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MUT/06/11

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

Background variation in chromosomal aberrations in peripheral blood lymphocytes; Discussion paper on risk factors.

Introduction

1. The COM identified the need for further evaluation of the factors affecting the formation of biomarkers of genotoxicity in peripheral blood lymphocytes a key guidance regarding the evaluation of results from biomonitoring studies of exposure to chemicals. (see statement on pesticide applicators <http://www.advisorybodies.doh.gov.uk/pdfs/pesapp.pdf>)
2. The basis for using cytogenetic approaches in peripheral blood lymphocytes (PBLs) as a biomonitor arises from the observations that most human carcinogens are genotoxic in-vivo and the findings of epidemiological studies suggesting a high frequency of chromosomal aberrations is predictive of an increased risk of cancer.¹⁻⁵ This discussion paper overviews the published literature specifically in relation to studies which provide information on the background variance of the formation of chromosomal aberrations in PBLs. The review includes information on a variety of assay procedures undertaken with PBLs including classical metaphase analysis using staining techniques such as Giemsa, the use of banding techniques such as G-banding to identify specific aberrations in individual or groups of chromosomes at metaphase, and the use of Fluorescence InSitu Hybridisation (FISH) techniques for individual and groups of chromosomes at metaphase and interphase. These different approaches vary in their suitability to detect different types of cytogenetic damage.
3. In contrast to the review on micronuclei formation (MUT/06/01), the review of chromosomal aberrations considers a range of end points such as stable and unstable chromosomal changes and hence there is a very brief overview of the types of chromosomal damage that may be identified.
4. The data are reviewed with respect to the impact of age, sex, smoking, diet, micronutrient level, and polymorphisms on the level of chromosomal aberrations in control populations. (A review of alcohol consumption on cytogenetic changes in PBLs is also needed see para 11 and the review by Carrano et al 1998, Annex 3.) For some aspects such as age and smoking there are as much data available as have been previously identified for the COM review of micronuclei formation.

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For other potential factors such as diet and micronutrients comparatively few data were retrieved. There are a number of papers presenting evaluation of combined data from several laboratories, although none of these are anywhere near as comprehensive as the HUMN project data were for micronuclei formation (see MUT/06/01). The review has also included a consideration the impact of different methods used on the background variance of chromosomal aberrations in PBLs in control populations.

5. The impact of background variation in risk factors for chromosomal aberrations in PBLs can be significant. Thus in an early review of biomonitoring studies of occupational exposure to a variety of genotoxic chemicals including vinyl chloride, ethylene oxide, epichlorhydrin, and epoxy resins, de Jong and colleagues reported that the use of metaphase analysis in exposed populations was not sufficiently sensitive for routine monitoring of cytogenetic effects in workers due to the variable and high background levels of chromosome aberrations in control populations.⁶ Literature searches identified 60 additional references which form the basis of this review paper.⁷⁻⁶⁷ [PUBMED and TOXNET searches using terms chromosomal aberrations, cytogenetics, FISH, PBL, biomonitoring, background, control. Retrieved references were cross referenced and additional papers identified. The extent of cross referencing could be increased but there was insufficient time to do this.] A summary of the identified papers is given as Annex 1 to this covering paper.

Cytogenetic end points in PBLs

6. A brief review of Cytogenetic end points is given to aid in the evaluation of the most suitable approaches which might be used for biomonitoring of exposure to genotoxic chemicals. The range of endpoints that can be detected using the available methods are outlined in the table below.²⁴ These are generally sub divided into chromosomal-type aberrations (induced in G₀/G₁ PBLs by ionising radiation and direct acting DNA mutagens which cause double strand lesions) and chromatid-type aberrations (in S and G₂) which are formed *in-vitro* when the damaged DNA template is replicated. Chemical mutagens predominantly form chromatid type aberrations.^{1,24} Cytogenetic yields in PBLs result from 3 competing processes, induction of lesions (through exposure, possibly prolonged), continual repair of lesions, and removal or redistribution of lymphocytes. It is assumed that at the time or shortly after exposure unstable and stable aberrations may be present, and subsequently the frequency of unstable chromosomal aberrations (e.g. dicentrics) begins to decrease. Numerical changes from the normal chromosomal complement in PBLs of 46 chromosomes can occur through abnormal replication and division of centromeres which can result in non-disjunction, and chromosomal loss and gain.

