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MUT/05/19

COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD CONSUMER PRODUCTS AND THE ENVIRONMENT.

BIOMONITORING STUDIES OF GENOTOXICITY IN PESTICIDE APPLICATORS:

DRAFT WORKING PAPER.

Introduction

1. The Medical and Toxicology panel of the Advisory Committee on Pesticides has asked the COM for advice on the genotoxicity in pesticide applicators. The referral statement is given below.

"To review investigations of mutagenicity and DNA adducts in pesticide applicators and workers exposed to pesticides (e.g. handling cut flowers) and factory (manufacturing) staff engaged in pesticide manufacture/formulation and produce a statement for the Advisory Committee on Pesticides and its Medical and Toxicology Panel. The review should include all studies identified by the MTP and any other relevant studies published, particularly those originating from the UK.

The review should consult COC epidemiologists with regard to the rigour of studies evaluated. this should include design, selection of controls, bias, confounding and use of multiple statistical comparisons.

The review should be initiated at the October 2004 COM meeting."

2. The DH Toxicology Unit in collaboration with the secretariat drafted a series of review papers considered at the October 2004, February 2005 and May 2005 meetings (listed below).

i) *Biomonitoring studies from EU (MUT/04/19). Annex 1 (overview of literature), Annex 2 Summary of individual studies and IPCS guidelines on biomonitoring studies of genotoxicity. Annex 3 tabular summary according to occupation, Annex 4 tabular summary of statistical approaches used to analysis of data.*

ii) *Review of biomonitoring studies of pesticide applicators from Croatia (MUT/04/20)*

iii) *Further information and follow-up of review undertaken in October 2004 (MUT/05/1). (Draft exclusion criteria Annexes I and II, Draft inclusion criteria Annexes III and IV, Magnitude of response Annexes V and VI, Exposure patterns documented in studies).*

iv) *Submitted published papers (for February 2005 meeting) (MUT/05/6), Addendum 1 MUT/05/6 review of studies from rest of world, Addendum 2 to MUT/05/6, tabulation of rest of world studies by occupation.*

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- v) *Cytogenetic changes following cumulative exposure to pesticides (MUT/05/9)*
- vi) *Revised criteria (MUT/05/10)*
- vii) *Epidemiological overview (MUT/05/11)*
- viii) *Discussion paper on evaluation of positive studies and control data (MUT/05/12) (Annex 1 Evaluation of positive response in biomonitoring studies of genotoxicity, Annex 2 Information on pesticide usage in UK).*

Topics considered in this paper.

3. A draft working paper was circulated during the summer for members consideration. An amended draft has been appended as Annex 1 to this cover paper. The Committee is asked to consider the text of the revised draft working paper in detail. A number of topics relating to the draft working paper where further evaluation has been undertaken have been briefly discussed below.

4. The Committee also asked for further information on whether there was any appropriate biomonitoring approach which might be of value in estimating absorbed doses of benzimidazoles.

5. The Medical and Toxicology Panel of the ACP considered a letter from the secretariat at the 14 July 2005 meeting of MTP which provided an overview of the COM review. The MTP made a comment regarding the literature search strategy (regarding the adequacy of identification of studies from the U.S.A.). The secretariat has replied to this point noting that the strategy used by MTP and COM resulted in identical literature being retrieved. The COM have used an assessment strategy which has identified relevant studies for detailed consideration. The MTP endorsed the provisional suggestion that a reasonable hypothesis for a first UK study would be to focus on approved uses of benzimidazoles. The MTP asked for guidance from COM on the possible approach to such a study.

Draft Working Paper (Annex1)

Data on Cancer patients and nurses

6. It was noted in the draft working paper that there would be some additional work on the evaluation of positive responses in nurses/patients exposed to cytostatic medicines. This approach had originally been suggested as one possible method of deriving a minimal positive control response in biomonitoring studies.

7. The COM compared the magnitude of response seen in the 24 studies of pesticide applicators with that reported for patients undergoing treatment with cytostatic medicines and nurses occupationally exposed to these medicines. Members were surprised at the small magnitude of response in the biomonitoring studies of nurses or patients exposed to cytostatic medicines. The mean fold increase in nurses (1.8, range 1.5-2.2) and in

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patients (mean 2.1, range 1.5-2.7) derived from studies for either micronuclei or chromosomal aberrations was similar to that reported for pesticide applicators in the studies reviewed by COM (1.7, range 0.8-5). Members noted that the higher maximum fold increase in pesticide applicators compared to nurses or patients exposed to cytostatic medicines might reflect differences in the extent of control for confounding between studies. The Committee agreed that this analysis provided valuable information on the outcome of biomonitoring studies for genotoxicity but that it was not possible to draw a definite conclusion on a minimum fold increase in genotoxicity indices associated with exposure to genotoxic compounds.

Additional information; chromosomal damage in cancer patients.

8. Following the May 2005 meeting, one COM member has identified a published biomonitoring study of cancer patients (Carbonell E et al *Anti Cancer Drugs*, 7, 514-519, 1996) treated with mephalan where it is evident that the background chromosomal aberration level in cancer patients before a cytostatic medicine treatment schedule is considerably higher than compared to control subjects with no previous exposure to cytostatic drugs. Thus the baseline frequency of CAs in cancer patients (n= 13), was (mean \pm SE) 12.27 ± 2.74 aberrations/100 cells (range 0-46) whereas the mean frequency in the controls (n =14) was 2.34 ± 0.05 . The authors reported that a wide variance in CA frequency between cancer patients had been previously reported and increased CA frequencies in cancer patients compared to controls before treatment have also been recorded. Thus a small increase on a relatively high background might suggest that patients were not as good a comparator group for pesticide applicators as previously thought during the drafting of papers for the COM.

9. This conclusion is supported by a recent small investigation of micronuclei in peripheral blood lymphocytes of six patients (three of whom also received radiotherapy) with small cell lung cancer undergoing a combination therapy with cisplatin and etoposide and 7 patients with ovarian carcinoma treated with taxol and cisplatin. The authors followed patients over 7 cycles of treatment analysing blood samples taken at the beginning of each treatment cycle. There was considerable inter-individual variation in pre-treatment levels of micronuclei and in the response to treatment. Generally the frequency of micronuclei increased during the first half of therapy and then reduced, reaching in some patients with ovarian cancer, values below the pre-treatment level. The authors suggested that repopulation of lymphocytes affected the measured micronuclei frequency (Padjas A et al, *Toxicology Applied Pharmacology*, online publication)

10. Osanto and colleagues reported that chromosomal damage induced by cytostatic medicines persisted for up to 9.3 years in a small group of cancer patients with testicular carcinoma patients. (Osanto S et al *Env Mol Mutagen*, 17, 71-78, 1991). Lambert and colleagues reported an increase in chromosome/chromatid aberrations in peripheral blood lymphocytes in patients (n=50) with ovarian cancer treated with mephalan (ca 5.4%

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compared to 2.3% in healthy controls (n=36). It was noted that 26 of the 50 cancer patients had received dual chemotherapy and some of these individuals had also received radiotherapy. Blood samples were taken from cancer patients between 4-132 months after the end of treatment (mean 57 months). Patients with a high total dose of mephalan (420 mg) and a long duration of therapy had a higher frequency of cells with aberrations (6.3% compared to patients with less than 420 mg mephalan and shorter duration of treatment 9ca 4.2%) (Lambert et al, Hum Genet, 67, 94-98, 1984.

11 Thus overall there is information available outlining the complexity of the evaluation of peripheral lymphocyte biomonitoring data on genotoxicity in cancer patients suggesting that response reflect the potency of cytostatic medicines used, duration of treatment and period after treatment before sampling. Thus cancer patients might not be as good a comparator group for pesticide applicators as had been initially thought.

