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## COMMENTARY

# Factors affecting the incidence of genotoxicity biomarkers in peripheral blood lymphocytes: impact on design of biomonitoring studies

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**A review of risk factors affecting background rates of micronuclei and chromosomal aberration (CA) formation in peripheral blood lymphocytes (PBLs) was undertaken with a view to aiding the interpretation of genotoxicity biomonitoring studies. Both endogenous factors and those due to methodological variation were evaluated. Background variation of other indices of genotoxicity in PBLs (specifically 8-hydroxy-deoxyguanosine and comet assays) were also considered as these data likely reflect overlapping causes of DNA damage and may provide some indicators for future research areas. A number of host risk factors, namely age, gender, smoking, vitamin $B_{12}$ and folate status, were identified for which there is strong or sufficient evidence that they impact on background levels of genotoxicity biomarkers. Evaluation of these factors should be routinely included in genotoxicity biomonitoring studies. Although data on the influence of smoking is somewhat inconsistent, because of its known association with cancer and DNA damage, it is also classified as a high-risk factor. A number of other factors were identified for which there is weak or insufficient evidence including alcohol consumption, disease conditions and infections, physical exercise, body mass index and genotype. The review shows that the evaluation of biomonitoring studies of genotoxicity is complex and there is a need to improve study designs by setting an *a priori* hypothesis, collecting good exposure data and stratifying groups appropriately, using appropriate power calculations before initiating biomonitoring studies, and collecting information on appropriate risk factors. There is a need for further collaborative work and the establishment of centres of excellence on genotoxicity biomonitoring. If these measures are achieved, then it would be possible to use the data from biomonitoring studies in risk assessments to derive risk management measures.**

## Introduction

The rationale for this review arose from our previous publication in which we performed a systematic assessment of studies reporting investigations of genotoxicity biomarkers in pesticide workers with a view to establishing whether there was evidence for any risk to those using pesticides approved in the UK (1). In this review, of the 70 studies initially identified, 24 were selected following a quality screening of which 17 reported positive findings. There was some limited evidence that benzimidazoles were more frequently used in the studies reported as being positive. However, as a result of the limited design, the relatively small fold increases reported in many studies of pesticide applicators (ca. $<$1.5-fold) in the absence of reliable information on exposure to genotoxic pesticides, and the observation that exposure to cytostatic medicines, which are considered to be potent human mutagens, also resulted in relatively small fold increases ($\sim$3-fold), no conclusions could be derived regarding the effects of exposure to specific pesticides from the studies reviewed. It was concluded that background variation in genotoxicity indices in the control population and a lack of awareness of factors which impact upon this variability confound interpretation of the true nature and significance of the reported positive findings. This review was conducted in an attempt to improve the understanding of this background variation.

There is accumulating evidence that micronuclei (MN) and chromosomal aberrations (CAs) in peripheral blood lymphocytes (PBLs) can independently aid in the prediction of risk of cancer development (2–7). Often papers investigating these genotoxicity end points provide commentary on the relevance of the findings to the development of cancer. However, a comprehensive assessment of the cancer studies in which genotoxicity biomarkers have been concurrently evaluated revealed that they are invariably constrained by the short periods of follow-up (ca. $<$10 years) and limited information on confounding factors in the investigations (2). This supports the need for a careful consideration of factors affecting the induction of MN and CAs in PBLs which, in turn, will aid in the interpretation of studies of environmental and occupational chemical exposures and cancer risk.

This commentary reviews the factors affecting the formation of MN and CAs in PBLs in humans and, to a lesser extent, other genotoxicity indicators such as 8-hydroxy-deoxyguanosine (8-OHdG) and the comet assay. It is partly based on reviews undertaken for the UK's Department of Health's Committee on Mutagenicity (COM) at the beginning of 2006 (8). Literature published since this review has also been considered for this commentary. Information on the search strategy used and evaluation process is given below.

There are a number of review articles which address factors affecting cytogenetic biomarkers and their usefulness in biomonitoring studies. Bonassi *et al.* (9) published a systematic review of human population studies with cytogenetic biomarkers which covered the period January 1, 1980 to December 31, 2003. Although they did not provide data on background frequencies, their commentary discussed the need for careful study and methodological design and indicated the importance of understanding the variables which impact upon the baseline incidence of genotoxicity biomarkers. There is comparatively more information available on risk factors for

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MN formation compared to CA formation in PBLs, much of which is derived from the HUman MicroNucleus (HUMN) collaborative project which has reported data on ~7000 individuals (10–13). More limited collaborative projects have been undertaken for CAs using metaphase analysis of PBLs (14–19) and fluorescence *in situ* hybridization (FISH) techniques using PBLs (20–23).

Cytogenetic biomarkers are the most frequently used end point in human biomonitoring studies and are used extensively to assess the impact of environmental, occupational and medical factors on genomic stability. The evaluation of MN in PBLs is the most commonly used technique, although cells such a buccal epithelia are also utilized. There was a rapid increase in the publication of studies, particularly with regard to investigations using MN, after 1985 when the cytokinesis-block (CB) method was first published (9,24). The scope and application of the CBMN assay in biomonitoring has also been expanded in recent years so that in addition to scoring MN in binucleate cells, there are proposals to evaluate MN in mononucleate cells (to provide a more comprehensive assessment of DNA damage), nucleoplasmic bridges (indicative of DNA misrepair, chromosome rearrangement or telomere endfusions) and nuclear buds (a measure of gene amplification or acentric fragments) (25–29). Fenech (29,30) has proposed that the CBMN assay can be used to measure chromosomal instability, mitotic dysfunction and cell death (necrosis and apoptosis) and has suggested the term 'cytokinesis-block micronucleus cytome assay'. Identification of the contents of MN (e.g. the presence and absence of centromeres) is now considered important in the evaluation of MN in biomonitoring studies, providing insight into mechanisms underpinning the positive results reported (i.e. to differentiate between clastogenic and aneugenic responses) (31). It is likely that biomonitoring studies using cytokinesis-block micronucleus cytome assay end points including centromeric probes to determine the mechanism of MN formation will become more widespread. This further emphasizes the need for good quality information on factors affecting MN formation in control populations.

Neri *et al.* have published systematic reviews of cytogenetic biomonitoring for exposure of children (0–18 years) to genotoxins in the environment, using both MN and CA assays. These authors reported the MN assay to be particularly sensitive to the measurement of genetic damage in children (32,33). One of the priority goals of the World Health Organization for Children's Environment and Health Action Plan for Europe is monitoring exposure of children to hazardous chemicals in a harmonized way (34). Thus, a priority will also be provision of background information on risk factors for MN and CA formation in PBLs from children.