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7. An estimate of the background rate of the different types of cytogenetic damage, where available has been included in the table, although it should be noted that the data should be viewed with caution in view of the limitations of the available studies (often of small size lacking statistical power considerations), limited information on whether first or second cell divisions have been included in the analysis, the variable classification and terminology used to describe cytogenetic damage, and experimental variance (e.g. culture conditions, and methods used to score end points).

A proposed classification of aberrations is given below²⁴;

Chromatid; (Generally stable)

- a) gaps/achromatic lesions, unstained lesions less than width of chromatid. (4.25%)²⁰
- b) chromatid breaks-breaks greater than the width of arm or displaced
- c) isochromatid-chromosome breaks involving both sister chromatids at the same position (can't distinguish from acentrics)
- d) chromatid exchanges- exchanges between chromatids of different chromosomes.
- e) chromatid intrachanges-exchange between sister chromatids at non-homologous points.

Chromosome (Generally unstable)

- a) acentric fragments (also called minutes depending on size, see isochromatid breaks); 0.037%¹², 0.016%²⁰
- b) dicentrics- exchanges between two chromosomes resulting in a structure with two centromeres and associated acentric fragment.; 0.078%¹², 0.016%²⁰ 0.095% (FPG)⁴⁷, 0.13% (FISH)³⁸ 0.16%²⁰
- c) ring chromosome- exchanges within one chromosome. Centric rings associated with a fragment. 0.02%²⁰

Numerical (Stability can vary with lesion)

Non disjunction, missing/additional chromosomes. (Can be affected by procedure for preparation of metaphase spreads. Can be assessed during interphase using FISH based approaches). Aneuploid females 8%, 4% males¹⁰; 4% males, 2-3% females (age 30-55y) (G-banding)¹¹
Hyperdiploid 2% females, 1% males.¹¹, 2.8% females, 3.0% males³⁰

Balanced translocations and inversions. Difficult to quantify without banding analysis or FISH. (Stable) Complete translocations (2 bi-coloured rearranged monocentric chromosomes), apparent incomplete (acentric material (b) attached to monocentric chromosome (A) (tiAb), or unpainted acentric material (a) attached to painted monocentric chromosome (B) (tiBa). 0.8% (FISH)³⁸ 0.748% (FPG)⁵⁸, 0.05%²⁰

Overview of available studies reviewed

8. The reviewed studies⁷⁻⁶⁷ were predominantly undertaken to investigate background levels of cytogenetic damage in populations. A small number were undertaken to determine background rates of specific cytogenetic damage in workers at first employment in the nuclear industry^{25,27,58}, or in individuals living in geographical areas considered to have differing levels of pollution.^{38,41,45,66} The main objective of most studies has been to investigate the effect of age^a, smoking^b and gender^c on overall and specific forms of cytogenetic damage, and the comparison of different methods to detect cytogenetic damage. The

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use of FISH to detect specific cytogenetic changes in biomonitoring studies^{38,40,46,49,51,54,57,59,63,67} such as translocations and aneuploidy was first introduced around 1994 as a technically easier to assess and more rapid approach to individual chromosome identification than banding techniques*.^{11,17,19,22,25,30,35,36,58} The use of FISH techniques in biomonitoring studies using PBLs allows the detection and quantification of a qualitatively different range of cytogenetic changes compared to metaphase analysis using Giemsa and thus one objective of the current review has been to compare these approaches regarding the most appropriate uses of each approach.