Additional information; chromosomal damage in nurses administering cytostatic medicines.

12. Information on micronuclei were reported in the COM discussion papers from two studies of nurses handling and administering cytostatic medicines. The fold increase in micronuclei frequency was 2.2x in the study of Burgaz et al (Mut Res, 439, 97-104, 1999) and 1.5x in the study by Kasuba et al (Journal Applied Tox, 19, 401-404, 1999). Kasuba et al reported that a statistically significant increase in micronuclei frequency was limited to nurses with 20-31 years of handling/administering cytostatic medicines. Burgaz reported that handling time, use of protective equipment and handling frequency had no effect on urinary excretion of cyclophosphamide or on cytogenetic parameters. This information is consistent with the conclusion reached from studies of cancer patients that it is difficult to use these data to help inform on the results obtained with pesticide applicators.

Section in draft working paper

13. Members are asked to consider the relevant entry in the draft working paper regarding data from cancer patients and nurses. Do members consider any changes should be made?

The COM compared the magnitude of response seen in the 24 studies of pesticide applicators with that reported for patients undergoing treatment with cytostatic medicines and nurses occupationally exposed to these medicines. Members were surprised at the small magnitude of response in the biomonitoring studies of nurses or patients exposed to cytostatic medicines. The mean fold increase in nurses (1.8, range 1.5-2.2) and in patients (mean 2.1, range 1.5-2.7) derived from studies for either micronuclei or chromosomal aberrations was similar to that reported for pesticide applicators in the studies reviewed by COM (1.7, range 0.8-5). Members noted that the higher maximum fold increase in pesticide applicators compared to nurses or patients exposed to cytostatic medicines might reflect differences in the extent

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of control for confounding between studies. The Committee agreed that this analysis provided valuable information on the outcome of biomonitoring studies for genotoxicity but that it was not possible to draw a definite conclusion on a minimum fold increase in genotoxicity indices associated with exposure to genotoxic compounds.

Additional information from positive studies where exposure to approved benzimidazoles has been cited

14. The Committee noted that with regard to pesticide active ingredients that were currently approved for use in the U.K. and which were also classified with regard to mutagenicity under EC/67/548, carbendazim and thiophanate-methyl were used in 5/11, and 1/11 positive studies respectively but not in the negative studies. This represented a very small and incomplete amount of information. Some brief additional information on the studies which documented exposure to carbendazim and thiophanate-methyl is given below. The information is intended to show that there is some very limited information which associates positive responses in the biomonitoring studies with pesticide products containing carbendazim and thiophanate-methyl (which are approved for use in pesticide products in the U.K. There is additionally some limited evidence to associate exposure to monocrotophos (which is not included in any approved pesticide products in the U.K.)

Study (Characteristics)	Biomonitoring indices	Evidence for exposure to benzimidazoles and other classified mutagens.
Bolognesi (ref 5)*. 52 floriculturalists/ 24 controls. MN distribution not reported. Unclear whether results adjusted for cofounders. Inadequate sampling strategy. No individual exposure assessment. Multiple comparisons	MN frequency in peripheral blood lymphocytes. Pan-centromeric probe used to distinguish centromere positive/negative MN. Statistically significant increase in C+ and C- MN. Percentage C+ MN was raised in subjects with reported use of benzimidazole fungicides.	Information collected by questionnaire (self administered) which included pesticides used (kg/year) and frequency of use (no of subjects). Use of pesticides not approved for use in the U.K. but classified as category 2 or 3 mutagens reported.) (monocrotophos and benomyl). Use of carbendazim and thiophanate-methyl (UK approved pesticides) reported. Order of usage by both kg/year and no of individuals, Monocrotophos>benomyl>carbendazim>thiophanate-methyl
Grover (ref 22). 54 pesticide production workers. 54 office workers employed on same site. Duration of exposure 3-13 years. Details of selection of controls unclear. Normality of data tested. Adjustments for smoking, age, gender. Multiple comparisons.	Alkaline Comet assay using (50 peripheral blood leukocytes)/person. Statistically significant increase in mean tail length in exposed.	Authors noted that all exposed subjects were simultaneously exposed to a range of pesticide active ingredients (eight were listed) and a wide range of formulations. This included monocrotophos and carbendazim (classified as mutagens)..
Lander (ref 31). 116 male floriculturalists, 29 non-smoking controls (from organic association). No information on sampling strategy. Data collected on many cofounders with modelling undertaken. Models inadequately presented.	Analysis of CAs both pre-season and post season. (Paired samples from 83 workers, pre-season March 94, post season Oct 94). Samples from referent collected March 95. An increase in total frequencies of cells with chromosome aberrations (excluding gaps) in exposed compared to pre-season and also to controls.	Authors report that up to 50 pesticide active ingredients may have been used by floriculturalists during the growing season. From the listed data, only carbendazim and benomyl are classified as mutagens. Authors reported that fungicide use was at a low rate throughout the year. Authors reported that most predominant effects were reported in floriculturalists who did

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	Increases were predominantly in gaps, but data excluding gaps was recorded.	not report using gloves.
Lebailly (ref 35) Blood samples from 29 French farmers collected. Subgroups (1-4) defined by period when samples taken reflecting different types of pesticide use. Each group comparatively small but consistent of a sample on the morning before application and one taken the morning the day after application. No enrolment strategy reported. Study design complex with many statistical comparisons reported. Presentation of data muddled.	Alkaline Comet assay using peripheral blood leukocytes. (200 cells/slide examined. Positive/negative controls included) An increase in DNA damage was reported for post application samples in groups 1 and 4 but not groups 2 and 3..	Information on pesticide use collected by personal interview. Only 3 of the farmers reported using carbendazim. (two were in group 1, and the remaining farmer was in group 4). All three farmers reported using at least protective gloves. However no other classified pesticide was reported in this study.
Peluso (ref 52) Blood samples from 26 floriculturalists and 22 matched controls. (age-gender). Very small study with limited details on subject recruitment. Analysis carefully reported. 0	³² P-postlabelling of DNA from white blood cells measured. Floricultural practice was associated with significantly higher DNA-positive rate.	Authors reported that pesticide products containing 10 active ingredients were the predominant products used. The only classified mutagens listed were monocrotophos and carbendazim. It is expected that carbendazim would not give a positive response in the ³² P-postlabelling assay.

* = ref number in draft working paper.

15. Members are asked to consider whether the section in the draft statement (working paper) regarding evidence of exposure should be amended.

The Committee noted that with regard to pesticide active ingredients that were currently approved for use in the U.K. and which were also classified with regard to mutagenicity under EC/67/548, carbendazim and thiophanate-methyl were used in 5/11, and 1/11 positive studies respectively but not in the negative studies^{5,22,31,35,52}. This represented a very small and incomplete amount of information. It was noted that two of these studies had used micronucleus or chromosomal aberration analyses which could potentially be affected by spindle inhibitors such as benzimidazoles.^{5,31} However it is unclear whether the results derived from the Comet assay^{22,35} can be related to benzimidazoles and there is no evidence for direct binding of benzimidazoles to DNA adduct and hence the results of the remaining study⁵² are unlikely to be related to benzimidazole exposure. It was noted that there were a number of other classified mutagens listed in the positive studies which were not approved for use in the U.K.

Approaches to biomonitoring of carbendazim.

16. An overview of carbendazim metabolism in the rat is given overleaf (abstracted from the EHC monograph). The only biomonitoring study retrieved from the literature investigated urinary excretion of 5-hydroxy-2-benzimidazole in a small number of volunteers given oral doses and in a number of individuals with back ground exposure. An HPLC method using reverse phase column chromatography combined with electro-chemical detection was used. A Limit of detection of 5 µg/l was described. The authors

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consider the method would be applicable to floriculturalists but noted that separate determination of 5-HBC conjugates had not been undertaken. No studies where biomonitoring had been applied to benzimidazole use by floriculturalists were found.