The current commentary is not intended as an exhaustive review of the entire genotoxicity biomarkers literature. We provide information on background variables principally for MN in PBLs, but also for CAs when data are available, and some studies of the use of the comet assay in PBLs and 8-OHdG in PBLs, urine and tissues and use these data to aid our discussion. First, we assess the utility of the different genotoxicity end points and then review the risk factors which may affect the background incidences of MN and CAs in PBLs (termed host factors). From our review of genotoxicity biomonitoring studies of pesticide applicators, 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 undertaking statistical analysis. It was noted that articles tended to focus on statistical significance even when the absolute difference between groups was very small (1). The second section considers information on methodological variation between and within laboratories regarding the background frequency of MN and CAs in PBLs (termed methodological factors). The final section discusses the potential impact of the sources of variation in biomonitoring studies of genotoxicity using MN and CAs in PBLs, comments on the interpretation of studies undertaken with pesticide applicators and makes some suggestions for the design of biomonitoring studies.

A number of proposals regarding the design and interpretation of studies and the value of developing expertise within the UK on genotoxicity biomonitoring of chemical exposure using MN and CAs are made, with particular emphasis on the future use of MN. The comments made here regarding recent developments in the application of genotoxicity biomonitoring have been made in this introduction with a view to providing context to the discussion given below.

## Biomarkers; sensitivity and relevance to genotoxicity

A summary of the various genotoxicity biomonitoring end points most commonly used in studies evaluating environmental or occupational risks associated with exposure to potential genotoxins is given in Table I.

Bonassi *et al.* (3,4) demonstrated that cytogenetic damage can be used to predict potential cancer risk using data from the screening of 6718 subjects for MN and 3541 subjects for CA. A recent review of the application of biomarkers of intermediate end points in environmental and occupational health concluded that MN in lymphocytes provided a promising approach with regard to assessing health risks but concluded that use of CAs in future studies was likely to be limited by the laborious and sensitive procedure of the test and lack of trained cytogeneticists (35).

Unfortunately, there are very few investigations where MN or CA formation in PBLs has been measured concurrently with other genotoxicity indicators such as oxidative DNA damage (36–38) and thus it is not possible to provide an overall comparison of sensitivities and specificities of the different

**Table I.** Overview of genotoxicity biomarkers and the end points evaluated by them

| End point | Measures | Marker for |
|---|---|---|
| MN | Chromosome breakage and/or chromosome gain or loss | Oxidative DNA damage, aneugens and clastogens |
| CA | Structural chromosome or chromatid aberrations | Variety of DNA effects (deletions, duplications, inversions and translocations) and oxidative DNA damage |
| Comet | Single and double DNA strand breaks | Oxidative DNA damage and DNA repair |
| 8-OHdG | Hydroxyl radical attack on C8 on guanine | Oxidative DNA damage |

biomarkers currently used. However, methodologies such as the comet assay and 8-OHdG in PBLs, urine and tissues are increasingly being used as markers of oxidative DNA damage. A review of the use of the comet assay in biomonitoring occupational exposure to genetic toxicants assessed possible confounding factors and confirmed the assay's usefulness for assessing DNA-damaging potential. The review recognized that careful consideration of factors influencing background levels was required (39). Similarly, a review of the utility of 8-OHdG in PBLs established its promise as an alternative biomarker to MN and CAs, but concluded that considerable research was required before a consensus on background levels could be attained (40).

Studies investigating correlations between end points used in genotoxicity biomonitoring studies have yielded inconsistent results. For example, Faust *et al*. (39) reported a good correlation between cytogenetics and comet in biomonitoring studies, while Koppen *et al*. (38) reported that there was no correlation between MN and CAs in PBLs or between comet (PBLs) and urinary 8-OHdG in adolescents (37). In another investigation, direct comparison of MN levels with CAs and 8-OHdG indicated large interindividual variability, although it was noted that the extreme values for each end point did not necessarily correlate within individuals, and subjects with highest levels of damage showed the greatest variation across time. Interestingly, in a study assessing DNA damage in the peripheral blood of smokers, neither the comet assay nor MN test gave significant differences between smokers and non-smokers (41).

However, the apparent lack of correlation between the variations in the incidences of the different biomarkers of genotoxicity is somewhat surprising. This may in part reflect the quality of the available studies but could also indicate that quite complex interactions are likely to exist between risk factors for endogenous oxidative DNA damage and MN and CA formation in PBLs. The relative sensitivities of the different end points discussed, together with the importance of other factors which influence the persistence of the biomarkers such as DNA repair, may plausibly impact on background levels in the studies considered and would need to be considered before the relationship regarding increases in genotoxicity end points with exposure to environmental chemicals or endogenous factors is explored.

Thus, as the genotoxicity end points summarized in Table I represent separate but overlapping biomarkers, we have tentatively used data from a spectrum of genotoxicity biomarkers to help inform on potential background variables affecting the formation of MN and CA in PBLs and to help reach conclusions on potential areas for future research in this regard. Furthermore, it is hoped that more studies which address the relationships of the different genotoxicity end points in PBLs to one another will be conducted, thus furthering our understanding of their potential use in chemical risk assessments.

## Risk factors for genotoxicity end points in PBLs

There is considerable variation in the way in which potential confounding factors for background levels of genotoxicity biomarkers are reported. Here, an attempt has been made to draw on data generated to specifically evaluate the background variables in addition to more general observations from occupational biomonitoring studies. It is noteworthy that some potential risk factors or combinations of risk factors for elevated MN or CAs in PBLs in unexposed populations might lead to persistent changes (e.g. smoking habit and altered vitamin status) (42,43), whereas others are likely to be transient (e.g. a bout of intensive exercise) (44,45) or not routinely screened for in biomonitoring studies (e.g. the presence of chronic viral infections) (46,47).

A series of literature searches (using PubMed) were undertaken using the risk factors for MN and CA formation in PBLs as set out in Table II and ~170 publications were retrieved for this commentary. Most studies were cross-sectional in design, although a small number of prospective, restrospective and intervention designs have been identified. Many of the studies reviewed were relatively small. It is noted that the systematic analysis undertaken for the HUMN project provides highly valuable evidence for this commentary.

Table II summarizes the information retrieved on risk factors for formation of MN and CAs in PBLs. The conclusions reached were derived from an assessment of all the published literature used in this review. We have ranked risk factors into strong/sufficient and weak/insufficient categories based on an assessment of the weight of the available evidence; factors deemed to have sufficient, convincing evidence for an association with baseline MN or CA incidence are those that should routinely be accounted for in biomonitoring studies; a wide range of factors were categorized as weak/insufficient weight of evidence, where an association has been suggested but further research is required to more accurately define the potential significance of routine incorporation in the design of genotoxicity biomonitoring studies. The decision to include low weight risk factors in a biomonitoring study would be a case-by-case decision depending on the research questions and study population under consideration and is a particular focus of this discussion paper.

