers used all available protective clothing (12,16). Interestingly, in the four studies reporting that a majority of workers took protective measures (>60%), all concluded that the results were negative (12,16,17,63). In contrast, seven of the eight studies in which workers wore little or no protective clothing reported significantly increased induced cytogenetic damage. This suggests some degree of efficacy of the PPE at preventing exposure.

Lander *et al*. (46) noted that cytogenetic effects were observed primarily in workers who did not use gloves, although data showing this were not presented. Several studies, reporting significant increases in CA and MN frequency, associated this with the lack of protective measures during pesticide use (2,46,57). Two studies reporting >4-fold increases in CA also reported a lack of PPE use (30,45).

Although many floriculturists allegedly use protective measures, in some cases, authors stated that due to humid conditions within the greenhouses, full protective clothing was not always used (16). In addition, floriculturists and agricultural workers may potentially be exposed to pesticides through re-entry activities and the handling of crops, respectively, when the use of PPE would be less likely. Pesticide sprayers also have a potentially increased exposure, as they handle pesticides throughout the year and those spraying chemicals from above head height are at risk of oral and percutaneous exposure if adequate measures are not taken (2).

Overall, from this limited review there appears to be a correlation between the absence of protective clothing and positive genotoxic endpoints.

Relationship to pesticide exposures

One of the original goals of the review was to identify any specific pesticides associated with increased indices of genotoxicity in exposed workers and to determine whether extended duration of exposure results in cumulative damage. Only 15 of the 24 studies provided information on the pesticides with which the study subjects were likely to have been in contact. However, in the majority of these studies (14/15), no attempts were made to measure systemic exposure to any of the pesticides listed.

The current evaluation centred on identifying pesticide active ingredients used by the workers that are classified as category 2 or 3 mutagens under the Dangerous Substances Directive EC/67/548 (i.e. *in vivo* mutagens or *in vivo* germ cell mutagens in animals), and attempting to relate exposure to such compounds with any increase in genetic damage observed. The list includes benomyl (category 2), carbendazim (category 2), cycloheximide, DNOC (4,6-dinitro-*o*-cresol), edifenphos, fenthion, atrazine, ziram, thiram, monocrotophos, phosphamidon and thiophanate methyl (all category 3). Table IV shows the studies in which the study subjects were exposed to these classified ingredients.

Of the 15 studies providing pesticide usage information 11 were reported as positive by the authors. At least one category 2 or 3 mutagen was cited as being used in 8/11 of the positive studies. Of the negative studies, Bolognesi *et al*. (12) and Lebailly *et al*. (47) reported the use of category 2 mutagenic active ingredients (carbendazim and benomyl), whereas workers in the study carried out by Falck *et al*. (4)

Table III. The recorded use of PPE in the selected studies

Author	Occupation	Endpoint	PPE use	Result*
Bolognesi *et al*. (24)	Floriculturists	MN	Not stated	Positive
Bolognesi *et al*. (12)	Floriculturists	MN	Most (86%) wore protection	Negative
De Ferrari *et al*. (29)	Floriculturists	CA	Not stated	Positive
Dulout *et al*. (30)	Floriculturists	CA	Little or no protective clothing used	Positive
Falck *et al*. (4)	Floriculturists	MN	One group wore full protection and one group wore only gloves and boots	Positive
Gomez-Arroyo *et al*. (35)	Floriculturists	MN	Not stated	Positive
Lander *et al*. (46)	Floriculturists	CA	Gloves worn by some workers	Positive
Munnia *et al*. (14)	Floriculturists	DNA adducts	Not stated	Positive
[a]Peluso *et al*. (62)	Floriculturists	DNA adducts	Not stated	Positive
Piperakis *et al*. (63)	Floriculturists	Comet	Some workers (62%) used some type of protective clothing (52% used gloves, 38% impermeable boots, 42% breathing masks)	Negative
Carbonell *et al*. (2)	Agricultural workers and farmers	CA	3% (1/29) wore all available protection, 76% wore some protection, 59% wore masks incorrectly	Positive
Hogstedt *et al*. (38)	Agricultural workers and farmers	CA	Not stated	Negative
Lebailly *et al*. (48)	Agricultural workers and farmers	Comet	Not stated	Positive
Lebailly *et al*. (49)	Agricultural workers and farmers	Comet	34% wore gloves. 15% wore gloves and masks	Positive
Lebailly *et al*. (47)	Agricultural workers and farmers	Comet	14% wore no protection, 14% wore masks/ protective clothes, 17% wore only gloves, 41% wore all protection	Negative
Pasquini *et al*. (57)	Agricultural workers and farmers	MN	Few workers (29%) wore protective clothing	Positive
Pastor *et al*. (17)	Agricultural workers and farmers	MN	Some workers (62%) wore protective clothing	Negative
Pastor *et al*. (16)	Agricultural workers and farmers	MN	Most workers (85%) wore protective clothing	Negative
Garry *et al*. (18)	Sprayers	CA	Not stated	Positive
[a]Kourakis *et al*. (45)	Sprayers	CA	None of the workers used protective clothing	Positive
Mustonen *et al*. (53)	Sprayers	CA	Not stated	Negative
Paldy *et al*. (56)	Sprayers	CA	Not stated	Positive
Garry *et al*. (18)	Foresters	CA	Not stated	Positive
Grover *et al*. (37)	Pesticide manufacturers	Comet	Not stated	Positive