17. Do members consider that such an approach would be useful in the proposed approach to a UK study?

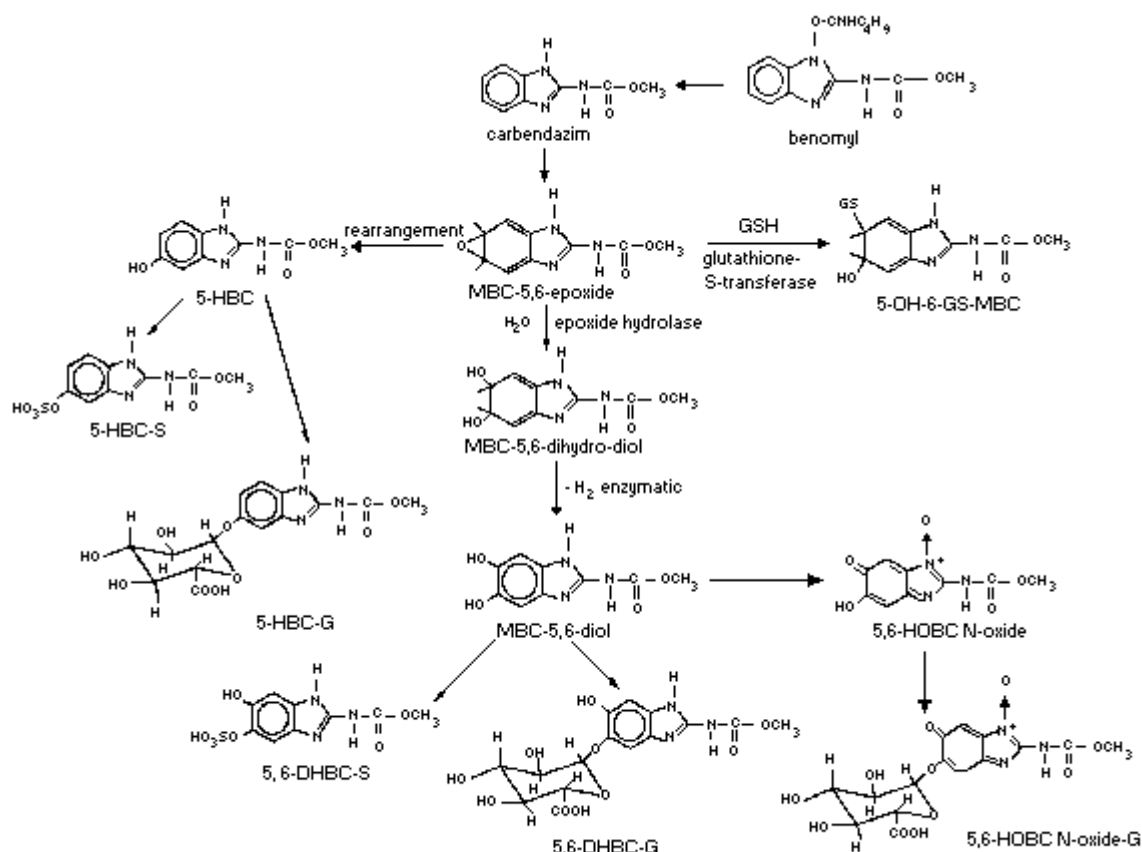


Fig. 1. Proposed metabolic pathway for carbendazim in rats (From: Monson, 1990)

Consideration of possible approach to a study in the U.K.

18. Both COM and MTP have suggested that some guidance should be provided on what might constitute an appropriate study for U.K. floriculturalists. One COM member provided an example paper which is appended as Annex 2 to this paper. The study was a biomonitoring study of occupational exposure to exhaust fumes using the binucleate MN assay of peripheral blood lymphocytes using whole blood cultures. Considerable effort was made to obtain information on potential confounding factors and to assess the impact of these on MN frequency. In addition quite extensive personal monitoring of exposure was undertaken. In the proposed study it would be presumably appropriate to study the presence of centromere positive and negative MN as the chemical under study (carbendazim) is a benzimidazole fungicide.

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19. During the epidemiology review one overall comment made was that most study designs were cross sectional with a single sampling time. Those studies that had used multiple sampling times did not report information on the critical window of exposure for the outcomes under study. Given that the proposed study focuses on carbendazim, what advice would members give in this respect?

20. A further comment from the epidemiology review was for the need for adequate modelling of the distribution of indices of genotoxicity used in biomonitoring studies and analysis of all potential confounding factors. Other relevant comments referred to the limited size of most studies and the inadequate evaluation of influence of protective equipment on outcome.

21. What further advice would the COM give with regard to the design of an appropriate study?

Conclusions

22. Members are asked to consider the revised draft working paper. Can this be agreed subject to members comments? It is intended that the finalised statement would be submitted to the November 2005 ACP meeting.

23. Members are asked to provide advice on the design of an appropriate biomonitoring study of carbendazim use by floriculturalists. Should biomonitoring for exposure and uptake of carbendazim be included? Should an appropriate section on these aspects be included in a revised draft working paper.

Secretariat. August 2005

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Annex 1 to MUT/05/19

1ST DRAFT WORKING PAPER

COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT. (COM)

REVIEW OF BIOMONITORING STUDIES OF GENOTOXICITY IN PESTICIDE APPLICATORS.

Background

2. The Medical and Toxicology panel of the Advisory Committee on Pesticides has asked the COM for advice on the genotoxicity in pesticide applicators. The referral statement is given below.

"To review investigations of mutagenicity and DNA adducts in pesticide applicators and workers exposed to pesticides (e.g. handling cut flowers) and factory (manufacturing) staff engaged in pesticide manufacture/formulation and produce a statement for the Advisory Committee on Pesticides and its Medical and Toxicology Panel (MTP) . The review should include all studies identified by the MTP and any other relevant studies published, particularly those originating from the UK.

The review should consult COC epidemiologists with regard to the rigour of studies evaluated. this should include design, selection of controls, bias, confounding and use of multiple statistical comparisons.

The review should be initiated at the October 2004 COM meeting."

2. The referral from ACP and the MTP came about through the ongoing routine review of epidemiology literature undertaken by the MTP. The MTP had considered in April 2004 that there were sufficient numbers of reports of biomonitoring studies in the published literature which had been retrieved and evaluated by MTP to request an independent view from COM on the available studies.

Evidence reviewed

3. The DH Toxicology Unit at Imperial college in collaboration with the COM secretariat drafted a series of review papers for the COM. The review

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considered published biomonitoring studies in the scientific literature up to December 2004. A comparison was undertaken with the literature search strategy used by the MTP in order to ascertain whether any published studies had been missed. A very good agreement between the MTP and COM literature searches was attained with only one study identified in the COM literature search which had not already been identified by MTP. (A total of 70 biomonitoring studies of genotoxicity markers in pesticide applicators was identified.¹⁻⁷⁰) A listing of the discussion papers considered by COM during the review period (from the October 2004 to the October 2005 meetings) is given below. All of these review papers are draft discussion papers and do not necessarily represent the views of the COM. A detailed evaluation was undertaken for all of the studies. The discussion papers can be accessed via the COM internet site under the "papers" section.