## Strong/sufficient evidence for association with genotoxicity end points

### *Age*

There are strong correlations between MN frequency and increasing age, although somewhat less evidence was retrieved for CAs (see Table II for citation of available studies). A clear increasing trend in MN incidence was evident in a large study designed specifically to assess the effect of ageing on chromosome damage (54). In the HUMN project, the inclusion of data from 25 different databases (nearly 7000 individuals) provides a robust assessment and all but two of the laboratories reported age-related increases in MN (10). The effect was more pronounced in females than in males, with the increase more marked after 30 years of age. The variation in frequency was >2-fold when comparing values for those under 20 with those over 50 years of age. Bukvic *et al*. (120) also demonstrated a strong correlation between age and MN frequency and suggested that chromosome loss is a determining factor in this increase. Despite a number of studies that have not demonstrated such a correlation (71), there appears to be a significant body of evidence necessitating accounting for age when designing biomonitoring studies in the adult population.

Although age is the most well-established factor impacting on MN incidence, and to a lesser extent CAs, in PBLs in adults, there is still some uncertainty with regard to incremental age-related increases in genotoxicity biomarkers in children (0–18

**Table II.** Risk factors for MN and CA formation in peripheral blood lymphocytes

| MN | CAs |
|---|---|
| Factors considered having strong/sufficient weight of evidence that supports an association, include in biomonitoring studies. | |
| Age:<br>Adults (12,48–53): limited evidence for mononucleate cells (26,54)<br>Children (0–18 years): inconclusive with both positive (32) and negative results reported (10,32,55) | Age:<br>Adults·(55,56) [breaks (15)˙ exchanges (57–59), aneuploidy (60–64), stable translocations (16,23,65,66)]Dicentrics [evidence less clear (67–69)]<br>Children: no evidence for age-related effect in studies with children (0–19 years) in a meta-analysis (70) |
| Gender:<br>Higher frequency in females (10,19,51–54,71)<br>Good evidence for X chromosome loss (72–74) | Gender:<br>See low evidence category below |
| Micronutrient status, vitamin $B_{12}$, serum folate:<br>Inversely correlated with MN while endogenous homocysteine correlates with MN (50,54,75–79) | Micronutrients:<br>See low evidence category below |
| Smoking:<br>>30 cigarettes/day; including data from HUMN project (12,80–82)<br>No evidence for association in pooled analysis of seven laboratories across all levels of smoking ($n$ = 646) (83) or in a pooled analysis from four laboratories ($n$ = 740) (14) | Smoking:<br>Studies reporting an association (84–89)>20/day; chromosomal aberrations (57,67,90)<br>Dicentrics (57,67,85,90,91), translocations (no significant effect) (66) |
| Factors considered having weak/insufficient evidence or more research required, include on a case-by-case basis. | |
| Gender:<br>See sufficient weight of evidence above | Gender:<br>Limited evidence from metaphase analyses (61) and for aneuploidy (48). No evidence from two interlaboratory assessments (14,19).<br>No evidence from FISH studies (16) and FISH studies of translocations (23,66,69,92) |
| Alcohol:<br>Increased MN in alcoholics but not correlated with duration of alcohol abuse (not in abstainers ≥ 1 year) with possible association with data on ALDH2*2 polymorphism (93–97)<br>Association reported for total MN and centromere-positive MN in one pooled analysis ($n$ = 50) where centromere staining was used (98) | Alcohol:<br>Increased CAs in alcoholics but not correlated with duration of alcohol abuse (not in abstainers ≥ 1 year) (no data on ALDH2*2 polymorphism (96,97) |
| Diet:<br>No evidence for vegan diet reducing MN (99–102,130) | Diet:<br>No evidence for vegan diet reducing CAs (100)<br>No effect of diet on CAs (including type of food or dietary profile) in dietary questionnaire study of polycyclic aromatic hydrocarbon-exposed workers (36) |
| Micronutrients:<br>Intervention studies vitamin C and E inconsistent (49,51,81)<br>A modest reduction in MN frequency of 13% reported in an intervention study using vitamin ACEZn supplementation (6 months) (51)<br>No effect in a study of middle-aged men [given vitamin C, E, carotene and selenium for 3 months (103)]<br>(For vitamin B12 and folate see good weight of evidence above) | Micronutrients:<br>No evidence for effect in limited design vitamin supplementation studies (104–106)<br>No investigations of folate or vitamin $B_{12}$ supplementation retrieved.<br>Significantly reduced CAs in a small intervention study of middle-aged men (given vitamin C, E, carotene and selenium for 3 months) (103) |
| Genotype:<br>Background formation of MN<br>*MHTFR* polymorphism (78,79), effect modification of genotoxin metabolism and exposure<br>*NAT2* rapid (smoking), *GSTM1*-null, *EPHXexon3 TyrlTyr*, *GSTT1*-posilive XRCC1 (107)<br>Combined XRCC1 codon 399 and *GSTM1* null. Reduced MN in *GSTT1* null (83) | Genotype:<br>Effect modification of genotoxin metabolism and exposure<br>*NAT2* rapid (smoking), *GSTM1* positive, *GSTT1*-posilive, *CYP1A1* mspI heterozygote (newborn), CYP1A1*2, *CYP2E1 wt*/*5B* and *EPHX* low activity (107–109) |
| Chronic diseases<br>Alzheimer's disease (110,111)<br>Belchets disease (112)<br>Cancer (3)<br>Cardiovascular disease (113)<br>Diabetes (114)<br>Chronic obstructive pulmonary disease (115)<br>Parkinson's disease (110)<br>Polycystic ovary syndrome (116)<br>Rheumatoid arthritis (117) | Chronic diseases:<br>Cancer (2,4,118)<br>Polycystic ovary syndrome (119) |
| Exercise:<br>Increased MN in trained athletes (44)<br>Exhaustive sprinting increased MN (45) | Exercise:<br>No data on CAs retrieved |
| BMI:<br>High BMI (52) | BMI:<br>No data retrieved |

*Includes data from metaphase analysis, G-banding studies and FISH investigations.

years). No effect of child age on MN was reported in an analysis of the HUMN database (10). However, when data were reanalysed by categorizing 448 children into four age groups, an effect of age on chromosomal damage, as measured by MN, was reported with increasing age from 0 to 18 years (32). Merlo *et al*. (70) did not find any association between age

in children (0–19 years) and CAs in PBLs from a systematic review of published studies using metaphase analysis and no significant differences were reported in a separate small study of 32 children (55). Thus, it is important to provide robust background data on risk factors in children such as age, for MN and CA formation in PBLs, particularly given the prospect of more studies being undertaken to evaluate the genotoxic effects of exposure of children to urban pollution, and for a further systematic review to be carried out.