* all present mainly G-banding and some C-banding, except²², R-banding.

a (Positive^{7,8,16,20,22,26,30,32,33,35,36,41,42,45,46,54,58,67}, negative^{21,31,49})
b(Positive^{13,14,18,19,29,32,36,39,47,51,58},negative^{28,31,38,46})
c (Positive (> in females, aneuploidy)^{8,10,30,54} positive (> in females, other aberrations)^{20,21}, negative (no differences between sexes)^{29,30,32,35,37,46,49,51,54,55,67}

9. Generally cross sectional or retrospective designs have been used with only two investigations identified which included prospective sampling at two different time points.^{20,36} A number of interlaboratory studies were identified which investigated various aspects of metaphase analysis in biomonitoring studies, some of which were retrospective in design.^{9,12,15,29,35,37,42} A smaller number of interlaboratory studies of the use of FISH were also identified.^{40,51,59,63} There is generally good agreement on the most appropriate approaches to undertaking metaphase analysis, although it is noted that the relative impact of certain aspects of the assay such as quality and type of PHA used to stimulate PBLs and the concentration and duration of colchicines (or colcemid) treatment have not been the subject of detailed investigation in the retrieved biomonitoring studies. There is comparatively less agreement on the most suitable approach to use for FISH analysis. None of the studies identified directly compared metaphase analysis and FISH using the same individuals. Several studies do report on the utility of metaphase analysis, G-banding and FISH at identifying dicentric chromosomes.^{13,15,20,22,35,36,46,47,49,51}
10. A relatively small number of studies have investigated the effects of vitamin supplementation on cytogenetic damage^{23,61,63} in comparison to the available studies on micronucleus formation in PBLs (reviewed in MUT/06/01). A small number of studies have investigated vitamin supplementation (predominantly vitamin C) or the effect of addition of vitamin C *in vitro* on the detection of cytogenetic damage induced by clastogens in PBLs.^{43,48,50,56,60} A small number of studies of genotype and chromosomal aberrations in PBLs have been included in this

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review.^{53,54,64} This aspect has been considered in more detail in the review by Norppa¹

11. The review by Norppa published in IARC scientific publications volume 157 in 2004¹ and the very informative article published by Carrano et al²⁴ in 1988 are included as Annexes 2 and 3 to this covering paper. The data on consumption of alcoholic beverages and effects on chromosomal damage in PBLs has not been considered in detail in this review and will be considered in a separate paper along with relevant information on micronucleus formation in PBLs.
12. Carrano²⁴ advised on the most appropriate approaches to statistical analysis of cytogenetic biomonitoring studies in 1988 and noted that since the exact distributions of aberrations were not known, statistical tests based on distribution free methods such as Mann-Whitney or the Kolmogorov-Smirnov tests were preferable. Another approach is to perform analysis using differences between populations assuming a Poisson distribution and to estimate standard errors. In general the data from the available studies suggests that biomarkers of cytogenetic damage in PBLs follows a Poisson distribution. Carrano provided advice on the approach to determining appropriate size of study groups for biomonitoring studies. In general study investigators have not provided details on the power of studies to determine cytogenetic damage occurring at low frequency. A number of collaborative studies are available which used relatively large group sizes.

Overview of risk factors for cytogenetic damage in PBLs. .

[Throughout this paper, culture times refer to 48h except where stated. Studies using standard metaphase analysis are reported first, followed by data from banding techniques and by data from FISH investigations. (Most authors assume that the chromosomes used in FISH studies are representative of the whole genome and scale results up to whole genome equivalents. There is some evidence from a study of reciprocal translocations in Chernobyl clean up workers and controls that this may not always be appropriate.⁷²) In all sections data on chromosome/chromatid aberrations are reported first with information on numerical changes reported towards the end of each section.]

Effect of Age

Metaphase analysis

13. An age related increase in the percent PBLs with chromosome aberrations (excluding gaps) was documented in metaphase analysis (54h culture) of 85 males living around Barcelona.⁴¹ An increase in chromosome breaks and exchanges with age was also documented. A highly significant correlation between age and chromosome aberrations (excluding gaps) was found in non-smokers in a study of

