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MUT/06/01

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

**Background variation in Micronuclei in peripheral blood lymphocytes;
Discussion paper on risk factors.**

Introduction

1. The COM recently identified the need for further evaluation of the factors affecting the formation of micronuclei in peripheral blood lymphocytes (PBLs) before the results of biomonitoring studies of environmental exposure to chemicals could be evaluated. (see statement on pesticide applicators <http://www.advisorybodies.doh.gov.uk/pdfs/pesapp.pdf>)
2. This discussion paper overviews the published literature specifically in relation to studies which provide information on the background variance of MN formation in PBLs.^{1-26,34} The review includes studies investigating the development of the cytokinesis block MN assay (CBMN assay) including measuring MN formation in mononucleated and binucleated cells and the identification of numerical chromosomal changes in the CBMN assay, and the effects of age, smoking, sex and micronutrients on CBMN. A small number of studies which primarily investigated MN formation in disease processes such as cardiovascular disease were also reviewed. A number of other studies reported data on the influence of methylenetetrahydrofolate reductase (MTHFR) genotype on the formation of MN in PBLs and the effects of cofactors for MTHFR activity on MN formation.
3. An important set of retrieved papers came from the Human Micronucleus project (HUMN) which was initiated in 1997.²⁷⁻³² The idea for HUMN was conceived by Dr Stefan Bonassi (Italian National Cancer Institute) and Dr M Fenech (CSIRO Division of Human Nutrition, Adelaide, SA Australia). Following an announcement there was direct approach to scientists and a workshop held during the ICEM in Toulouse 1997. Forty-five scientists expressed an interest in participating. The main objectives were;
 - i) A comparison of the main culture methods to investigate the effect of such methods on base-line MN frequency.
 - ii) A comparison of results accumulated for base-line MN frequencies from various laboratories. (Information on the normal range for different laboratories/countries).

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iii) A prospective study linking the accumulated MN index data from each laboratory to provide

4. A total of 32 publications which contributed information on background variance of MN in PBLs were identified through PubMed literature searches and cross referencing retrieved papers. Additional papers which contribute to the discussion including a meta-analysis of MN levels in children were also identified.³³ number of other studies which report effects of exposure to both mutagenic and non-mutagenic chemicals and MN formation in disease states are also briefly considered where these help to provide information on the variables which affect the background MN frequency in PBLs in humans.³⁶⁻⁴⁰ However there hasn't been sufficient time during the drafting of this discussion paper to explore all potential aspects of MN formation in PBLs fully, e.g. aspects of a vegetarian diet which might influence MN formation.⁴⁰ The primary objective of this discussion paper is to identify risk factors for MN formation in PBLs which should be considered in any adequately conducted biomonitoring study of chemical exposure. A further objective is to assess whether the influence of such risk factors can be quantified. The evaluation reached in this review is briefly compared with the guidance published by Albertini in 2000.³⁵
5. A short discussion of risk factors for MN formation in PBLs is given below. A summary of identified papers can be found in Annex 1 (published papers) and Annex 2 (data from the HUMN project) and the Albertini guidance (Annex 3). An appended paper on transient increases in MN due to exposure to TCDD and other dioxins³⁶ is appended as Annex 4. A number of relevant papers from the published literature are appended as Annex 5. Most authors report the MN frequency in CB peripheral blood lymphocytes (No of micronucleated cells/1000 CB PBLs; MN ‰).

Overview of risk factors for MN formation in PBLs

Interindividual variance

Effect of age

6. A clear correlation between MN frequency in PBLs and age of donor (adults from 16 y+ up to and including centenarians) has been reported in several population and vitamin/micronutrient intervention studies where it has been investigated^{3,5,11,16,25}, although no correlation was found in some population studies which have included group sizes of up to 200^{2,4,6,20}. The proportion of BN cells reduced with age.¹¹ The data from the HUMN project included information from 25 databases (6583 individuals, group sizes 1-1637, mean 263) found that, out of the factors evaluated, age (based on data from 4899 un-exposed individuals) had a prominent