*Gender*

A gender difference in the background incidence of MN in PBLs is also well documented, with the frequency being consistently higher in females. A large, comprehensive study assessing MN, CA and sister chromatid exchange (SCE) showed highly significant elevations in MN in PBLs of women (29% when adjusted for age and smoking) whereas CA and SCE remained unchanged, confirming previous reports (see Table II) (19). This may reflect aneuploidy detected in MN assays. Similarly, Fenech (48) reported a 1.2- to 1.6-fold greater frequency of MN in females than in males. A large population-based study addressing sources of variability also reported gender as the principle differential following adjustment for age (71). As for age, the HUMN project provides robust data based on a large number of subjects and laboratories and clearly demonstrates a gender difference (with females showing a higher incidence of MN compared to males), the effect being more manifest with increasing age (10). There are several reports that link this later observation to an elevated loss of X chromosomes (72). However, as noted in Table II, overall the data from metaphase analysis and for hypoploidy and FISH studies of translocations are limited with respect to a gender effect. In children, a small pilot study showed a significantly lower MN frequency in PBLs of males (aged 5–11 years) in families living in the Czech Republic (121). However, in the significantly more comprehensive analysis by Neri *et al.* (32), a gender difference was not observed. These data suggest that gender effects become more pronounced with increasing age. Thus, it would be pragmatic to include gender as a variable in all biomarker studies investigating genotoxicity using MN or CAs, particularly when study subjects are taken from a wide age range.

*Micronutrient status (vitamin $B_{12}$ and folate)*

There is a body of strong evidence that folate and vitamin $B_{12}$ deficiency is associated with increased chromosome damage and MN incidence in PBLs (75–77), which is clearly supported by mechanistic data. The functions of both micronutrients are associated with DNA repair and synthesis fidelity. Briefly, folate acts as a methyl donor in the process of thymidylate synthesis (dUMP to dTMP) which is required for DNA repair and synthesis. In addition, vitamin $B_{12}$ is required for the conversion of homocysteine to methionine, which generates *S*-adenosyl methionine needed for DNA methylation (75). In two studies, Fenech showed a positive correlation between plasma homocysteine levels and MN, and an inverse correlation between vitamin $B_{12}$, folate status and MN formation (48,76). Consumption of a vitamin–antioxidant mixture reduced spontaneous MN frequency in PBL and susceptibility to *in vitro* γ-radiation-induced damage (49). Moreover, an intervention study by Titenko-Holland indicated that post-menopausal women with low folate had increased MN frequencies in PBL (50).

In conclusion, a number of well-conducted studies, including intervention studies, clearly demonstrate the link between endogenous levels of vitamin $B_{12}$, folate or homocysteine levels and MN frequency and thus represent a factor that should be monitored in biomonitoring studies when MN is used as an end point. Although there are no data to assess the effect of endogenous levels of vitamin $B_{12}$, folate and homocysteine on CA formation in PBLs, it is reasonable to postulate an association.

*Smoking*

Although the link between smoking and cancer is strong and exposure to genotoxic carcinogens present in tobacco smoke has been convincingly demonstrated (122), interestingly the same convincing association is less apparent when assessing biomonitoring studies of genotoxicity. Two studies have reported increased frequencies of MN in smokers. Fenech (80) showed that, after adjustment for age and sex, individuals with high cigarette usage (>30) had statistically greater MN compared to non-smokers and another small study (12 smokers and 12 non-smokers) also demonstrated the association between smoking and significantly higher MN levels in circulating lymphocytes (81). However, a comprehensive analysis of 24 databases (3501 non-smokers, 1409 current smokers and 800 former smokers) for the effects of smoking on MN induction was conducted as part of the HUMN project (12). Most laboratories showed no significant differences between smokers and non-smokers and the pooled analysis, interestingly, indicated an overall decrease for all smokers compared to controls. When looking at numbers of cigarettes smoked, significant increases were seen in those who smoked >30 cigarettes/day (9).

Structural CAs in lymphocyte cultures from smokers have been demonstrated by various techniques including FISH and G-banding, although in general effects were only statistically significant in heavy smokers (57,67,90). In the Nordic Study group, three out of nine laboratories revealed significant increases in CAs in smokers, but the MN frequency was not increased (56). More recently, the comet assay has been utilized widely to investigate the genotoxic effects of smoking and to compare with other genotoxic biomarkers. Kopjar used the comet assay and CA test in a general assessment of healthy volunteers [76 regular smokers (29F/47M) and 94 non-smokers (47F/47M)]. It was shown that smokers had increased levels of primary DNA damage as indicated by comet tail length and the number of long tailed nuclei. Furthermore, Kopjar also reported a statistically significant increase in CAs in the PBLs of smokers compared to non-smokers (84). A meta-analysis of smoking and DNA effects in the comet assay reported that there was, overall, a higher level of DNA damage in smokers (123). In an investigation of the effects of environmental tobacco smoke (passive smoking) in children, comet analysis was undertaken in the PBLs taken from two groups of 23 children from high (>20 cigarettes/day) and low (<20 cigarettes/day) smoking homes and compared with 20 non-exposed controls. A highly significant increase in mean comet length was observed in the passive smoking groups, and exposure was inversely related to glutathione peroxidase activity and tocopherol levels which were included as measures of induced oxidative stress (124).

In contrast, a number of authors reported that there were no significant changes in DNA migration in the comet assay of PBLs from heavy smokers (>20 cigarettes/day) (41,125),

although it is noteworthy that these sample sizes were very small ($n = 6–20$). Moller *et al*. reviewed 23 studies in order to investigate the impact of confounding factors on the outcome of comet assays in occupational investigations. Large discrepancies were noted with regards to the effects of smoking, as the numbers of positive and negative studies were approximately equal. Interestingly, positive studies appeared to cluster in southern Europe. It is suggested that low statistical power may have influenced the detection of a positive effect (126).

To explore the reasons why cigarette smoking, so strongly associated with an increased cancer risk, does not induce equally robust elevations of genotoxicity biomarkers is beyond the scope of the current review. However, with respect to this we note that the effect of smoking in studies of biomonitoring indices of genotoxicity in PBLs appears to be relevant only at levels of smoking in excess of 20–30 cigarettes/day. It is possible that modified study designs to estimate better measures of the cumulative effects of smoking on MN and CAs in PBLs could optimize detection of the response to smoking, and further investigations of genetic polymorphisms may be important but nevertheless information on smoking should be routinely gathered in biomonitoring studies of genotoxicity.

## Weak/insufficient evidence for association with genotoxicity end points

There are wide range of potential risk factors for MN and CA formation in PBLs for which the evidence is limited or inconsistent and thus there is a need for further research to substantiate whether there is a need to routinely take account of such factors in biomonitoring studies. These include alcohol consumption, diet, micronutrient status (other than vitamin $B_{12}$ or folate), a range of chronic diseases, infections and exercise. One possible theme which might underlie many of these factors is the induction of, or protection against, endogenous oxidative DNA damage.