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Italian subjects from different regions.³⁹ An increase in dicentric chromosomes with age was reported in a study of 353 individuals (male and females) employed at the Brookhaven National Laboratory in the U.S.A.²⁰ An increase in chromosome aberrations was also reported in a further small study in males (although it is unclear whether gaps were excluded in this analysis).³³ An increase in chromosome exchanges was documented in a small metaphase analysis (72h) study of nine elderly (>65y) donors compared to healthy hospital personnel (aged 20-35y).¹⁶ All donors were non-smokers. An increase in hypoploidy was reported in a number of early metaphase analysis studies of males and females.^{7,8}

14. The most consistent finding in the Nordic Study group evaluation of metaphase analysis of data from nine laboratories was an age related increase in chromosome aberrations (percent cells with aberrations excluding gaps 5/9 positive).²⁹ Bolognesi reviewed data from 40 studies and also reported on an interlaboratory trial with 12 participating laboratories (using 48 and 72 h protocols) An age related increase in chromosome aberrations (excluding gaps) was reported.⁴² The increase was particularly evidence in the 30-59 y age group. [The evaluation of age-related effects on the occurrence of dicentric chromosomes is influenced by the size of the biomonitoring study, the technique used to assess dicentrics, and confounding by smoking see summary of study by Presl et al^{46,47} in para 17 below]

G-Banding Techniques

15. A possible increase in chromosome exchanges was reported in a small study of new entrants to the Sellafield Nuclear reprocessing plant in individuals aged over 40 y compared to those aged 20-40y.¹⁹ (data were available for 51 individuals with no previous exposure to clastogens, 13 of whom were smokers) In a further study using new entrants to the Sellafield plant, Tawn and colleagues reported data for 162 individuals (some of whom were included in the earlier study¹⁹). In non-smokers, a significant increase in chromosome translocations and symmetrical aberrations was associated with age. There was no age-related effect on asymmetrical aberrations.
16. A number of early studies using G-banding reported an age-related aneuploidy in PBLs (related to X-chromosome loss in females and Y-chromosome loss in males).^{10,11} Nowinska et al undertook a retrospective analysis of 15-30 G-banded metaphase spreads from relatives (male and female) of patients with Down Syndrome and women who had multiple miscarriages, or other inherited conditions.³⁰ There were 174 control females and 158 control males also included in the study. In total 47,361 cells from 2408 individuals were evaluated. Overall in female X-chromosome hypoploidy and hyperploidy increased with age. There was evidence for Y-chromosome gain in

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males but the numbers identified were too small to make any definite conclusions.³⁰ Prieur evaluated R-banded PBLs (using 48 or 72h cultures) from a small number of individuals (n=2 or 4) and reported an increase in reciprocal rearrangements in elderly individuals aged >70y compared to individuals aged in their 20's.²² It is difficult to draw any conclusions from this very small study.

Studies using FISH analysis

17. An age-related increase in stable aberrations was documented in a study using probes for chromosomes 1,2, and 4 in 47 subjects (3300 metaphase equivalent genomes/individual studied).³⁵ In a follow-up study by the same group of investigators, an increase in stable aberrations was recorded in 91 individuals (using the equivalent of 1000 metaphases/individual. An abrupt increase was noted at 50y).³⁶ These authors controlled for a wide variety of confounding factors including smoking. An increase in dicentrics and acentrics with age was also reported in this study. Presl et al reported an increase in reciprocal translocations in a study of 43 individuals using FISH analysis for chromosomes 2,4,8.⁴⁶ In a follow up study, the same investigators used FPG (Fluorescence Plus Giemsa) analysis of metaphases from 53 individuals and reported that the age related effect on dicentrics and acentrics was not statistically significant if heavy smokers were removed.⁴⁷ Lucas et al reported an age-related increase in translocations (complete and incomplete) using FISH analysis of PBLs from 35 healthy controls in a study where a large number of metaphase equivalent genomes were evaluated per individual (average 5822).⁴⁹ Information on the chromosomes investigated was not provided and probably varied between individuals. An increase in non-disjunction of chromosome 21 was reported in adults compared to children in a study of 68 individuals (non smokers and non-drinkers). There was no evidence for an age-related effect on chromosome 21 loss.
18. A retrospective evaluation of FISH data from 8 laboratories (data on 436 individuals used) reported an age-related increase in complete (two-way), incomplete and total translocations.⁵¹ A recent collaborative evaluation of control data for translocations in PBLs using data from 385 individuals from 7 laboratories (at least 300 genome equivalents/individual) reported a clear association between chromosome translocations and age. The authors reported an upward curvature of the association with age and suggested this represented an ageing process.⁶⁷