*Statistically significant difference between exposed and non-exposed groups (positive).

and Pastor *et al*. (16) did not report the use of any of the category 2 or 3 listed pesticides. Carbendazim and benomyl were used in 4/11 and 5/11 positive studies and 1/4 and 2/4 negative studies listed, respectively. Four of the positive studies measured MN or CA which are potentially the outcome of damage by spindle inhibitors such as benzimidazoles (2,12,46,57). However, it is unclear whether the damage detected when using the Comet assay (37,49) is consistent with exposure to benzimidazoles as there is no evidence for binding of such compounds directly to DNA (83) and exposures were presumably below levels likely to be cytotoxic. Furthermore, benzimidazoles do not appear to form DNA adducts and hence the results of the Peluso *et al*. (62) study are unlikely to be related to exposure to this class of pesticide.

Garry *et al*. (18) was the only group who attempted to measure exposure. They showed that CA frequency showed no relationship to urinary 2,4-dichlorophenoxyacetic acid, although the former did correlate with total volume of herbicide applied. The test population also used other pesticides, but they were not listed in the publication.

There were limited data on estimated duration of exposure to pesticides in 6 out of the 24 selected studies (12,24,37,45,56,57). Four of these studies reported a positive correlation between duration of exposure and increased indices of genotoxicity (24,37,56,57) whereas the remaining two studies reported a negative correlation (12,45). In all studies, the magnitude of the increase in genotoxic response was small relative to the duration of exposure and therefore it is not possible to draw any definite conclusions based on these data. Additionally, it must be noted that the incidence of genotoxicity biomarkers such as CA and MN increases with age (84) and it is likely that those who had worked the longest were also among the oldest subjects. However, few attempts were made to control for this potential confounding factor.

Epidemiological study quality and statistical issues

As part of this review, the selected studies were scrutinized by an epidemiologist for adequacy, and to suggest if studies could be ranked according to their quality, so that more weight could be placed on some studies rather than others.

The principle conclusions reached in the epidemiology overview were that all of the selected studies had limitations in their design, particularly with regard to the study size, the assessment of subject selection and the potential recruitment biases. Many of the studies provided information on demographics, medical history, lifestyle factors, potential occupational exposures to materials other than pesticides (e.g. solvents, radiation), and also information on the type of pesticides used, duration and frequency of exposure and use of protective measures. However, these data had generally not been used in the analyses reported and the majority of the articles did not provide a specific analysis of exposure to individual pesticides.

It was noted that the majority of studies were not sufficiently large to allow an evaluation of all the variables for which data might be available. Study designs were generally cross sectional, although a few had taken multiple samples (e.g. at different time points in a growing season). The time interval between exposure and sampling thus varied considerably

Table IV. The recorded use of pesticide products in the selected studies (emboldened names are classified as category 2 or 3 mutagens)