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effect on MN frequency. In general a stronger effect of age has been documented in women. There was no evidence from the HUMN project that the increase in CBMN frequency levelled off in the oldest age group.³² Data from Fenech's laboratory reported that the slope of the regression line for the relationship with age is 0.314 ($P < 0.0001$) and in females the slope is 0.517 ($P < 0.0001$).¹⁰ In a population study of 196 volunteers, Fenech reported that the CBMN frequency in PBLs in individuals aged 60+Y was 359% higher than the MN frequency in the 20-29 y age group.²⁶ Fluorescence-in-situ-hybridisation (FISH) identification of X and Y chromosome loss in PBLs using interphase whole blood cultures was undertaken as part of a study of ageing on MN formation in a group of 33 males and 22 females (38, aged 16-63, and 16 centenarians).¹⁶ The authors reported that chromosome loss is an important factor in determining the increase in MN in PBL associated with ageing. This conclusion was based on an analysis of chromosome loss in centenarians compared to a small number of controls (3/sex) aged about 30y.

7. The meta-analysis of studies involving children (0-18y) also identified a clear effect of age on the formation of MN within the individuals studied (based on 13 studies involving 440 individuals).³³
8. A population study of effects of ageing and gender using data derived for 791 individuals for whom PBL MN data were available between 1984-1994 reported an age related effect for MN in BN cells but not in mononucleated PBLs. However in review by Kirsch-Volders and Fenech published in 2001 which included some additional data from Dr Elhajouji on the distribution of MN in mononucleated and BN cells from 240 donors, the frequency on MN in both mononucleated and BN cells was age-dependent.¹⁸
9. Thus overall there is evidence for an increase in MN frequency in PBLs with age, both in males and females, which is apparent in all age groups. The effects is in part is due to numerical changes in chromosomes. There is inconsistent evidence as to whether an age related effect of MNs also occurs in mononucleated PBLs.

Effect of gender

10. Statistically significant higher MN frequency in CB PBLs was reported in females in a number of population, occupational and vitamin/micronutrient intervention studies.^{3,4,12,21,26} A study of 54 age matched married couples reported the CBMN frequency in PBLs was 1.4x higher in females. In a study of 791 individuals (aged 20-77 y with analyses undertaken at three laboratories), the increased CBMN frequency was most evident between 30-59y.¹² A number of studies have not reported an effect of gender.^{2,6,18} It is noted that two of these studies also failed to report an effect of age.^{2,6} The HUMN project reported that the effect of gender on CBMN frequency in PBLs was less evident than age with 9 out of the 25

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databases evaluated reporting a higher MN frequency in males. Overall there was a 19% (95% CI 14%-24%) higher MN frequency reported in females. The authors noted that the inclusion of two studies with higher MN frequency in males had focused on children where the gender effect was not expected to be so apparent.²⁹

11. Thus overall the evidence supports a higher background MN frequency in PBLs in females of approximately 20-40% which is most evident between 30-59y of age.

Effect of smoking

12. Fewer published studies of background MN frequency in CB PBLs have reported on the effect of smoking. Two studies retrieved have reported that tobacco smoking increased CBMN frequency.^{1,19} The population study published by Fenech et al in 1995 evaluated CBMN frequency in PBLs from 225 donors of which only 29 were active smokers (no data from 69 individuals). A statistically significant higher MN frequency was reported for high levels of smoking but not for duration of smoking.¹ Higher MN numbers in PBLs (total MN/500 cells/individual) was reported in a small vitamin C and E intervention study in smokers, although this may in part be due to lower levels of plasma vitamin C and E in these individuals.¹⁸ (see Annex 1 for detailed summary) A number of other studies which investigated smoking failed to find an association with CBMN frequency in PBLs.^{2,17} Lower levels of MN frequency were documented in smokers in one study.⁶ A study of 24 databases was undertaken as part of the HUMN project.³² This comprised 5710 subjects, with 3501 non smokers, 1409 current smokers and 800 former smokers. The authors suggested there was a small decrease in the MN frequency ratio in current smokers (FR= 0.97 (95% CI 0.93-1.01), and in former smokers (FR= 0.96, 95% CI=0.91-1.01) when compared to non smokers. MN frequency was not influenced by the number of cigarettes per day among subjects in the HUMN project exposed to genotoxic agents. In non exposed smokers, there was a significant increase in MN frequency at ≥ 30 cigarettes/day. (FR = 1.59 (95% CI 1.35-1.88) (adjusted by sex, age, calendar year of test, exposure to genotoxic agents, and laboratory.) The authors caution against excluding data from smoking from biomonitoring studies, particularly with regard to possible interaction between smoking and chemical exposure and also with regard to heavy smokers.
13. Thus overall, the effect of tobacco smoking on CBMN frequency in PBLs appears to be only evident at high levels of smoking (>30 cigarettes/year) and is possibly confounded by nutrition in smokers. (A review of nutrition in smokers is outside the scope of this review, but there is evidence available to indicate altered vitamin requirements (e.g vitamin C and E) in smokers.^{41,42})