<http://www.advisorybodies.doh.gov.uk/com/> ;

- i) *Biomonitoring studies from EU (MUT/04/19). Annex 1 (overview of literature), Annex 2 Summary of individual studies and IPCS guidelines on biomonitoring studies of genotoxicity. Annex 3 tabular summary according to occupation, Annex 4 tabular summary of statistical approaches used to analysis of data.*
- ii) *Review of biomonitoring studies of pesticide applicators from Croatia (MUT/04/20)*
- iii) *Further information and follow-up of review undertaken in October 2004 (MUT/05/1). (Draft exclusion criteria Annexes I and II, Draft inclusion criteria Annexes III and IV, Magnitude of response Annexes V and VI, Exposure patterns documented in studies).*
- iv) *Submitted published papers (for February 2005 meeting) (MUT/05/6), Addendum 1 MUT/05/6 review of studies from rest of world, Addendum 2 to MUT/05/6, tabulation of rest of world studies by occupation.*
- v) *Cytogenetic changes following cumulative exposure to pesticides (MUT/05/9)*
- vi) *Revised criteria (MUT/05/10)*
- vii) *Epidemiological overview (MUT/05/11)*
- viii) *Discussion paper on evaluation of positive studies and control data (MUT/05/12) (Annex 1 Evaluation of positive response in biomonitoring studies of genotoxicity, Annex 2 Information on pesticide usage in UK).*

Pesticide applications considered in the review.

4. The papers retrieved identified a wide diversity of occupational pesticide exposures. The authors described investigations in occupational groups such as floriculturalists, green house workers, agricultural workers and farmers, pesticide sprayers and applicators (which included agricultural/horticultural, amenity, fumigators), production workers (e.g. manufacture of pesticides) and forestry workers. The extent of information provided on occupational exposure to pesticides (e.g. during handling, diluting, applying), the duration of exposure and use and adequacy of personal protective clothing varies considerably between the different

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accounts. The Committee considered it was difficult to evaluate such a diverse data set. It was agreed that the most appropriate approach would be to assess the adequacy of the studies with regard to investigation and evaluation of genotoxicity indices and with regard to overall adequacy of design, analysis and interpretation of results. With respect to overall adequacy, the COM sought an opinion from an independent epidemiologist.

Indices of genotoxicity used in the reviewed biomonitoring studies

5. A short overview of the indices of genotoxicity used in biomonitoring studies is provided to assist in evaluating the significance of findings. Almost all the studies considered in this review provided data for investigations using *in-vitro* culture of peripheral blood lymphocytes derived from blood samples. A small number of studies used epithelial cells from the buccal cavity. The committee had access to the general guidance published by a WHO IPCS working group on use of genotoxicity indicators in biomonitoring studies.⁷¹ The Committee agreed that in general the genotoxicity indices measured in samples (predominantly peripheral blood lymphocytes) including micronuclei, chromosomal aberrations, COMET assay, ³²P-postlabelling assay results indicate uptake and exposure to DNA damaging chemicals. The evidence suggested that there may be an increased risk of mutagenicity and possibly carcinogenicity but it is not possible to be certain that there is a risk or to quantify this risk because of the poor quality of many of the studies and frequent contradictory findings.

Micronuclei frequency

Micronuclei are small, extranuclear bodies that arise from acentric chromosome fragments or from whole chromosomes that are excluded from the nucleus during mitotic cellular division. They can be a consequence of DNA breakage, replication on a damaged DNA template or inhibition of DNA synthesis, failure of any of the mitotic apparatus or alterations in cellular physiology and mechanical disruption^{71,77}. In most cases, the cytokinesis-block MN method is used, in which scoring only takes place in cells that have only divided once in culture.¹⁶ Micronuclei analysis can be used for a number of cells, both *in vitro* and *in vivo*, including lymphocytes^{5,9,72} and buccal epithelial cells.⁴⁹⁻⁵⁰ Micronucleus induction is an indirect indicator of mutagenicity. It is unclear however, whether MN formation has a specific role in carcinogenesis.⁷¹

Chromosome aberrations

Structural chromosome aberrations arise from direct DNA breakage, replication on a damaged DNA template or inhibition of DNA synthesis and may involve both chromatids of the chromosome (chromosome-type CA), or only one chromatid of the chromosome (chromatid-type CA).^{71,73} Chromosome aberration analysis has been commonly performed on human peripheral blood lymphocytes to assess DNA damage.^{11,14,16,29,42,44} To ensure that only first-generation metaphase cells are scored for CA, bromodeoxyuridine is commonly added to the culture medium prior to DNA replication *in vitro*²⁹. Both structural and numerical chromosome aberrations may cause alterations of in the structure or arrangement of oncogene and tumour suppressor genes of somatic cells, and hence are involved in the induction of cancer in humans.⁷⁴

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Sister chromatid exchange

Sister chromatid exchanges arise from equal exchange of DNA replication products between two identical sister chromatids of a duplicated chromosome⁷⁵ They are thought to arise as a consequence of “error free” homologous recombinational repair or bypass of DNA lesions during replication on a damaged DNA template, possibly at the replication fork.⁷⁵ In the most commonly used method of SCE analysis, DNA replication is required for two consecutive cell cycles, hence bromodeoxyuridine is added to the culture medium and cells are scored in the second division metaphase.^{14,61} Although the induction of SCE has been widely used as an indicator of DNA damage following exposure to pesticides,^{12,26,32,36,45,61} the mechanism of formation and biological significance of SCEs are still unknown.⁷⁵ The COM agreed that biomonitoring studies using SCE analysis were not informative with regard to evidence for genotoxicity.

Comet assay

The comet assay, or single cell gel electrophoresis technique is a more recent technique established as a sensitive method for detecting DNA single strand and double strand breaks, alkali-labile sites, DNA cross linking and incomplete excision repair events.^{34,64,76,77} The comet assay can be carried out with a number of cells, both *in vitro* and *in vivo*, including peripheral blood leukocytes, bladder, liver, buccal, gastric and sperm cells. To date, peripheral blood lymphocytes are mainly used for human biomonitoring studies following occupational exposure to an array of chemicals.^{34,53,64,77} However, the relevance of the endpoint measured in the comet assay has yet to be established, as it is usually the result of a temporary strand breakage, which, is repaired within a few hours under normal circumstances and may or may not become fixed as a mutation.⁷⁷

DNA adducts

A DNA adduct is a chemical entity covalently bound to DNA⁷¹, and is usually formed following the interaction of an electrophilic molecule with a nucleophilic site of DNA⁷⁸. They are often the initial DNA lesion following exposure to a genotoxic chemical and may lead to mutation and altered gene function if not repaired. In epidemiological studies DNA adducts are particularly useful as they provide information on the exact chemical exposure of the individual.⁷⁸ ³²P-DNA postlabelling technique has been widely used to measure non-radioactive carcinogenic DNA adducts in humans, due to it being a highly sensitive technique.⁷⁸

The COM guidance on a strategy for testing chemicals for mutagenicity recognised that artifactual positives may be obtained in the cell assays that do not reflect intrinsic mutagenic activity. Factors such as hyperthermia, hypothermia or induction of erythropoiesis may produce MN or CA⁷⁹ or exercise immediately prior to sampling may lead to increased DNA damage measured by the comet assay.⁷⁹