### *Alcohol consumption*

Drinking alcoholic beverages has been causally associated with cancer at a number of sites (e.g. head and neck cancer) (127). The mechanism of alcohol-induced head and neck cancers has not been fully elucidated and may be related to impurities in beverages rather than the alcohol itself. Alcoholic beverages have not been reported to induce mutagenic effects in rodents (128). The evidence regarding an effect of drinking alcoholic beverages on increased MN or CA formation in PBLs is inconclusive. However, an increase in MN formation has been reported in drinkers of alcoholic beverages who also have the ALDH2*2 polymorphism (which is associated with slower metabolism of acetaldehyde) (93–95,98,129). An increase in MN and CAs in PBLs has been observed in alcoholics consuming alcoholic beverages but not in abstainers of a year or more (96,97). The alcohol-induced disease processes may be relevant to induction of MN in PBLs sampled from alcoholics.

### *Diet and micronutrients (other than vitamin $B_{12}$ and folate)*

Several groups have published studies that were designed to assess the role of dietary micronutrient status (in addition to folate and vitamin $B_{12}$) and how it impacts on oxidative DNA damage. However, the results are far from conclusive or indeed indicative of any such effect.

With regards to diet, small studies comparing vegetarians with non-vegetarians did not provide any evidence that diet alters MN or CA frequency in PBLs (99,100,130). Furthermore, studies carried out to investigate the effect of particular food types, such as vegetables and fruit, on oxidative DNA damage are inconsistent with, at most, only small effects reported (131,132). Recently, Raimondi *et al*. (36) used a detailed dietary questionnaire during the examination of polycyclic aromatic hydrocarbon-exposed workers in the Czech Republic; no effects of diet on a number of indicators of DNA damage including CAs were found, although smoked and fried foods were associated with an increase in DNA adduct levels.

Thomson *et al*. measured biomarkers of oxidative damage in women previously treated for breast cancer who were included in a diet intervention trial resulting in reduced fat, polyunsaturated fat and saturated fat intakes and increased fruit and vegetable consumption. A significant increase in intakes of vitamins E, C and beta-carotene, corresponding to increased fruit and vegetable consumption, were reported during the year-long follow-up period. In this study, vitamin E intake was inversely associated with 8-OHdG levels (133). In a separate dietary recall and supplement inventory study of African Americans and Whites aged 20–45 years in North Carolina, USA, vitamin E intake was positively associated with oxidative DNA damage measured by the comet assay (134). Other intervention studies using different micronutrient mixes but including vitamin C and/or E gave inconsistent results regarding effects on MN and CA formation in PBLs (49,76,81). However, in a small intervention study of middle-aged men given vitamin C, vitamin E, carotene and selenium for 3 months, a significantly reduced incidence of CAs was reported, but no effect on MN formation was seen in the same study (103). Other published reviews of the effects of dietary intervention on DNA damage have concluded that there is insufficient evidence to draw conclusions regarding effects of vitamins C and E (135,136).

Fenech has reviewed the information on micronutrients on genome stability and also undertook an intervention study (vitamins A, C and E and zinc supplementation) from which it was concluded that micronutrients may have a possible impact on genomic stability, such as chromosome breakage and DNA hypomethylation (51,75,137).

It might be proposed that information regarding diet and micronutrient intakes during biomonitoring studies will aid in the interpretation of outcomes. However, apart from folate and vitamin $B_{12}$, it is unclear which dietary factors would significantly affect the outcome of biomonitoring studies of genotoxicity of chemical exposure on MN and CAs.

In summary, the available studies which use biomarkers of genotoxicity do not allow any clear conclusions regarding effects of diet and micronutrients (other than vitamin $B_{12}$ and folate) on oxidative DNA damage to be drawn, which might help with formulating further research in PBLs.

### *Disease conditions and infections*

A range of chronic illnesses have been identified as factors which impact upon MN or CA incidence in PBL. For example, Miglore *et al*. (138) found a significantly elevated comet response in PBLs from individuals with mild cognitive impairment (a clinical condition between normal ageing and Alzheimer's disease) and consider a possible association between oxidative damage and the onset of the disease.

Demirbag *et al*. (139) reported that the severity of coronary artery disease correlated with DNA damage using the comet assay, again possibly associated with oxidative damage.

There are some reports regarding the impact of bacterial or viral infections on MN or CA formation in PBLs. Both acute and chronic bacterial infections have been reported to be associated with increased MN and CA formation in PBLs (140–142). However, it is unclear whether this is a generic response to all bacterial infections involving a common mode of action (e.g. production of reactive oxygen species) which has been reported, for example, following infection by *Helicobacter pylori* in gastric epithelia (143) or whether there are differences in potency and mode of response between different bacterial infections. An increase in CAs in PBLs has been associated with viral infections (144), although there is general agreement that viral infections can induce non-specific cytogenetic damage in host cells (145).

A number of studies have demonstrated that oxidative DNA damage in PBLs was correlated with hepatitis B or C infection and the extent of DNA damage was proportional to the severity of infection and associated liver disease (46,47,146). It is postulated that the mode of action for viral-induced cytogenetic changes involves disturbances of the cell cycle induced by viral genes (144,145,147).

It is highly likely that any individual with clearly overt signs or symptoms of bacterial or viral infections would be screened out of a biomonitoring study, although screening would not necessarily exclude individuals with subclinical disease resulting from acute or chronic bacterial or viral infection. It would be expected that for widely prevalent infections, there would be similar numbers of individuals in exposed and control groups with ongoing or developing infection, but other factors such as the healthy worker effect or the onset of a contagious disease in a study population at a particular time might be relevant to the outcome of biomonitoring studies of chemical exposure and MN and CA formation. Neri *et al.* (32,33) concluded that screening for infections would be an important part of the design of genotoxicity biomonitoring studies in children.

Interestingly, although changes in core body temperature (hypo- and hyperthermia) are recognized as variables which impact on control incidence of MN in rodent studies (148), there do not appear to be any published studies that examined factors such as fever on background incidences of PBL genotoxicity biomarkers. Overall, it could be postulated that hyperthermia associated with an infection contributes to a transient increase in DNA damage in PBLs.

*Physical exercise; workload*

High-intensity physical exercise in untrained athletes has been shown to increase MN formation in PBLs whereas there was no reported increase in trained athletes (44,45). Similarly, Peters *et al.* (149) reported that prolonged treadmill running (for 2.5 h) did not affect DNA damage in PBLs from trained athletes, assessed by comet assay. No studies investigating physical exercise using CAs in PBLs were retrieved.