Effect of diet

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14. Two studies have specifically investigated whether a vegetarian diet has an effect on MN in PBLs. A small comparative study of 24 vegetarians (13 men, 12 women) matched for age and sex with 24 volunteers with a traditional diet (Slovakia).⁴⁰ There were 13 lacto-ovo vegetarians (8 women, 5 men who consume both dairy products and eggs) with an average length of vegetarian diets of 10.8y (5-26y), and 11 lacto-vegetarians (5 women, 6 men who consume dairy products but not eggs) with an average length of vegetarian diet of 8.2y (3-15y). There were no differences between vegetarians and the traditional diet regarding MN or chromosomal aberrations in PBLs. The authors did report a higher level of oxidative DNA damage in PBLs (strand breaks and oxidised purines, $P=0.005$) in the traditional diet.⁴⁰ Fenech reported on the results of an analysis of CBMN in lymphocytes where data were compared between vegetarians/vegans and non vegetarians. Although there were significant differences in folic acid, vitamin B₁₂ and C status between vegetarians and non-vegetarians, there was no significant difference in MN frequency.¹⁰ One small study of 10 individuals reported that a high level of CBMN was associated with a high level of lipid peroxidation in one individual. The authors suggested that dietary fat might be factor for increased MN in PBLs worthy of further investigation.¹³
15. Thus the influence of a vegetarian diet has been investigated in a relatively small number of individuals. There is no evidence to indicate that a vegetarian diet has an effect on the background MN frequency in PBLs. There are no data available from the HUMN project on the influence of diet on background frequency of MN in PBLs.

Effects of micronutrients

16. A number of studies which include population studies of plasma/serum levels of folate, vitamin B₁₂, and homocysteine have evaluated the influence of these factors on MN in PBLs. A number of studies have investigated the association between plasma vitamin C and MN in PBLs.
17. In brief, folate (FA) (following conversion to methyltetrahydrofolate (MF) via a number of steps including the reduction of 5,10-methylenetetra hydrofolate to 5-methyltetrahydrofolate by methylenetetrahydrofolate reductase (MTHFR)) acts as a methyl donor for conversion of homocysteine to methionine. Methionine is needed for the formation of S-adenosyl-methionine which is involved in DNA methylation. This is compromised when vitamin B₁₂ (a co-factor for methionine synthase) is deficient. Levels of homocysteine subsequently increase. Methyltetrahydrofolate (MF) also donates methyl groups for thymidine synthase-mediated

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conversion of dUMP to DTMP which are needed for DNA repair and synthesis.

18. In addition intervention studies have also been undertaken to investigate the influence of antioxidant vitamins on MN in PBLs. (Some additional studies on the influence of micronutrients on the conduct of the in-vitro CBMN assay are considered below in the section on Assay variables, para 24 below). There are no data available from the HUMN project on the influence of micronutrient status on the background frequency of MN in PBLs.

19. A brief tabulation of the main results from the available studies is given below; (more details are presented in Annex 1). No data were available from the HUMN project.