Overview of approach used by COM

6. A flow diagram outlining the approach used by COM is shown below.

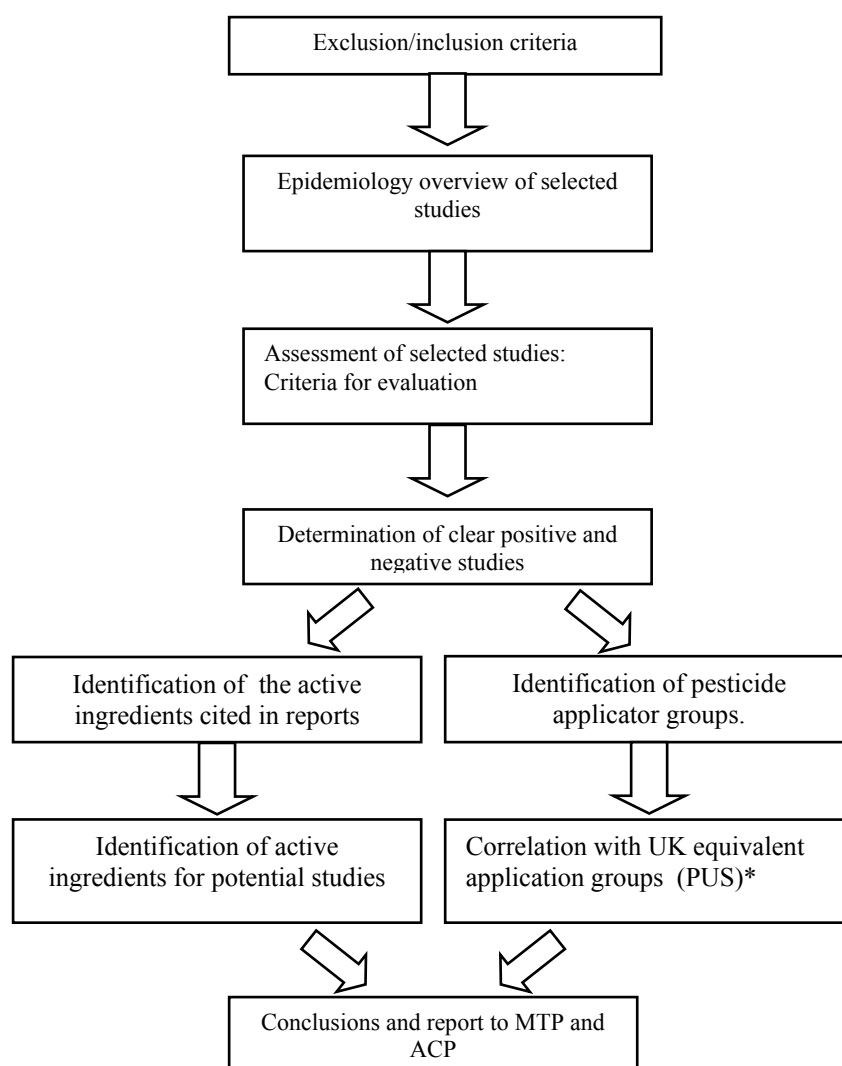
7. The COM took its remit from the referral from MTP and agreed to consider data originating from applications of formulated pesticides and pesticide mixtures. COM did not consider studies where a single pesticide active ingredient with known mutagenic potential was used (e.g methyl bromide fumigation). The COM agreed to eliminate a number of publications arising from studies undertaken in Croatia because of irregularities in the reported data. A number of papers from Zeljezic and Garaj-Vrhovac from the

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laboratory of Mutagenesis, Institute for Medical Research and Occupational Health, Zagreb, Croatia were published during 2000-2 and were identified during literature searches.⁶⁵⁻⁷⁰ It was noted that there were apparent discrepancies between the different publications in the reporting of the demographic data on exposed and controls and reporting of results of mutagenicity studies and no confidence could be attached to the results.

8. The remaining 65 studies were undertaken from all parts of the world but the literature search did not find any published study which had evaluated UK pesticide applicators. The 65 studies were subject to a review procedure for the adequacy of the genotoxicity assessment. The COM discussed the exclusion/inclusion criteria at the October 2004 and February 2005 meeting and following a postal consultation after the February 2005 meeting. The criteria and selected studies are outlined in Annex 1 to this statement.

Figure 1: Flow Diagram of approach used by ACP.



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* = Pesticide Usage Survey data

Epidemiology overview of biomonitoring studies

9. The 24 studies selected by COM using the exclusion/inclusion criteria were subjected to an epidemiology overview (see para 3 vi above). The full report can be accessed from the COM internet site. One objective of the epidemiology overview was to attempt a quality ranking of studies.

10. The Committee noted the conclusion reached in the epidemiology overview that all of the studies were limited in design, particularly with regard to study size, the assessment of selection and recruitment biases. Many of the studies provided information on demographics, medical history, lifestyle factors, potential occupational exposures to materials other than pesticides (eg solvents, radiation), and also information on 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 papers did not provide a specific analysis of individual pesticides. It was noted that the majority of studies were not sufficiently large enough 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 between studies and this might affect the conclusions which could be drawn. There were limitations in the statistical approaches used in many of the studies. Thus for example many did not consider normality. The reporting of modelling was variable and in most cases was not adequate. It was noted that the papers tended to focus on statistical significance even when the absolute difference between groups was tiny. Overall it was not possible to identify any particular study that was clearly better in design and reporting than the other papers in the 24 studies identified by COM.

11. The COM considered the epidemiology overview at its 26 May 2005 meeting. Members agreed that the review had highlighted and confirmed their views on the limitations of the data set. However members agreed to review the data in order to reach the most appropriate conclusions possible.

COM Review of selected studies.

12. The COM considered the full published reports and a narrative summary of the selected studies.

Criteria for evaluation

13. The COM undertook an evaluation of the control data from the 24 studies for micronuclei and chromosomal aberrations in peripheral blood lymphocytes. Such an evaluation might aid in the assessment of the data from studies and help to decide what magnitude of response was suggestive of a positive result. Modelling of the data from the 24 selected studies suggested

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that MN data were normally distributed whilst there was evidence for a skewed distribution of CA as would be expected. Members felt the available data suggested that the distribution of micronuclei and chromosomal aberrations in human peripheral blood lymphocytes was consistent with an approximately binomial distribution, whilst distribution of micronuclei was much more dispersed than would be expected for binomial/Poisson distributions. However, for both endpoints multi- (tri-) modal distributions were a distinct possibility. It was considered that more data would help to resolve the actual distribution of these indices in peripheral blood lymphocytes. The large overall variation in the negative control (reference) data (approximately 16 fold for micronuclei and chromosomal aberrations) suggested that it was not possible to define a single historical control range. Members agreed that statistical significance from adequately conducted studies in combination with magnitude of response represented the most appropriate approach to evaluating the results of studies. Members agreed that the distribution of data should be assessed prior to consideration of the most appropriate statistical approach to analysis and that the effect of confounding factors should be clearly evaluated

14. The COM compared the magnitude of response seen in the 24 studies of pesticide applicators with that reported for patients undergoing treatment with cytostatic medicines and nurses occupationally exposed to these medicines. Members were surprised at the small magnitude of response in the biomonitoring studies of nurses or patients exposed to cytostatic medicines. The mean fold increase in nurses (1.8, range 1.5-2.2) and in patients (mean 2.1, range 1.5-2.7) derived from studies for either micronuclei or chromosomal aberrations was similar to that reported for pesticide applicators in the studies reviewed by COM (1.7, range 0.8-5). Members noted that the higher maximum fold increase in pesticide applicators compared to nurses or patients exposed to cytostatic medicines might reflect differences in the extent of control for confounding between studies. The Committee agreed that this analysis provided valuable information on the outcome of biomonitoring studies for genotoxicity but that it was not possible to draw a definite conclusion on a minimum fold increase in biomonitoring indices of exposure to genotoxic compounds.

15. Members agreed that consideration of statistical significance and magnitude of effects from adequately conducted studies was the most appropriate approach to evaluating the available data. Members confirmed that the available information was severely limited and hence no definite conclusions could be drawn with regard to any U.K. agricultural applications of pesticides or to individual pesticide active ingredients. However the COM agreed that it would be appropriate to derive appropriate tentative conclusions supported by the available evidence.