Several studies have been identified that support the hypothesis that intense physical exercise is associated with DNA damage, measured by using the comet assay in PBLs (150,151). Similarly, studies using 8-OHdG in urine also suggested that intensive physical exercise, particularly in untrained athletes, is associated with DNA damage (152,153). Kasia reported that moderate physical exercise reduced urinary excretion of 8-OHdG while physical labour (presumably high-intensity activity) was associated with increased urinary excretion of 8-OHdG (153).

The only occupational study of work activity and DNA damage retrieved related to riskshaw pullers in India and reported increased DNA damage in PBLs measured by the comet assay (154). This particular occupation is associated with strenuous physical activity. There is insufficient quantitative information regarding physical demand and level of training from these studies to draw any inferences regarding mild or moderate physical activity associated with work practices or with intermittent occupational physical activity to judge how physical workloads, work patterns or worker experience for many occupations might affect baseline MN or CA formation in PBLs. However, this would seem an area where further investigations and review are warranted.

*Body mass index*

A number of studies have addressed the association between body mass index (BMI) and genotoxicity biomarkers, with variable results. BMI was positively associated with MN formation in PBLs in a study of environmental exposure to benzene (52). Moreover, Demirbag concluded that there was a significant correlation between obesity and DNA damage using the comet assay in PBLs, but it is unclear how DNA damage in coronary artery disease, which is also a risk factor for MN formation, affected this finding (139). Collins found a correlation between BMI and DNA damage (using comet assay) in a small study of insulin-dependent diabetics (155). In addition, intervention studies in healthy smokers and non-smokers, using urinary 8-OHdG as a marker of DNA damage, noted an inverse correlation between BMI and urinary 8-OHdG and it was concluded that weight loss was associated with DNA damage (156,157). These studies are in contrast to the randomized intervention study undertaken by Heilbronn *et al*., which involved calorie restriction (25 or 12.5% with or without an exercise regime) for 6 months or a low-calorie diet (890 kcals) until 15% weight loss was achieved. In the study, all the intervention groups showed a significant reduction in DNA damage in PBLs as assessed by comet assay (158). In a further small study, self-reported moderate weight increase ($>5$ kg) and increased body fat in 17 non-obese individuals were associated with increased 8-OHdG levels in muscle tissue and also increased markers of inflammation (159).

Few studies were retrieved that investigated DNA damage related to BMI in children. Peng *et al.* investigated the oxidative DNA damage in adolescents of families with hepatocellular carcinoma, using 7,8-dihydro-8-oxoguanine (8-oxoG) as a marker of oxidative DNA damage in PBLs. A significant negative correlation between BMI and 8-oxoGP was reported, suggesting that BMI could influence levels of oxidative damage in adolescents (160). In contrast, Tondel reported that there was no statistically significant effect of BMI on urinary 8-OHdG in children living in urban areas of Belarus, compared with those living in rural areas (161).

Overall, these data suggest a complex interaction between BMI and changes in body weight with regard to biomarkers of genotoxicity in both adults and children. The potential impact of such factors on MN or CA formation in PBLs is unclear and thus there is a need for further research and systematic review of studies. For example, it is unclear what the significance of weight gain or loss in the period before inclusion in a biomonitoring study would be, even if there were no

recorded weight changes during the study. No equivalent data on CA formation in PBLs was retrieved.

*Genotype*

The objective of this section of the review is to identify whether there is evidence to indicate that the routine screening for genetic polymorphisms that affect background DNA damage should be considered for inclusion in biomonitoring studies of genotoxicity end points and chemical exposure rather than to undertake a detailed review of how genotype impacts upon biomarkers. Thus, polymorphisms which have significant effects on the background production of MN or CAs in PBLs are potentially relevant.

Iarmarcovai *et al*. recently published a thorough, systematic review of the literature pertaining to the impact of genotypes on MN formation. The authors examined how particular genotypes (in particular those affecting xenobiotic metabolism, DNA repair proteins and folate metabolism) may impact on chromosome damage and genome instability and discussed how these genetic variations may modulate the effects of exposure to environmental genotoxins. The heterogeneity reflecting multiple external and internal exposures and the large number of chromosomal alterations leading to MN formation suggest that the effect of genotype will be complex and particularly difficult to investigate (98).

Briefly, there is evidence that methylenetetrahydrofolate reductase (MTHFR) genotype is implicated in genomic stability and impacts upon the formation of methylenetetrahydrofolate from folic acid as it catalyses the conversion of 5,10-methylene-tetrahydrofolte into 5-methyl-tertahydrofolate, the precursor for methylation of homocysteine to methionine. The C677T polymorphism in MTHFR is associated with reduced activity (162). Two studies indicate an increase in MN in polymorphic TT individuals, which positively correlated with homocysteine levels (78,79,163). However, a larger population study of 191 individuals did not find any statistically significant differences in MN frequency between different MHTFR genotypes, although the association with higher homocysteine levels was observed (79).

Studies of DNA repair genes have also been highlighted as being of particular significance, for example a population study indicated that polymorphisms of DNA repair (ERCC2 751 Gin/Gin) significantly affected DNA damage in PBLs, as assessed by SCE, although MN and comet assays were unaffected (164). Although there is a biological rationale whereby polymorphisms of folate metabolism and/or DNA repair could affect the background rate of MN and CA formation in PBLs, the current evidence is not sufficient to draw a definite conclusion regarding routine incorporation of these polymorphisms in biomonitoring study designs.

In addition, the impact of glutathione *S*-transferase polymorphisms on genotoxicity induced by smoking was investigated and indicated a correlation between MN induction and tobacco smoke exposure and the combination of the genotypes *GSTM1* and *GSTT1*. However, the sample size was small (165). In another study, *GSTM1* and *GSTT1* polymorphisms were investigated together with potential confounding factors: it was demonstrated that *GSTT1*-null subjects had lower MN frequencies, but the same was not shown for *GSTM1*-null subjects (12,83).

The Committee on Carcinogenicity (COC; sister committee to the COM) has reviewed the design of gene–environment studies regarding effects of polymorphisms on cancer risk (166). The committee reached a number of overall conclusions that have generic relevance to the design of biomonitoring studies of genotoxicity and chemical exposure and are therefore pertinent to the current review. First, inclusion of polymorphisms needs to be considered on a case-by-case basis and that one important consideration is that there should be an *a priori* hypothesis (biological rationale) for studying the polymorphism. The COC recommended a tiered approach, initially looking at strength and establishing the credibility of a link and that the assessment should ideally also include information on phenotype as well as genotype.