Author	Endpoint	Results*	Pesticides used
Bolognesi *et al*. (24)	MN	Positive	Metham sodium, dodemorph, zineb, antracol, captan, dazomet, dichloropropane, dichloropropene
Bolognesi *et al*. (12)	MN	Negative	Dichlorvos, ethoprophos, glyphosphate, methamidophos, methidathion, **monocrotophos**, omethoate, parathion methyl, deltamethrin, aldicarb, methomyl, dazomet, mancozeb, zineb, ethiophencarb, **benomyl**, **carbendazim**, **thiofanate methyl**, paraquat, captan, endosulfan, dodemorph, buripimate, vinclozolin
Carbonell *et al*. (2)	CA	Positive	Acephate, chlorpyrifos, dimethoate, fenitrothion, **fenthion**, fosetyl, isofenphos, methamidophos, naled, pyrazophos, cypermethrin, deltamethrin, fenpropathrin, fenvalerate, methiocarb, methomyl, oxamyl, mancozeb, propineb, zineb, **benomyl**, diquat, paraquat, captan, folpet, procymidone, endosulfan, abamectin, kasugamycin, iprodione, oxadixyl, buripimate, metribuzin, linuron, methabenzthiazuron, triforine, vinclozolin, bitertanol, fenbutatin oxide, amitraz, propargite, dithiocarbamate
De Ferrari *et al*. (29)	CA	Positive	Diazinon, dichlorvos, dimethoate, malathion, methylazinophos, **monocrotophos**, parathion, parathion methyl, phorate, prothoate, terbufos, trichlorofon, cypermethrin, fenpropathrin, permethrin, maneb, **thiram**, dazomet, mancozeb, zineb, **ziram**, thiabendazole, paraquat, captan, folpet, endosulfan, dodemorph, chlorothalonil, iprodione, acetic metaldehyde, barium polysulfide, copper oxychloride, copper sulphate, sulphur, white oil, dinocap, **DNOC**, alachlor, simazine, MCPA, linuron, vinclozolin, phenmedifam, methalaxyl, ethofumesate, 2,4-D, dicofol
Dulout *et al*. (30)	CA	Positive	Dimethoate, mevinphos, **monocrotofos**, parathion, parathion methyl, aldicarb, maneb, dazomet, propineb, zineb, captan, endosulfan, aldrin, aramite, chlordimeform, heptachlor, tetradifon
Falck *et al*. (4)	MN	Negative	Acephate, dichlorvos, dimethoate, trichlorofon, methiocarb, methomyl, mancozeb, metiram, propineb, captan, procymidone, endosulfan, chlorothalonil, iprodione, vinclozolin, methaloxyl, azocyclotin, benfuracarb, dicofol, fenopropathrin, toclofos-methyl
Garry *et al*. (18)	CA	Positive	2,4-D (others had been used but were not listed)
Gomez-Arroyo *et al*. (35)	MN	Positive	Diazinon, dichlorvos, fosetyl-aluminium, malathion, methamidophos, parathion methyl, cypermethin, carbaryl, methomyl, mancozeb, pirimicarb, **benomyl**, captan, endosulfan, lindane, diuron, 2,4-D, aldrin, ametrina, BHC, DDT, dacomil, dieldrin, di-syxtox, endrin, furadan, gusathion, javelin, metalaxyl, nuvacron, oxidemeton methyl, talstar, tordon.
Grover *et al*. (37)	comet	Positive	Acephate, chlorpyrifos, dimethoate, **monocrotophos**, phorate, cypermethrin, fenvalerate, **carbendazim**
Lander *et al*. (46)	CA	Positive	Chlorpyriphos, cypermethrin, deltamethrin, fenpropathrin, methomyl, **thiram**, pirimicarb, **benomyl**, **carbendazim**, endosulfan, chlorothalonil, iprodione, buprofezin, **atrazine**, triforine, vinclozolin, cyhexatin, fetin acetate, carboxin, 2,4-D, chloridazon, defenamide, oxadiazon, propargyl
Lebailly *et al*. (49)	comet	Positive	Dimethoate, ethephon, omethoate, oxydemeton-methyl, thiometon, befenthrin, b-cyfluthrin, deltamethrin, mancozeb, **carbendazim**, endosulfan, chlorothalanil, iprodione, diflufenicanil, λ-cyhalothrin, pyrimethanil, fluroxypyr, cyproconazole, epoxyconazole, flutriafol, tebucanazole, **atrazine**, MCPA, isoproturon, 2,4-D, amidosulfuron, bentazon, bifenox, bromoxynil, clopyralid, fenopropadin, imazamethabenz-methyl, ioxynil, mecoprop, sethoxydim
Lebailly *et al*. (47)	comet	Negative	Dimethoate, glyphosate, methidathion, phosalone, pyrazophos, vamidothion, carbaryl, fenoxycarb, dazomet, perimicarb, **benomyl**, captan, folpet, endosulfan, abamectin, amitraz, buripimate, pyrifenox, flufenoxuron, teflubenzuron, dinocap, flusilazole, aramite myclobutanil, penconazol, triadimefon, simazine, diuron, imidaclopride, tolyfluanide
Pasquini *et al*. (57)	MN	Positive	Deltamethrin, carbaryl, mancozeb, propineb, **benomyl**
Pastor *et al*. (17)	MN	Negative	Dichlorvos, fosetyl-aluminium, malathion, methamidophos, permethrin, tralomethrin, methomyl, oxamyl, propamocarb, mancozeb, propineb, pyriproxyfen, procymidone, endosulfan, kasugamycin, cymoxanil, buprofezin, cyromazine, imidaclopride
Peluso *et al*. (62)	DNA adducts	Positive	Glyphosate, methamidophos, **monocrotophos**, parathion methyl, methomyl, metam-sodium, dazomet, zineb, **benomyl**, **carbendazim**, paraquat, captan, folpet, endosulfan