Consideration of available data on exposure from selected studies

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16. The Committee noted the limited information on exposure. The only direct exposure measurements were reported in the study by Garry et al¹⁸ for exposure to 2,4-dichlorophenoxyacetic acid. Information had been provided on the identity of pesticides which applicators had used in 14 (11 reporting positive results and 3 reporting negative results) out of the 24 studies reviewed.^{5,11,14-16,18,20,22,31,33,35,45,49,52} This information had been reviewed in the context of information from the Pesticide Usage Survey regarding information on use over the period from 1993 up to 2002 and also with regard to the available information on classification status under Directive EC/67/548 with regard to mutagenicity which had been provided by HSE. Members noted that apart from the study published by Garry et al¹⁸ the magnitude of pesticide exposure in these studies had not been recorded and information on use of personal protective clothing had not been documented in many reports.

17. A number of the published papers selected by the COM had reported information which supported the view that the lack of protective clothing used by pesticide applicators/workers was associated with evidence of increased genotoxicity indices in biomonitoring studies.^{11,15,22,32,45} It was noted that there was some limited evidence to suggest that work practices in green house (such as avoiding use of protective clothing in humid conditions and on re-entry after pesticide applications) might be potential sources of pesticide exposure.^{32,81,82} It was also uncertain to what extent the application practices cited in the published reports were relevant to UK agricultural practice. In addition data from 2003 onwards on pesticide use was not available at the time of the COM consideration

18. The reported use of pyrethroids, thiocarbamates and benzimidazoles was higher in the positive studies (n=11) compared to the negatives studies (n = 3). At least one pyrethroid and/or benzimidazole was cited in 73% of the positive studies compared to 33% of the negative studies and at least one thiocarbamate was cited in 36% of the positive studies whereas none were used in the negative studies. Using information from the Pesticide Usage Survey, the amount of metam sodium and carbendazim increased over part of the period 1993-2001 in floricultural and green house applications. The area of outdoor bulbs and flowers sprayed with carbendazim was reported to increase. Increases in the use of bifenthrin, metam sodium and thiram were reported in agricultural practices. The Committee noted that with regard to pesticide active ingredients that were currently approved for use in the U.K. and which were also classified with regard to mutagenicity under EC/67/548, carbendazim and thiophanate-methyl were used in 5/11, and 1/11 positive studies respectively but not in the negative studies^{5,22,31,35,52}. This represented a very small and incomplete amount of information. It was noted that two of these studies had used micronucleus or chromosomal aberration analyses which could potentially be affected by spindle inhibitors such as benzimidazoles.^{5,31} However it is unclear whether the results derived from the Comet assay^{22,35} can be related to benzimidazoles and there is no evidence for direct binding of benzimidazoles to DNA adduct and hence the results of the remaining DNA-adduct study⁵² are unlikely to be related to benzimidazole

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exposure. It was noted that there were a number of other classified mutagens listed in the positive studies which were not approved for use in the U.K.

19. There were limited data on estimated duration of exposure to pesticides in 6 out of the 24 selected studies.^{5,7,22,30,44,45} Four of these studies reported a positive correlation between duration of exposure and increased indices of genotoxicity^{7,22,44,45} whereas the remaining two studies reported a negative correlation.^{5,30} The stratification into groups according to exposure was generally based on periods of 10 years or more. No rationale was given in the studies for the stratification of exposure groups according to duration of exposure. The magnitude of increased indices of genotoxicity with duration of exposure was small in all of the studies. The Committee was aware that biomarkers of genotoxicity (such as chromosome aberrations and micronuclei) increase in frequency with age and that this potential confounding factor had not been considered adequately in the analyses reported. The Committee agreed it was not possible to draw any definite conclusions based on these data, although it is noted that three out of four of the studies which reported on duration of exposure and which also reported a positive response had documented evidence for exposure to benzimidazole pesticides.

20. The COM discussed whether any proposed study should focus on an occupational group (such as floriculture) or on specific pesticides. Members acknowledged that the evidence was limited particularly with regard to the design, conduct, reporting and analysis of the available studies both with respect to identifying either an occupational category or specific pesticides. It was noted that a limitation in the available published literature concerned relevant information on the mutagenicity of mixtures of pesticides. The secretariat referred members to the COT report on mixtures of pesticides (the WIGRAMP report <http://www.food.gov.uk/science/ouradvisors/toxicity/COTwg/wigramp/>).⁸³ The COT working group had identified benzimidazoles as a possible common mechanism group of compounds for further evaluation.

Consideration of use of Personal Protective Equipment (PPE)

21. None of the selected studies specifically investigated the effect of use of PPE on biomonitoring indices of genotoxic effects in pesticide applicators. The extent of PPE usage, where reported, varied considerably. Thus in some reports, no PPE was used,^{29,30} whilst other reports describe conditions in which most pesticide applicators use PPE.^{5,47} Several studies reported significant increases in chromosome aberrations and micronuclei correlated with a lack of PPE use during pesticide application.^{11,15,31,33} It is noted that floriculturalists might report using PPE, but some investigators note that due to humid conditions within green houses appropriate PPE is not always worn. The Committee noted that a correlation between the lack of use of PPE and increased biomonitoring indices of genotoxicity but concluded that no definite conclusions could be reached with regard to exposure to pesticides based on the available data.

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COM conclusions

22. Members confirmed that the available information was severely limited and hence no definite conclusions could be drawn with regard to and U.K. agricultural applications of pesticides or to individual pesticide active ingredients. The COM concluded;

i) The COM review was based on 70 retrieved published studies of biomonitoring of genotoxicity in pesticide applicators. The evidence covered a large number of types of applications and a wide diversity of pesticide mixtures. The COM selected 24 studies which had been adequately undertaken with respect to genotoxicity evaluation. An independent epidemiological overview of these 24 studies reported that all had significant design and evaluation faults. The COM agreed that any conclusions reached on this evidence would be limited by the poor quality of the available studies.

ii) The COM agreed that following a review of the 24 selected studies there was limited evidence supporting increased biomonitoring indices of genotoxicity in biomonitoring studies of pesticide applicators. The Committee agreed that it was not possible to make any conclusions regarding duration or amount of exposure to pesticides thus it was not possible to correlate degree of exposure with observed genotoxic endpoint in support of any positive findings. The Committee noted that there was no published study of pesticide applicators using pesticide mixtures in the U.K.

iii) The COM agreed that it is very difficult to draw conclusions on what might be the most appropriate biomonitoring study for U.K. pesticide applicators. There was some limited evidence to suggest that an appropriate study of floriculturalists using benzimidazoles (e.g carbendazim and thiophanate-methyl) might represent a reasonable first hypothesis to examine whether genotoxicity could be identified in a biomonitoring study of pesticide applicators in the U.K. In reaching this conclusion the COM was aware that these compounds were *in-vivo* mutagens inducing effects via a threshold related mechanism.

iv) The Committee was aware that a threshold approach to the risk assessment of benzimidazole mutagenicity had been agreed by the COM in 1996. Such a study of benzimidazole use would also aid in the evaluation of the adequacy of personal protective clothing and the adequacy of overall risk assessment for these pesticide active ingredients.

Secretariat September 2005.