A number of research groups have begun to undertake pooled analyses of biomonitoring studies of genotype to provide improved power to detect significant effects on MN and CA formation in PBLs. Mateuca *et al.* have recently described a significant increase in MN with smoking in occupationally exposed individuals of Arg/Gln XRCC1 genotype. This evidence suggests effect modification of smoking on MN formation by a DNA repair genotype, but would need independent confirmation and studies of phenotype before a definite conclusion can be drawn (167). Moreover, Gerogiadis *et al.* (108) reported a significant effect modulation of CYP1A1*2A genotype on environmental tobacco smoke-induced formation of CAs in PBLs.

Overall, the data for MHTFR, polymorphisms of DNA repair, xenobiotic activation and folate metabolism were not judged to be adequate enough to recommend routine inclusion in biomonitoring studies of genotoxicity.

## Methodological variation (between and within laboratories)

Methodological variables and interindividual variation in the scoring and assessment of MN formation in the CBMN assay are also factors which impact on the robustness of biomonitoring studies and the ability to directly compare studies from different laboratories.

As part of the HUMN project, scoring criteria were closely defined (168) and a large trial examined interlaboratory variation in analyses and staining of slides (11). Background and radiation-induced CBMN frequencies in PBLs were reported, using slides prepared from one individual (male aged 30 years) with *in vitro* exposure to gamma rays. Those laboratories with two scorers ($n = 10$) showed interscorer differences $<25\%$. There was more heterogeneity in laboratories with three or more scorers ($n = 4$). The authors suggest that the estimated intrascorer median coefficient of variation could be used as a standard for quality acceptance criteria for future studies. These results were consistent with an earlier population study of 126 males and 166 females that reported significant interscoring and sampling error in the determination of CBMN in PBLs (48) Similarly, a marked intraindividual variation (sampling error) of MN, measured using the CBMN assay in isolated lymphocytes, was noted in a small population study ($n = 56$) of individuals living near to a uranium plant. Such variation was greater than the interindividual variation (71).

In a recent study investigating the use of the CBMN in an epidemiological study of radiosensitivity in cancer patients and controls, the authors reported that there was a clear decline in the maximum MN frequency for all scorers approximately half way through the 18-month period. When using automated counting techniques, more consistent results were reported as

similar decreases in MN frequency were not observed. It was suggested that an inadvertent switching in scoring criteria might have been responsible and that the use of reference slides was warranted throughout studies where cultures and MN determinations would be undertaken over an extended period of time (169). In contrast, another study reported that there was no evidence for intraindividual variation over time in 53 volunteers from which CBMN in PBLs were determined four times equally spaced over a year (48).

Automated image counting of MN in PBLs has been used by a number of research groups and procedures have been developed which can give reproducible results, although the data may differ from concurrent manual scoring (170–172). Automated procedures for evaluation of the end points used in the 'cytome' assay are currently being developed which will include the evaluation of the sources of variation, for example between laboratories and scorers (30).

With regard to CAs in PBLs, using experimental and/or photomicrograph data from metaphase analyses, interlaboratory trials report considerable variation in results for a number of reasons including individual scorer selection of metaphases and scoring of aberrations with a low frequency (in particular unstable aberrations) was recorded (17,67,173).

Overall, it is suggested that even after standardizing culture and scoring conditions it would be necessary to calibrate scorers and laboratories if the CBMN assay data are to be compared among laboratories and populations. The COM concluded that there is a need to calibrate scorers to include predetermination of cell selection and scoring criteria and also standardization of the scoring procedure at the start of the study and implement evaluation and assessment of reference slides during the conduct of biomonitoring studies using the CBMN assay in PBLs. Subsequent retraining of analysts to ensure consistency may be necessary during the course of a study. Moreover, the automation of MN assessment could help considerably reduce variation in the scoring in MN assays (174).

## Discussion: sources of variation in biomonitoring studies of genotoxicity using MN and CAs in PBLs

The current review aimed to evaluate factors which might affect the formation of MN and CAs in PBL's in man and thus impact on the intrinsic variability in control ranges of these biomarkers as identified in our review of pesticide applicators (1). We have considered a large body of data and evaluated the evidence for the impact of both host factors and methodological factors on the variation of background incidence of MN and CA in biomonitoring studies utilizing PBLs, and how these two sources of variation might impact on the design and interpretation of studies. The majority of the reliable studies available were conducted using the CBMN assay which reflects the recent move away from using CAs as a genotoxicity end point in biomonitoring studies.

Overall, there were a number of factors for which we recommend there was strong or sufficient evidence to suggest an association with genotoxicity end points in PBLs, such as age, gender and smoking. It is therefore suggested that it would be prudent to routinely incorporate such factors when conducting biomonitoring studies.

Age and gender were the host factors identified most frequently as impacting on background incidence of biomarkers of genotoxicity and have previously been highlighted as being of particular significance when considering confounding factors when designing biomonitoring studies (10,48). Clear correlation of increasing incidence of both MN, and to a lesser extent CA, with increasing age have been demonstrated in most studies and it has been suggested that this is a consequence of enhanced chromosomal instability with age, which manifests as numerical changes or chromosome loss (120). It is also well documented that baseline MN frequency is consistently higher in females than in males although there is less evidence that CAs are similarly affected. Interestingly, the sex trend is more pronounced with increasing age and it is postulated that this may be a consequence of altered hormonal status and loss of sex chromosomes in women (19,175). The significance of the variation observed due to these two factors was addressed by Fenech who indicated that 25th and 75th percentiles of MN frequency varied 1.4- to 2.3-fold for a subset of healthy individuals depending on age and gender (48). This observation was one that contributed to realisation of the need for a collaborative project to evaluate variables and thus the initiation of the HUMN project.

The impact of micronutrient status was recognized and has since been investigated by Fenech and Rinaldi (53). The logical causal correlations between vitamin $B_{12}$, folate, homocysteine and MN formation are robustly supported by data from dietary intervention, supplementation and questionnaire type studies (25,51,75,77). This line of investigation has instigated informative speculation with regard to the importance of micronutrients in genome stability and how nutritional intervention should be considered as part of disease prevention strategies (51,176). However, the most appropriate approach regarding how vitamin $B_{12}$ and folate status can be accounted for in the design and interpretation of biomonitoring studies is less widely discussed. Ideally, these factors clearly contribute to the formation of MN and CAs and therefore need to be controlled for in biomonitoring studies. We suggest that the most practical way of incorporating this would be to measure endogenous vitamin $B_{12}$ or folate levels at the time of blood sampling for the CBMN assay and then include the data in a multivariate model rather than trying to match groups for nutrient intake.

In agreement with the review of the effect of smoking habits on the frequency of MN undertaken as part of the HUMN project (12), we concluded that despite lack of consistent evidence for the impact of smoking, it should be routinely evaluated to the same extent as factors identified as strong or sufficient evidence for the induction of MN and CAs in PBLs. Additional research might include better measures of the cumulative effect of smoking, the possible interaction between genotype and smoking and possible role of antioxidant status.