*Statistically significant difference between exposed and non-exposed groups (positive).

between studies and this might affect the conclusions that can be drawn.

There were limitations in the statistical approaches used in many of the studies. The reporting of distribution modelling was variable and in most cases was not adequate. For example, many did not consider normal distributions of control data prior to statistical analysis. It was noted that the articles tended to focus on statistical significance even when the absolute difference between groups was very small.

Overall, the study by Garry *et al*. (18) was considered to have been reasonably well designed with appropriate use of statistical techniques. This study reported an association between genotoxicity and total volume of herbicide applied. However, it was based on a small number of individuals and had not adequately accounted for potential confounding. The study by Peluso *et al*. (62) had included adequate control data modelling and reported odds ratios (OR) for exposure and confounding factors, but again the number of subjects was

very small and hence only limited conclusions could be reached. Overall, it was emphasized that the lack of adequate evaluation of individual exposures severely limited any conclusions that could be reached with regard to specific pesticide active ingredients. Moreover, it was not possible to highlight any particular studies on which a greater weight of evidence could be placed on the basis of consideration of overall quality.

Discussion

Several groups of workers are potentially exposed to pesticides on the basis of their occupation and the possible health effects of these chemicals are constantly being scrutinized. Although the genotoxicity testing strategies employed prior to product registration are designed to identify potential *in vivo* genotoxins, concerns that exposure to pesticides may result in long-term adverse effects still exist. This report has focused on published studies that evaluated genetic damage in those exposed to pesticides as floriculturists and greenhouse workers, agricultural workers and farmers, pesticide sprayers and applicators, production workers and forestry workers. The aim of the current review was to extract systematically detailed information from the selected studies and attempt to draw conclusions based on generalized observations, rather than to focus on individual study outcomes.

The exclusion criteria used were rigorous with 66% of identified studies being excluded from further analysis. Studies using only SCE as the endpoint, where the period for MN incubation was considered too long or in which chromosome/chromatid gaps were included in the overall analysis made up a large proportion of those rejected (66% of rejected studies). Although these are not criteria detailed in the IPCS recommendations (8), the COM regarded data from these studies to be potentially unreliable or uninformative.

There is evidence supporting an increase in biomonitoring indices of genotoxicity in pesticide applicators. Of the 24 studies selected for closer evaluation, in 17 of these the authors concluded that there was an increase in indices of genotoxicity in pesticide workers compared to matched controls. The proportion of studies in farmers yielding positive findings was smaller than in floriculturists (4/8 and 8/10, respectively), but in the absence of exposure assessments it is not possible to relate this to any differences in exposure. It has been widely reported that floriculturists are potentially at increased risk of cytogenetic damage because of increased exposure as a consequence of their working conditions (1,13). They might also be relatively highly exposed during loading, mixing and applying pesticides as well as during manual activities resulting in continuous contact with flowers and ornamental plants (14). Sprayers are likely to be the most highly exposed group being directly in contact with pesticide formulations during mixture and applications (1).

One factor which is likely to have a significant impact on worker exposure to pesticides is the use of protective clothing during application or when handling crops (flowers) which have been sprayed. Nevertheless, there were a large number of studies in which this confounding variable was not considered. Furthermore, there is a surprising lack of discussion regarding the potential significance of its use, and there were no studies in which the differences between those who wore PPE and those who did not were investigated. The observation that those working in humid greenhouse conditions may be less inclined to wear the appropriate protection seems pertinent in considering which occupations may be at greater risk, but the lack of detailed information makes it impossible to address the issue at present. However, there is evidence from this limited review of an association between the absence of protective clothing and the positive genotoxic endpoints. Notably, in a study conducted by Bolognesi *et al*. (12), despite the use of three different category 3 mutagens, there were no statistical differences in indices of genotoxicity in exposed subjects, suggesting the possible effectiveness of PPE. However, the authors of this study do not appear to have considered this possibility.