Study	Main findings (Correlations)	Comments
Population study of 113 males and 52 females (non-smokers, aged 20-87) ³	Positive for plasma vitamin C and negative for vitamin B ₁₂ (males). Negative for combined folate and vitamin B ₁₂ in females.	The authors suggest that MN frequency in human lymphocytes can't be assessed without data on vitamin C, folic acid and vitamin B12 status
4month intervention using vitamin A,C,E, folic acid in individuals aged (23-30 (13), 56-83 (35), 63-82 (12)) ⁵	Consumption of antioxidant vitamins decreased MN by 25% in older donors, no effect on younger donors.	Information on vitamin status (e.g deficiency) not provided.
8 week intervention in 20 individuals (matched for age, smoking chronic illnesses) with 20 controls, using vitamin E at 5, or 50X RDI. ⁸	No effect on MN frequency.	Relatively small study. Evidence suggests vitamin E is not a significant modulating factor for MN in PBLs.
Study of vitamin deficiency in 64 healthy men (50-70) and intervention study of folate (3.5 or 10x Australian RDI) in 20 males for periods of 8 weeks (total intervention 16 weeks). ⁹	MN was positively correlated with plasma homocysteine and negatively correlated with serum vitamin B ₁₂ . There was no correlation with serum or red blood cell folate.	Before supplementation, 56% of men had abnormal levels of folate, vitamin B ₁₂ , or homocysteine. Overall it was concluded that elevated homocysteine in the absence of vitamin deficiency, and low but not deficient vitamin B ₁₂ status were important risk factors for MN formation in PBLs.
Intervention study in 9 postmenopausal women to investigate induced moderate folate deficiency and repletion effects on PB MN. Study investigated kinetochores positive and negative MN ¹¹ .	Boderline statistically significant reduction and increase in MN in PBLs during depletion and repletion. These changes were just outside statistical significance, which was achieved if the data were expressed as total MN/1000 cells. Both kinetochores –positive and kinetochores-negative MN (expressed as total/1000 cells) were increased after depletion, but after repletion only the change in kinetochores positive MN was statistically significant.	The main variables affecting MN during the depletion phase were negative associations with vitamin B ₁₂ (P=0.02 and for MN+ P=0.009) level, plasma folate level (P=0.06). During repletion a positive association was seen with vitamin B ₁₂ (P=0.02-0.03) while the changes in MN+ and B ₁₂ were negatively correlated. The authors observed that the subjects initially had quite different MN, folate, B ₁₂ and homocysteine levels, the difference in micronutrients decreased during the study while MN variability did not change appreciably. The authors noted that individual plasma folate measurements and MN frequencies were not associated at any collection time.

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<p>Intervention study in smokers (>20/d) of vitamin C (1g/d, 7 days) and vitamin C and E combined for next 7 days(0.34 g/d) (n=12, aged 19-33y).¹⁹</p>	<p>Baseline MN levels (No/500 BN cells) were higher in smokers than age, sex matched non-smokers. Intervention led to increased plasma vit C and E in smokers, (but not in non-smokers). MN were non-significantly significantly reduced during vitamin C and significantly reduced during the combination intervention period.</p>	<p>MN increased in washout period.. Smaller non significant changes seen in non-smokers in response to intervention.</p>
<p>Population study of 191 individuals (140 males, 51 females, mean age 41.9y)²²</p>	<p>CBMN in isolated lymphocytes reported that plasma folate was positively correlated with MN. Plasma homocysteine was negatively correlated with MN. There was no correlation with plasma vitamin B₁₂.</p>	<p>Authors also reported some limited evidence for genotype status of methionine synthase reductase being associated with DNA damage.</p>
<p>Study of 46 patients with coronary artery disease.²³</p>	<p>CBMN frequency (whole blood cultures) was negatively correlated with plasma vitamin B₁₂ concentration and negatively correlated with plasma homocysteine concentration.</p>	<p>Authors acknowledged the limitations of the study (small subgroups studied)</p>
<p>Population and Intervention study in approximately 90 individuals with ACEZn (6 months). (approx 90 individuals, age matched controls). Food Frequency questionnaire administered.²⁶</p>	<p>Increasing vitamin E, retinol, folate, preformed nicotinic acid, and calcium were associated with dose-related reductions in MN frequency (-28 to -49% highest v lowest tertile). B-carotene was associated with a more modest reduction in MN frequency (-18%). In contrast riboflavin and panthothenic acid intakes were associated with increases in MN frequency (41% and 69% respectively (lowest v mid tertile, effect not so evident at highest tertile). An increase in MN frequency was also documented at the highest biotin tertile intake (65%).</p>	<p>In the intervention study, dietary administration of ACEZn for 3 months resulted in a statistically significant increase in plasma vitamins/micronutrients. At the end of the intervention period there was a non significant drop in MN frequency in the control group and a larger significant decrease (25%) in the supplemented group relative to baseline figures. The final net effect after adjustment for individual characteristics, dietary habit and baseline values was a significant 13% reduction in MN frequency (95% CI= -1% - -24%)</p> <p>The authors considered the data on folate was consistent with previous studies. The finding of an association between calcium and MN frequency had not been previously reported. The authors noted that in a previous study, they had not seen any correlation between plasma vitamin E and MN but considered an effect occurred when supplementation above the RDA was used.</p> <p>The results of this study support the view that micronutrient status affects MN frequency</p>