Conclusion; age

19. Thus overall there is good evidence for an age related increase in chromosomal aberrations (excluding gaps). This includes breaks,

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exchanges and aneuploidy. There is good evidence from studies using FISH that stable translocations also increase with age. The evidence regarding unstable chromosomal changes such as dicentrics is unclear with both positive and negative findings reported which may also be affected by the method used to score dicentrics (see also section on assay variables below). It is also noted that smoking may be a risk factor for dicentric formation.

Effect of smoking.

20. Studies are available using metaphase analysis, G-banding and FISH. Both case-control and cross sectional designs have been used. There is a variable amount of information provided on extent and duration of smoking. Generally a cut off of 20 cigarettes per day is used to define heavy smoking. The Nordic Study Group collaborative evaluation of metaphase studies reported an effect of smoking in 3/9 laboratories.²⁹ The retrospective evaluation of FISH data from 7 laboratories did not find an association between smoking and translocations, although there was a suggestion of an effect in certain age groups who smoked.⁶⁷ The available data from studies has been summarised in the following table.

Reference, study group (no of smokers/controls)	Main result (Statistically significant increases reported)	Data on smoking/comments
METAPAHSE ANALYSIS		
Ref13, 193/140	Combined chromatid translocations/dicentrics. Combined rings/minutes	Data not given
Ref 14, 55/43	Chromosome/chromatid interchanges. Chromosomal fragments/exchanges	Majority were light smokers <10/day
Ref 18, 6/6	Increased chromosome type aberrations	Ca 20/d for 20y
Ref 20, 353 (cross section)	Cigarette smoking did not contribute to variance	Not given
Ref 21, 106 (cross section)	No effect of smoking identified	Not given
Ref 25, 13,38	Increase in exchanges	Not given
Ref 28, 8/7 never, 9 ex-	Increase in chromosome type aberrations in ex-smokers	Not given
Ref 31, 48 (cross section)	No effect of smoking identified	Not given
Ref 32, 127 (15 somkers/15 non smokers age matched selected)	Increase in percent aberrations (excluding gaps)	>15/d
Ref 39, 310 (cross section)	Increase in aberrations (excluding gaps)	>5/day
Ref 47, 53 (Study undertaken to follow up negative FISH study, ref 46) FPG used to evaluate 1 st division metaphases.	Increase in dicentrics . No significant increase in acentrics	>30/d (7 heavy smokers)
G-BANDING		
Ref 19, 66 (entrants to Sellafield)	Increase in dicentrics	Significant in heavy smokers (n=7, >20/d)
Ref 27, 12,12 (retrospective data on Sellafield entrants)	Increase in dicentrics (not statistically significant)	Significant in heavy smokers >20/d
Ref 58, 72 curent/ex/90 non	Increase in translocations/symmetrical	Not given. Age is risk factor for translocations.

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	aberrations in smokers aged 40-49 and 50-59	
FISH		
Ref 36 , 91 (Ch 1,2,4)	Smoking a significant variable in overall aberration frequency	Not given
Ref 38 , 12/30 (Up to 10 chromosomes)	Increase in dicentrics/rings (2x, but not statistically significant. No increase in dicentrics alone or translocations	Average of 20/d for 26 y.
Ref 46 , 42 (Ch 2,4,8)	No significant increase in dicentrics (authors noted limited number of individuals studied)	Stratified into <20/day and >30/day. (8 heavy smokers)
Ref 51 , Retrospective evaluation of data from 8 laboratories (436)	Suggestion of an effect (translocations).	Not given
Ref 67 Retrospective evaluation of data from 7 labs (385)	No effect on translocations. Suggestion of effect in heavy smokers aged 30-39y, and 60-69y	Not given