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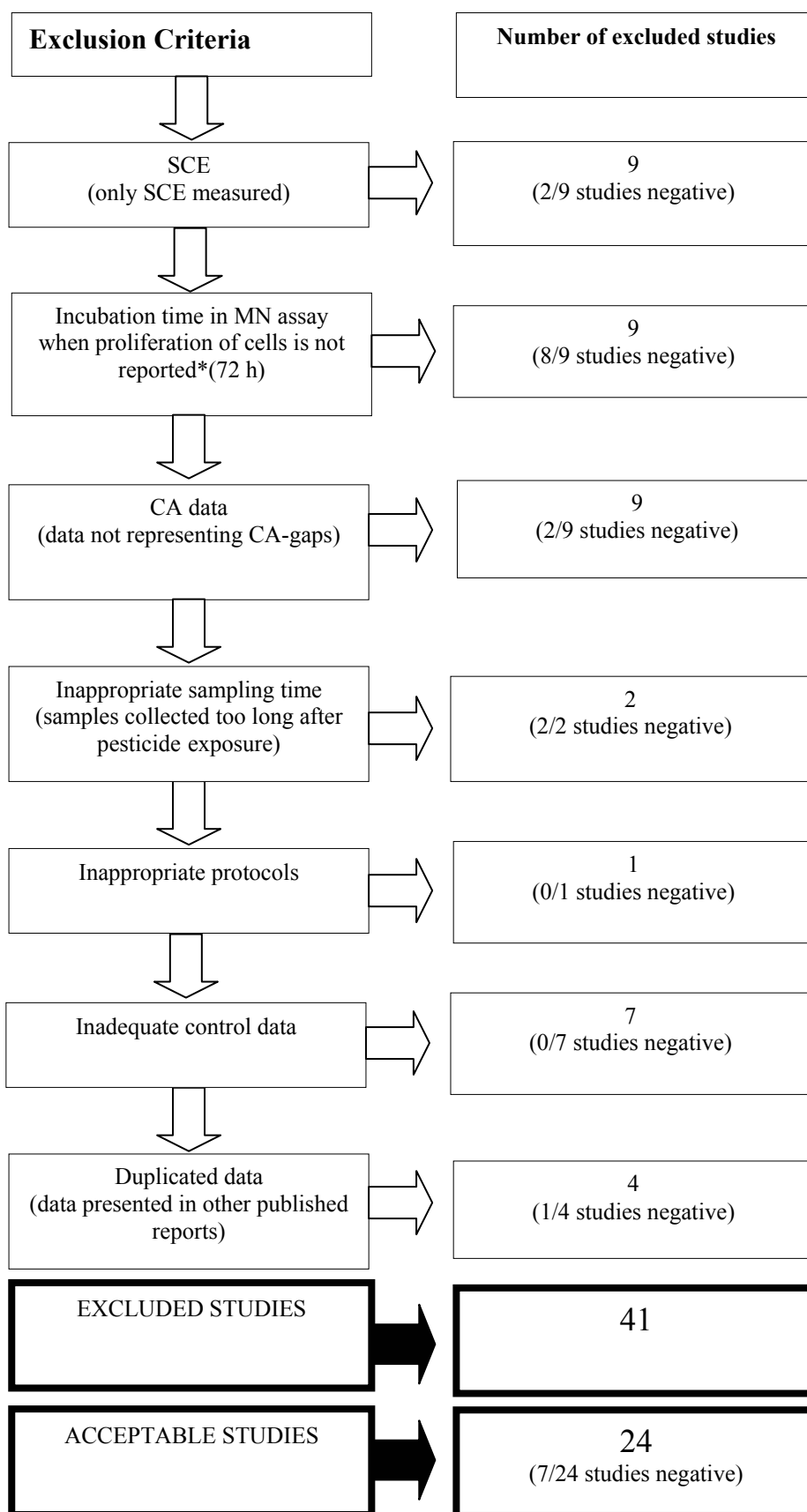
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ANNEX 1 TO DRAFT WORKING PAPER ON BIOMONITORING STUDIES OF GENOTOXICITY IN PESTICIDE APPLICATORS JULY 2005

Flow chart of excluded studies.

The flow chart demonstrates how many studies were excluded from further analysis due to various selection criteria, and the data reported in such studies.



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ANNEX 2 TO COM STATEMENT ON BIOMONITORING STUDIES OF GENOTOXICITY IN PESTICIDE APPLICATORS:

Data reported in selected studies

Author	Results	Data for controls	Data for exposed subjects	Fold increase over controls
^a Bolognesi <i>et al.</i> , 1993b	+ MN	MN frequency = 6.67 ± 3.12	MN frequency = 8.57 ± 5.02 ¹ (mean / 1000 cells ± SD) RR = 1.25; 95 % CI = 1.11 – 1.41	1.3-fold increase
^a Bolognesi <i>et al.</i> , 2004	- MN	Total MN (C+MN) = 2.18 ± 6.31 Total MN (C-MN) = 1.32 ± 3.38	Total MN (C+MN) = 2.79 ± 12.21 ² Total MN (C-MN) = 1.56 ± 6.00 ³ (mean / 1000 cells ± SD)	1.2-fold increase 1.3-fold increase
^a Carbonell <i>et al.</i> , 1995	+ CA	Spring/summer Cells with aberrations = 4.56 ± 2.53 % Chromatid-type aberrations = 3.14 ± 2.76 % Chromosome-type aberrations = 1.90 ± 1.51 % Total aberrations = 5.04 ± 2.85 % Autumn/winter Cells with aberrations = 3.39 ± 2.4 % % chromatid-type aberrations = 2.57 ± 2.0 % Chromosome-type aberrations = 1.32 ± 1.96 % Total aberrations = 3.90 ± 3.23 %	Spring/summer Cells with aberrations = 6.27 ± 2.96 % Chromatid-type aberrations = 5.31 ± 3.12 % Chromosome-type aberrations = 1.63 ± 1.56 % Total aberrations = 6.93 ± 3.5 % ⁴ Autumn/winter Cells with aberrations = 3.69 ± 2.14 % chromatid-type aberrations = 2.49 ± 0.56 % Chromosome-type aberrations = 1.21 ± 1.83 % Total aberrations = 3.70 ± 2.15 % ⁵ (mean ± SD)	1.4-fold increase 1.7-fold increase 0.9-fold increase 1.4-fold increase 1.1-fold increase 1.0-fold increase 0.9-fold increase 1.0-fold increase
^a De Ferrari <i>et al.</i> , 1991	+ CA + SCE	Chromatid-type aberrations = 4.44 ± 3.06 Chromosome-type aberrations = 1.08 ± 1.28 Complex rearrangements = <0.02 Total aberrations = 5.52 ± 4.12	Exposed subjects Chromatid-type aberrations = 7.46 ± 6.22 Chromosome-type aberrations = 2.72 ± 1.58 Complex rearrangements = 0.12 ± 0.12 Total aberrations = 10.30 ± 7.18 ⁶ Exposed subjects with bladder cancer; Chromatid-type aberrations = 5.07 ± 3.90 Chromosome-type aberrations = 2.65 ± 0.26 Complex rearrangements = 0.30 ± 0.62	1.7-fold increase 2.5-fold increase 6.0-fold increase 1.9-fold increase 1.1-fold increase 2.5-fold