18. It is difficult to assess the available data drawn from a wide variety of study designs (population and intervention studies) with variable data available on the baseline nutritional status of the individuals examined and a number of approaches used to ascertain endogenous levels of micronutrients.

19. Overall the data are consistent with endogenous levels of vitamin B₁₂, folate and homocysteine affecting the background MN frequency in PBLs. The magnitude of the effect of endogenous folate and vitamin B₁₂ on MN cannot be quantified in relation to the effect of age/gender. It is suggested that micronutrients are important in the evaluation of chemical exposure-response biomonitoring studies of MN frequency in PBLs.

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20. There are also some data from population and intervention studies to suggest that other micronutrients, in particular endogenous levels of vitamin C also affect MN frequency. The most recent publication retrieved by Fenech et al²⁶ also reports dietary intake data and an intervention trial with ACEZn to suggest that micronutrients which may be involved in maintaining oxidant status and DNA integrity (e.g niacin) may also affect the background MN frequency in PBLs. However overall, there are insufficient data (for example from a number of well control blind intervention studies) to draw definite conclusions on the significance of these micronutrients for background MN frequency in PBLs. It is noted that the overall effect of ACEZn supplementation was an approximate 13% reduction in MN frequency in PBLs.

21. Toxicological data on a range of vitamins and minerals were evaluated by the U.K. Expert Group on Vitamins and Minerals which considered the Safe Upper levels for Vitamin and Minerals. However, this review did not extend to the influence of micronutrients on the background MN frequency in PBLs.⁴³

Intraindividual variance

22. There is evidence that Methylene tetrahydrofolate reductase (MTHFR) genotype may affect the formation of Methylene tetrahydrofolate (MF) from folic acid (FA). Thus wild type MTHFR C677T polymorphism TT individuals have lower MTHFR activity than wild type CC individuals. A small study of 46 individuals with coronary artery disease found that homocysteine levels were higher in TT individuals compared with CT and CC genotypes and that the MN frequency in PBLs was statistically higher in TT genotype compared with CC to CT genotypes. 15.7 ± 2.4 , compared to 8.9 ± 1.7 and 9.9 ± 0.8 , ($P=0.02$).²³ A larger population study of 191 individuals did not find any statistically significant differences in MN frequency between different MTHFR genotypes.²² Kimura et al undertook *in vitro* CBMN assays in 9 day lymphocyte cultures from donors who had been genotyped for methylene tetrahydrofolate reductase (MTHFR) (wild type CC ($n=7$), and seven homozygous TT individuals with lower MTHFR activity c677T polymorphism). MN frequency was 21% higher in TT cells than CC cells ($P<0.05$).²⁴ Methionine synthase reductase (MTRR) polymorphism was found to affect approximately 15% of the background variance in MN in one small study.²²

23. Thus there is some limited evidence to suggest that MTHFR polymorphism affects the background MN frequency in PBLs. One author has suggested that the impact of MTHFR polymorphism is less than the availability of folic acid in *in-vitro* test systems for CBMN in isolated lymphocytes.²⁴

Background variation in MN frequency in PBLs due to CBM assay.

24. A large interlaboratory trial was undertaken as part of the HUMN project to examine interlaboratory variation in analyses and staining of slides reporting background and radiation induced CBMN in PBLs using slides