Conclusion smoking

21. Thus, overall, the results of metaphase analysis studies are consistent with an effect of smoking on chromosomal aberrations, although it is difficult to assess the level of smoking required for an effect on chromosomes in view of the limitations of the smoking consumption data from the available studies. Overall the increase in unstable aberrations (e.g. dicentrics) was evident in heavy smokers (>20/d). G-banding studies supported this conclusion. There is less evidence for a cytogenetic effect of smoking from FISH studies. The retrospective evaluation of data from a number of laboratories concluded that there was no a statistically significant association between smoking and translocations (some evidence was presented for certain age groups). The differences between the data from metaphase analysis, G-banding and FISH may relate to the adequacy of the methods for evaluating unstable chromosomal changes, the size of FISH studies and in particular the limited number of heavy smokers included in the FISH studies. It is noteworthy that the limited data on multi vitamin intervention does report an effect of vitamin C,E and Se intervention (12 weeks) on metaphase analysis for chromosomal aberrations.⁶¹ (see section below). Thus the comment made in the COM paper on micronuclei formation (MUT/06/01) regarding the possible association of heavy smoking and poor nutrition may also be relevant for cytogenetic damage in PBLs.

Effect of Gender

Metaphase Analysis

22. The predominant effects reported relate to aneuploidy and hence these data have been summarised first. Aneuploidy was reported in early studies in both males and females.^{8,10} The frequency of aneuploidy was reported to be higher in females in these studies (cf 8% compared 4% in males).¹⁰ Subjects in this study were aged between 18-87 y, with

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10-13/sex in each of three age groups. Fitzgerald reported 307/3199 aneuploid metaphases in males compared to 188/307 in females. 188 metaphases from the 307 aneuploid metaphases in females had 45 chromosomes. Aneuploidy was positively correlated with ages in both sexes.¹⁰ Criteria for selection of metaphase spreads were generally not reported in these early studies and thus there should be some caution regarding the reported frequency of aneuploidy. A higher frequency of acentric fragments was reported in females compared to males in an early metaphase study.¹¹ Premature centromere division (PCD) of the X chromosome was reported to be relevant.^{11,20} Anderson reported a higher frequency of overall chromosomal aberrations (total excluding and including gaps, deletions and exchanges) in females (n=33) compared to males.²¹ This observation has not been reported in other studies. Thus the Nordic Study Group reported a significant gender effect on chromosomal aberrations in 1/9 participating laboratories. Ganguly et al found no gender effects in an investigation of 69 males and 58 females using both 48h and 72 h cultures.³² No effect of gender was reported in the collaborative study of 7 Italian laboratories (959 females, 1539 males).³⁷

Banding techniques

23. G-banding and C-banding techniques were applied to identify chromosomes in one early study.¹⁰ Limited data from relatively few G-banded metaphases (n=14) showed that hypoploidy in males involved Y-chromosome loss. PCD was reported to be the predominant cause of aneuploidy in both males and females.¹⁰ In a separate study Galloway and colleagues showed X-chromosome loss to be a predominant change in hypoploidy in females.¹¹ Hypoploidy in females between 30-55 y was reported to be 3-5% and in males 2-3%. Hypoploidy rose to 8% at 60 y in males and 70 y in females. Galloway also noted a slight decrease in hypoploidy in females using a 72h culture compared to a 48 h culture. (no effect in males).¹¹ Hyperploidy increased with age in females but not in males.¹¹ Sinha AK et al investigated chromosomal disintegration leading to the formation of supernumerary chromosomal elements (70h) in males but presented no data regarding females and thus there is no G-banding equivalent investigation to confirm the finding of higher acentric fragments in females.^{11,20}

Studies using FISH analysis

24. Limited evaluation of the effect of gender has been undertaken using FISH analysis. No gender effects were reported in an evaluation of 14 males and 33 females using probes for chromosomes 1,2,4 (equivalent to 1134 G-banded metaphases/individual).³⁵ Presl et al did not report any gender related differences in translocation frequency in 24 males/18 females (using chromosomes 2,4,8; an average of 2953