A number of other risk factors have been identified as having weak or insufficient evidence for an association with genotoxicity end points, including alcohol consumption, micronutrients (other than B12 and folate), exercise, infection and early stages of chronic diseases, BMI and genotype, although comparatively little data were available. Such risk factors were not evaluated in the HUMN project. Herein, we have reviewed the literature and identified many factors which, at present, are principal areas of research interest and thus discussion of their potential impact upon the outcome of biomonitoring studies was not considered during the conduct and analysis of the individual investigations. However, it is clear that the factors we highlighted; namely, some genetic polymorphisms, disease and infection, antioxidant

micronutrients, physical activity and BMI could all contribute to the results of genotoxicity biomarker investigations. Therefore, it is recommended that investigators should be aware of the potential impact of these contributory factors on study outcomes and if appropriate, they should be accounted for on a case-by-case basis. Furthermore, the interplay between these potential confounding variables is likely to be complex, and thus controlling for them is likely to present considerable problems. However, if study investigators are alerted to such factors and thus are able to take them into consideration, study designs will improve.

Investigation of genetic polymorphisms such as those that impact upon DNA repair or xenobiotic metabolism is clearly an area from which an understanding of variability will emerge. It is possible that polymorphisms may modulate the effect of smoking or potential effect of drinking alcoholic beverages on DNA damage in PBLs. We recommend that any studies take into account the advice from COC to derive suitable prior hypotheses for study and include information on genotype and phenotype (166).

Substantial analyses were conducted as part of the HUMN project to establish the relative importance of individual factors on the overall variability of background incidence of biomarkers and thus provide a basis for evaluating the impact of these modulating factors (10). A model was designed to quantify the sources of variability in the data analysed as part of the HUMN project. It was acknowledged that many of the factors affecting the formation of MN in PBLs were closely related and the analysis was presented in terms of four general sources of variability, which in order of priority were methodological parameters > criteria for identification of BN cells scoring MN = exposure to genotoxic agents > host factors (age, gender, etc.). The total variability explained by this model was 75%, although micronutrient status was not included in the analysis.

Standardization of methodological protocols has been addressed in the IPCS guidelines for monitoring genotoxic effects of carcinogens in humans (177). It was these procedures which were used as the basis to reject or accept the studies investigating genotoxicity of pesticides in our original review (1). It is acknowledged that limitations in the statistical approaches used may have resulted in the reporting of weak or false positives, as some increases determined to be statistically significant were <1.5-fold in magnitude. It is therefore plausible that methodological factors such as interscorer variation in MN scoring could wholly account for the reported results in a number of the pesticide studies.

It is possible that automation of MN assessment could help considerably to reduce variation in the scoring in MN assays. The development of new approaches to investigating MN such as the cytome assay (30) and the reticulocyte MN assay (178) may offer more appropriate biomonitoring approaches. The use of MN in reticulocytes may offer the prospect of investigating DNA damage occurring a short period after exposure and in one preliminary study of cancer patients, a 10- to 20-fold increase 1–4 days after chemotherapy or radiation treatment was reported indicating the potential for a very sensitive response to exposure to genotoxicants (77). However, to date, there are currently insufficient data to assess the utility of using reticulocytes. Iarmarcovai has assessed the utility of scoring centromere-positive and -negative MN and those with two or MN (129). They conclude that the routine use of such an approach provides additional information on the mechanism of action of genotoxic agents, and although we concur with this concept, we are of the opinion that the additional work involved is only warranted if the chemical under investigation is expected to induce aneuploidy or chromosome loss (for example, carbendazim or benomyl, aneugens has an affect highlighted in our previous review 1).

It is apparent that the comet assay is increasing in popularity as a method to evaluate DNA damage in occupational and environmental exposure biomonitoring studies. From the limited assessment of the literature conducted here and the studies evaluated as part of the pesticide applicators review (1), it is clear that some of the factors impacting on MN and CA also need to be accounted for when designing comet studies (126). As its utility is expanded and developed, it is hoped that investigations of the variability of the background levels of DNA damage will take place concomitantly.

With regard to the evaluation of genotoxicity biomarkers in children, little information was retrieved. There is a basic need to collect information on the risk factors affecting MN and CA formation in PBLs in children (i.e. from 0 to 18 years). It is notable that the only systematic review retrieved did not find an association between MN formation and age (0–18 years) or gender (32). Overall, data from a well-designed prospective study investigating age, gender and foltate/vitamin $B_{12}$ on MN and CA formation in PBLs from children would provide valuable information.

Approaches to the assessment of exposure to environmental chemicals or genotoxins under investigation in biomonitoring studies are outside the scope of the current review. However, during our review of genotoxicity in pesticide applicators, we found that even when information on pesticide exposure was collected through questionnaire data, it was usually not possible to extract detailed information on formulation constituents or to quantify exposures or exposure duration to pesticide active ingredients. This severely limits how the studies can be interpreted as in the event of a positive response, it is unlikely that an effect can be assigned to a single chemical. We suggest that in order to improve study designs, a number of factors should be considered, in addition to providing evidence for a potential exposure to an *in vivo* genotoxin, including setting an *a priori* hypothesis to be tested, collecting good exposure data, stratifying groups according to exposure levels, using appropriate power calculations before initiating biomonitoring studies, and collecting information on appropriate background risk factors.

It is hoped that increasing knowledge in this area will lead to improved study designs where 'risk factors' can be accounted for when selecting and allocating study subjects, and also as an aid in interpreting the significance of very small increases (ca. 1.5-fold) in genotoxicity biomarkers that are often reported in these studies. It is evident that understanding and knowing how to account for such variables is critical in good study design and experts within a centre of excellence are most likely to be able to perform a function such as this.

Within the UK, there is no central body of information on background risk factors for genotoxicity indices used in biomonitoring studies and therefore we suggest there is a clear need for a centre of excellence to be developed. The value of undertaking such a venture would be that it might be possible to base policy decisions (such as decisions to reduce exposure or advise on chemical incidents) on good quality biomonitoring studies but at present many such studies are not acceptable for use in policy-based decisions.

In conclusion, we are aware that genotoxicity biomonitoring studies using MN or CA formation in PBLs are widely undertaken. Our review highlights the risk factors for MN and CA formation in PBLs for which there is strong or sufficient evidence for an effect but importantly we also show that there may be a range of other potential risk factors for MN and CA formation in PBLs where the evidence is less convincing (i.e. weak or insufficient) and where there is need for further research. In particular, we highlight the paucity of information on children. There is a clear need for more collaborative work and the establishment of a centre of expertise in the UK on genotoxicity biomonitoring studies. In our view, many of the biomonitoring studies using genotoxicity end points currently available are not of sufficient quality to be used in risk assessments or to derive risk management strategies.

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