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			Total aberrations = 8.02 ± 4.98 ⁷ (Mean / 100 metaphases \pm SD)	increase 15.0-fold increase 1.5-fold increase
^b Dulout <i>et al.</i> , 1985	+ CA	Abnormal cells = 2.65 ± 1.01 % Gaps = 1.56 ± 2.09 Chromatid breaks = 1.70 ± 0.74 Chromosome breaks = 0.54 ± 0.62 Dicentric chromosome and ring chromosome = 0.10 ± 0.21	Abnormal cells = 2.71 ± 0.36 % Gaps = 2.43 ± 1.62 Chromatid breaks = 1.51 ± 1.26 ⁸ Chromosome breaks = 0.95 ± 1.08 ⁹ Dicentric chromosome and ring chromosome = 0.43 ± 0.84 ¹⁰ (CA / 100 cells - gaps \pm SD)	1.0-fold increase 1.6-fold increase 0.9-fold increase 1.8-fold increase 4.3-fold increase
^a Falck <i>et al.</i> , 1999	+ MN	MN frequency 0.5 μ g/ml BrdU = 7.4 ± 3.1 1 μ g/ml BrdU = 7.4 ± 3.1	0.5 μ g/ml BrdU = 7.8 ± 2.4 ¹¹ 1 μ g/ml BrdU = 8.0 ± 2.7 ¹² (mean / 1000 cells \pm SD)	1.1-fold increase 1.1-fold increase
^b Garry <i>et al.</i> , 1996	+ CA	Rearrangement frequency = 0.4 ± 0.57	Rearrangement frequency Fumigant = 1.4 ± 1.44 ¹³ Insecticide = 1.4 ± 1.28 ¹⁴ Herbicide = 1.0 ± 1.34 ¹⁵ (mean \pm SD)	3.5-fold increase 3.5-fold increase 2.5-fold increase
^b Garry <i>et al.</i> , 2001	+ CA	Translocations/inversions/deletions = 0.65 ± 1.12	Translocations/inversions/deletions Low volume (1-100 gall) = 1.20 ± 1.13 ¹⁶ Mid-range (100-1000 gall) = 1.00 ± 1.13 ¹⁷ Heavy (>1000 gall) = 2.22 ± 1.14 ¹⁸	1.9-fold increase 1.5-fold increase 3.4-fold increase
^b Gomez-Arroyo <i>et al.</i> , 2000	+ MN	MN frequency = 0.38 ± 0.021	MN frequency = 1.01 ± 0.03 ¹⁹ (mean / 100 cells \pm SD)	2.7-fold increase
^b Grover, <i>et al.</i> , 2003	+ comet	Smokers Comet tail length = 7.03 ± 11.46 Non-smokers Comet tail length = 10.34 ± 13.25	Smokers Comet tail length = 18.26 ± 9.76 ²⁰ Non-smokers Comet tail length = 19.75 ± 14.48 ²¹	2.6-fold increase 1.9-fold increase
^b Hogstedt <i>et al.</i> , 1980	- CA	Cell with aberrations = 4.6 %	Cell with aberrations = 4.2 % ²²	0.9-fold increase
^a Kourakis <i>et al.</i> , 1992	+ CA	Chromosome-type aberrations = 0.2 ± 0.37 chromatid-type aberrations = 0.34 ± 0.60 Total aberrations = 0.54 ± 0.90 %	Chromosome-type aberrations = 1.34 ± 1.62 chromatid-type aberrations = 0.80 ± 0.81 Total aberrations = 2.14 ± 1.62 % ²³ (mean / 100 metaphases \pm SD)	4.6-fold increase 6.1-fold increase 5.0-fold

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				increase
^a Lander <i>et al.</i> , 2000	+ CA	Pre-season Chromatid-type aberrations-gaps = 1.03 ± 0.82 Chromosome-type aberrations-gaps = 0.28 ± 0.45 Total aberrations-gaps = 1.31 ± 0.85 %	Pre-season; Chromatid-type aberrations-gaps = 0.87 ± 0.95 Chromosome-type aberrations-gaps = 0.45 ± 0.74 Total aberrations-gaps = 1.32 ± 1.23 % ²⁴	0.84-fold increase 1.6-fold increase 1.0-fold increase
			Post-season Chromatid-type aberrations-gaps = 1.04 ± 0.99 Chromosome-type aberrations-gaps = 0.34 ± 0.56 Total aberrations-gaps = 1.37 ± 1.20 % ²⁵ (Mean / 100 metaphases ± SD)	1.2 (1.0) -fold increase 0.8 (1.2) -fold increase 1.0 (1.0) -fold increase compared with pre-season (compared with controls)
^a Lebailly <i>et al.</i> , 1998	+ comet	Beginning of spraying season = 30 Beginning of spraying season = 30	Middle of spraying season = 43 ²⁶ End of spraying season = 36 ²⁷	1.4-fold increase 1.2-fold increase
^a Lebailly <i>et al.</i> , 1998b	+ comet	DNA damage Before spraying Mixture of pesticides = 48 % Herbicides on wheat = 30 % Fungicides on wheat = 43 % Fungicides & insecticides on peas = 36% Tail moment Before spraying Mixture of pesticides = 3.21 Herbicides on wheat = 2.30 Fungicides on wheat = 3.64 Fungicides & insecticides on peas = 2.39	DNA damage After spraying Mixture of pesticides = 56 % ²⁸ Herbicides on wheat = 28 % ²⁹ Fungicides on wheat = 35 % ³⁰ Fungicides & insecticides on peas = 39% ³¹ Tail moment After spraying Mixture of pesticides = 3.92 ²⁸ Herbicides on wheat = 1.93 ²⁹ Fungicides on wheat = 3.58 ³⁰ Fungicides & insecticides on peas = 3.16 ³¹ (mean)	1.2-fold increase 0.9-fold increase 0.8-fold increase 1.1-fold increase 1.2-fold increase 0.8-fold increase 0.9-fold increase 1.3-fold increase 1.3-fold increase
^a Lebailly <i>et al.</i> , 2003	- comet	DNA damage Morning before pesticide use = 10 % (2-21 %) Tail moment Morning before pesticide use =	DNA damage Evening after pesticide use = not measured Following morning = 13 % (5-49%) ³² Tail moment	1.3 -fold increase (compared to before pesticide use)

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		4.35 ± 1.11 (2.16-5.85)	Evening after pesticide use = not measured Following morning = 4.80 ± 2.57 (3.18-12.76) ³³ (Mean ± SD)	1.1-fold increase (compared to before pesticide use)
^a Munnia <i>et al.</i> , 1999	+ DNA adducts	DNA adducts = 2.17 x 10 ⁹ ± 5.75 RAL	DNA adducts = 8.50 x 10 ⁹ ± 14.95 RAL ³⁴ (Mean ± SD)	3.9-fold increase
^a Mustonen <i>et al.</i> , 1986	- CA	Aberrant metaphases-gaps Non-smokers; 1.5 ± 0.73 Smokers; 1.9 ± 1.2	Aberrant metaphases-gaps Non-smokers; 1.2 ± 1.5 ³⁵ Smokers; 1.8 ± 1.26 ³⁶ (Mean ± SD)	0.8-fold increase 1.0-fold increase
^b Paldy <i>et al.</i> , 1987	+ CA	Chromosome aberrations = 1.1 ± 0.36	Years of exposure Chromosome aberrations 0-5 years = 2.96 ± 0.36 ³⁷ 6-10 years = 3.55 ± 0.75 ³⁸ 11-15 years = 4.28 ± 0.76 ³⁹ (Sum of aberrations / 100 cells without gaps ± SD)	2.7-fold increase 3.2-fold increase 3.9-fold increase
^a Pasquini <i>et al.</i> , 1996	+ MN	MN frequency = 13.30 ± 5.35 Overall MN frequency = 13.30 ± 5.35	MN frequency (>19 year) = 18.30 ± 7.22 ⁴⁰ Overall MN frequency = 15.98 ± 7.65 ⁴¹ (Mean / 1000 cells ± SD)	1.37-fold increase 1.2-fold increase
^a Pastor <i>et al.</i> , 2001b	- MN	MN frequency = 16.38 ± 12.19	MN frequency = 12.20 ± 6.58 ⁴²	0.7-fold increase
^a Pastor <i>et al.</i> , 2002a	- MN	MN frequency = 10.3 ± 7.06	MN frequency = 10.22 ± 7.06 ⁴³	1.0-fold increase
^a Peluso <i>et al.</i> , 1996	+ DNA adducts	DNA adducts = 9	DNA adducts = 42 ⁴⁴	4.7-fold increase
^a Piperakis <i>et al.</i> , 2003	- comet	DNA damage Male non-smokers = 82.3 ± 14.1 Female non-smokers = 81.1 ± 16.12	DNA damage Male non-smokers = 83.2 ± 14.02 ⁴⁵ Female non-smokers = 82.1 ± 13.14 ⁴⁶ (mean ± SD)	1.0-fold increase 1.0-fold increase

^a Studies from EU ^b Studies from rest of world

Mean fold increase of positive studies over controls ± SD (SE) = 1.73 ± 1.07 (0.15)

Figures in bold denote the total or mean fold increase of the study.