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metaphase equivalents/individual).⁴⁶ Lucas reported no gender effects on translocations, dicentrics and complex rearrangements in a study of 20 males and 15 females using varying probes sets.⁴⁹ There were no gender effect reported in an interlaboratory evaluation using data from 436 individuals from 8 laboratories.⁵¹ Whitehouse reported on an interlaboratory evaluation of data from 385 individuals from 7 laboratories using 385 individuals and found no overall evidence for a gender effect on translocations (a higher rate for males aged 30-39y was reported).⁶⁷ Catalan examined chromosome lagging in a single female and noted that that X-chromosome was over represented.⁵⁴ Shi et al did not report any gender differences for malsegregation of chromosome 21.⁵⁵

25. A number of additional studies using micronuclei support the findings for X-chromosome malsegregation.⁶⁸⁻⁷¹ These are summarised here as they were not retrieved for the COM paper on MN formation and support the observation of X-chromosome loss in females during aging. Surralles J et al measured X-chromosome loss in 4 females (aged 24, 27, 57 and 60y) in cytochalasin B blocked PBLs (following a 44 h culture) using centromeric specific X-chromosome DNA probe and immunolabelling for acetylated histone H4 acetylation to detect X-inactive and X-active chromosomes. The authors reported that X-chromosome loss occurred at a greater rate in aged females and this involved both X-inactive and X-active chromosomes.⁷⁰ Catalan and colleagues reported over-representation of X-chromosomes in MN formed in PBLs in older women (>50y) compared to younger women (<30y).⁶⁸ Hando et al reported the X-chromosome to be present in 72.2% of MN scored (based on a group of 38 females aged 19-77 and 8 newborn females). An age related increase in MN containing X-chromosomes was documented.⁶⁹

Conclusion; Gender

26. Thus overall, there is good evidence for sex chromosome non-disjunction and X-chromosome loss or gain in females which is age related. There is evidence for sex- chromosome non-disjunction and Y-chromosome loss in males, but insufficient evidence regarding Y-chromosome gain in males. It is difficult to conclude whether the overall rate of aneuploidy differs between females and males based on the available metaphase analyses and G-banding studies. There is insufficient evidence to conclude whether there is any gender related differences in cytogenetic changes (e.g the frequency of unstable chromosomal changes). There is no evidence from FISH studies for any gender related cytogenetic effects with the possible exception of malsegregation of the X-chromosome in females; an observation supporting the finding of aneuploidy in metaphase analyses and G-banding studies.

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Effect of diet

27. The only available study investigated chromosomal aberrations in 13 lacto-ovarian vegetarians (8 women, 5 men), 11 lacto vegetarians (5 women, 6 men) compared to aged matched controls. BMI was significantly higher in non-vegetarians. There were no significant differences between the groups regarding the frequency of chromosomal aberrations.⁶²

Effects of micronutrients

Metaphase analysis

28. There were only three studies retrieved which investigated the effect of vitamin supplementation on background levels cytogenetic damage in PBLs using metaphase analysis.^{23,61,65} None of these studies used a blind or cross-over design. Two studies were retrieved where the effect of vitamin supplementation on cytogenetic damage induced by bleomycin or dixidine was investigated.^{43,60} One of these trial used a double blind approach.⁴³

Effects on background levels of cytogenetic damage

29. The effect of vitamin supplementation in 38 chemical laboratory workers exposed to newly developed cytostatic drugs and 18 non-exposed, matched, (librarians/clerks) on cytogenetic analysis was investigated.²³ Individuals took effervescent tablets at 1g ascorbic acid (AA)/day for 5d/week from November 1984 till may 1985. Blood samples were taken at initiation of vitamin treatment and at termination. Intakes were checked using urinary AA determinations in a number of individuals during the study. The number of aberrant cells was significantly higher in cytostatic drug exposed individuals. It is not clear whether this included an assessment of gaps. There was no effect of vitamin supplementation on the number of aberrant cells, chromatid breaks, isochromatid breaks, exchanges, number of breaks/cell and number of gaps/cell (52h culture).²³
30. A supplementation trial in 40 myocardial patients (MI group) average 53, (35-66y), 60 controls (RC group 44y (34-56y) living in Pezinok, Bratislava. Supplementation of 100 mg vit C, 100 mg vit E, 6mg β -carotene, 50ug Se for 12 weeks.⁶¹ A significant decrease in the frequency of chromosome/chromatid breaks was reported in the RC supplementation group when compared to placebo. (0.63% cf 0.27%, $P=0.03$). The changes in RC non-smokers were non-significant. In smokers a highly significant reduction in percentage of cells with aberrations (0.8% cf 0.2%) was reported ($P<0.001$) and frequency of cells with chromosome breaks ($P=0.0002$).