Exposure to the pesticides was in general, poorly documented. The mixtures of pesticides used comprised some that have been demonstrated to have genotoxic effects *in vivo* in animals (i.e. category 2 and 3 mutagens; benomyl, carbendazim, DNOC (4,6-dinitro-*o*-cresol), methyl bromide, monocrotophos, phosphamidon) and others considered devoid of genotoxic activity. Although no individual pesticides could be identified as clearly associated with the observed genotoxicity, attention is drawn towards the use of benzimidazoles, for example, carbendazim was used in 4/11 positive studies in which specific pesticide usage was given. It is reasonable to assume that exposure to benzimadazoles at a concentration above that required for spindle disruption [its documented mode of action (84)] may result in the appearance of MN or CA in the blood of exposed workers. There are pesticide products containing carbendazim which are currently approved for use in the United Kingdom for agriculture and floriculture. It is noteworthy that the COM agreed a threshold approach to benzimadazole mutagenicity in 1996 (86). A key question is whether occupational exposure would ever exceed this threshold. Also of note is that carbendazim usage was reported in 1/4 of the negative studies which listed pesticide usage (12).

It was noted that some authors concluded that the magnitude of the genotoxic response correlated with duration of exposure. However, it is also well documented that age is significantly associated with an increase in the occurrence of MN and CA and this was demonstrated in some of the studies considered in this report (4,23,24). Therefore, without correcting for this variable, a positive association with duration of occupation duration is of equivocal significance.

Overall, it was concluded that there were weaknesses in all of the published studies examined and that the available information for exploring potentially causal associations with pesticide exposure was severely limited. A similar conclusion was reached in a recent review of the COMET assay as an occupational biomonitoring tool, where it was also noted that the available studies had many shortcomings, including the observations of low numbers of subjects and selection bias (87). Assessment of associations with individual pesticide exposure is very difficult as most occupations involve the regular use of a large number of different pesticides, together with other chemicals such as co-formulants, which vary greatly in their potential toxicities and potencies. Furthermore, measurements of systemic exposure to the pesticides were not undertaken and therefore correlations of increased genotoxicity biomarkers with degree of exposure were not possible.

It is regrettable that no clear conclusions can be reached regarding the available biomonitoring studies of genotoxicity following occupational exposure to pesticides. One factor, which appreciably influenced this conclusion, is the extreme variation in the biomonitoring indices frequency in the control populations examined in the selected studies. In this respect,

it is very difficult to infer causality for the small magnitude of responses seen in the positive studies. Furthermore, it is concluded that factors affecting variance in genotoxicity endpoints are not adequately understood and there is a clear need for more data on the background variability in the general population.

The pre-marketing registration testing of pesticides provides reassurance that non-threshold, DNA-reactive genotoxins are not marketed as the testing strategies used are generally regarded as being highly efficient at identifying *in vivo* mutagens (88). However, there is always some uncertainty in extrapolating from toxicity tests to the exposed population. Hence, appropriately designed post-market monitoring studies, where practicable, are of considerable value in the continuing evaluation of the risks of pesticide exposure. Unfortunately, the studies available to date are inadequate for this purpose, despite the time and resources evidently expended in their conduct. Furthermore, it is noteworthy that no study of UK workers has been published to date, thus indicating a need for the consideration of appropriate, hypothesis-driven biomonitoring studies. In the first instance, it is suggested that examination of workers following exposure to benzimidazoles should constitute the first suitably designed UK study. However, an improved understanding of the factors affecting the background variance of each genotoxic endpoint would dramatically enhance the quality of such studies. Furthermore, the relatively small increases in genotoxic biomarkers seen in the studies examined herein coupled with this intrinsic variability in control ranges suggests that the detection of an effect would likely require assessment of a very large number of subjects.

Post-market monitoring studies such as those reviewed in this article should provide appropriate platforms to substantiate the pre-marketing assessments. In this respect, the available studies are inadequate despite the large amount of resources used. However, it is accepted that there is some evidence of genotoxic effects in those who work with pesticides and that hypotheses-driven biomonitoring studies in the United Kingdom are needed, once background variance of genotoxicity indices have been more fully elucidated.

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