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prepared from one individual (male aged 30y) with in-vitro exposure to gamma rays.³⁰ Data from 33 laboratories were available. The authors assessed the MN frequency in control and following exposure to radiation with respect to the total number of slides scored by each scorer over lifetime and reported that experienced technicians had a better capability to discriminate the effect of irradiation. Those labs with two scorers (n=10) showed inter-scorer differences of <25%. There was more heterogeneity in labs with 3 or more scorers (n=4). The coefficient of variation (CV) for between spot estimates was 14% and 11% at 1 and 2 Gy, but was 29% in controls. All labs found an increasing MN frequency by irradiation dose. The effect for 1 Gy varied from 7.4-29.2 fold, (50% most frequent estimates were between 10.7-16.6). At 2 Gy the range was 16.0-60.7 fold with 50% most frequent estimates between 28.3-46.4. The authors concluded the estimated intra scorer median coefficient of variation could be used as standard for quality acceptance criteria for future studies. The results suggested that even after standardising 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. These results were consistent with an earlier population study of 126 males and 166 females undertaken by Fenech et al¹⁰ which reported significant interscoring and sampling error in the determination of CBMN in PBLs. However there was no evidence for intra-individual variation over time (in a study of 53 volunteers with CBMN in PBLs determined four times equally spaced over a year).¹⁰

25. Raddack et al⁴ reported a marked intra individual (sampling error) variation greater than the inter-individual variation in a small population study where 20 samples of 100 cells from each individual (n= 56 living near to a uranium plant and 56 controls) were scored using the CBMN assay in isolated lymphocytes.

26. There are limited data from *in-vitro* studies to indicate that the level of folate in the test system may affect the background rate of aneuploidy and MN formation.^{25,44} Methionine dependency of cultures was reported to vary considerably between individuals, but did not affect the background MN frequency in PBLs. The concentration of cytocholasin B was not reported to be a significant influence on determination of CBMN in PBLs.

Discussion: Impact of factors affecting CBMN in PBLs.

27. A model based on the data from the HUMN project using a negative binomial function of probability has been used to attempt to quantify the sources of variability in the data analysed as part of the HUMN project. It was acknowledged that many of the factors were closely related and the analyses was presented in terms of four general sources of variability, namely,

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| i) host factors (age, gender, continent (surrogate for ethnic group) | 31% |
| ii) exposure to genotoxic agents | 45% |
| iii) methodological parameters | 65% |
| iv) criteria for identification of BN cells and scoring MN | 47% |

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28. The total variability explained by this model was 75%. The sum is greater than 100% as the parameters evaluated were closely correlated and had been combined for ease of assessment. The HUMN project did not evaluate the influence of diet or micronutrients probably because there were too few studies with appropriate data in a form which could be analysed. It is not possible to quantify the relative effect of micronutrients compared to age/gender on the background frequency of Mn in PBLs. It is not possible to identify all the potentially relevant micronutrients which might affect MN formation.

29. The information summarised in this paper is consistent with the general advice given by Albertini et al³⁵ on the conduct of MN biomonitoring studies (Annex 3), although it is possible that more information on micronutrients and assay aspects such as the influence of scorer are now available.

30. The magnitude of effects reported in studies of cytostatic medicines in patients undergoing chemotherapy and in nurses administering such medicines suggested relatively small increases in the MN frequency (ca 2 fold). Members were surprised at the small magnitude of the increase, given the potential exposure to mutagenic substances. Members are also asked to consider a recent paper (Valic et al³⁶ appended as Annex 4) where much larger increases in MN frequency in PBLs was documented in two individuals with exposure to TCDD and other dioxins. Although its not possible to attribute the cause of the transient increases in MN frequency of approximately 8 fold to any particular chemical exposure, it is interesting to comment on what changes in physiology could have resulted in such an effect. Does this have relevance for the consideration of factors controlling the background frequency of MN in PBLs?

30. The Committee suggested in the statement on pesticide applicators <http://www.advisorybodies.doh.gov.uk/pdfs/pesapp.pdf> that a longitudinal approach was likely to be more preferable to a cross sectional approach to biomonitoring studies.

31. Members are asked to consider the following questions for discussion which are designed to help draft a statement on guidance for consideration in evaluating biomonitoring studies of MN in PBLs.

i) What are the factors which influence the background MN frequency in PBLs?

ii) Is the available information adequate to identify all the relevant factors relating to individuals consider in biomonitoring studies, or is more information required?

iii) Is the available information adequate to identify all the relevant factors relating to the CBMN assay in PBLs as used in biomonitoring studies, or is more information required?

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iv) What is the value of measuring MN in mononuclear cells and using specialist procedures to identify whole chromosomes or centromere with regard to biomonitoring studies?

Secretariat January 2006

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