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31. No effects on the frequency of chromosomal aberrations was reported in an intervention trial where a group of 15 donors (mean age of 8 females, 32.4 ± 2.1 y and of 7 males 32.7 ± 2.7 y) consumed a vitamin mineral complex was administered every morning and evening (gingko, biloba, garlic, Q10 coenzyme, grape seed extract, ginseng root, guarana, Echinacea, spirulina, royal jelly, alfalfa powder, and chlorella. This provided a large number of vitamin and minerals (including vitamin B₁₂ (100 ug/day) B₆ 12 mg, niacinamide, biotin folic acid, vitamin B₁, B₂, C, E, A, D, K, Cr, Ca, Cu, I, Fe, Mg, Mo, Ni, K, Se, Si, V, Mn, P, Zn) for 14 or 30 days.⁶⁵

Conclusion; Micronutrients

32. Thus overall there was no evidence from the available limited trials that vitamin supplementation independently affected cytogenetic damage in PBLs. However the studies retrieved did not include a specific investigation of folate or vitamin B₁₂ supplementation and thus the data cannot be compared to the available data for MN formation in PBLs.

Effects on bleomycin/dioxidine induced cytogenetic damage.

33. A double-blind intervention study with cross over was used to assess the effect of vitamin supplementation on bleomycin mutagen sensitivity 22 volunteers (16 female, 6 male) Group 1 took 15 mg β -carotene and 400 IU of α -tocopherol 2x/day, for 6 weeks after baseline blood sample was obtained. Group 2 took placebo. Subject used their own diets but were asked not to take any vitamin supplements. Week 6-12 was a washout period. Cross over was undertaken from week 13-18. There was a clear effect on vitamin levels with supplementation. There was no effect on the reported levels of bleomycin chromatid breaks.⁴³
34. In the second retrieved study, peripheral blood lymphocytes were isolated from healthy volunteers (5 female, 6 male, age 26.8 ± 1.9 y). The donors received vitamin complex twice/day (60 mg vit C, 1.2 mg vit B₁, 1.2 mg B₂, 1.2 mg B₆, 0.002 mg B₁₂, 13 mg PP, 1mg vit A, 7 mg vit E, 3000 IU vit D₃, 6 mg pantothenic acid, 0.4 mg folic acid, 0.14 mg biotin and 2.0 mg β -carotene for 2 weeks. Cultures were maintained for 54h. The percentage of damaged cells after dioxidine treatment significantly reduced after vitamin therapy. Thus therapy improved resistance to dioxidine induced CAs. With regard to bleomycin, the authors reported that mean CAs did not alter with vitamin therapy, but there were some increases and decreases in sensitivity in donors reported.⁶⁰

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Conclusion: Effects of micronutrients on sensitivity to clastogens

35. There is thus some limited evidence that vitamin supplementation may affect sensitivity of PBLs to chemically induced cytogenetic damage, but the data are inadequate to draw any firm conclusions particularly with regard to specific vitamins that might be relevant with regard to reduction of chemically induced cytogenetic damage.

Individual variation

36. A relatively small association has been reported between slow *NAT2* acetylator genotype and cytogenetic damage assessed by metaphase analysis⁶⁴ and FISH analysis (using chromosomes 1,2,4)⁵³ in PBLs. Pluth reported that this finding was particularly evident in smokers (data on smoking consumption reported indicates that the smokers in this study included several (3 out of 9 >30/d) who were heavy smokers. Norppa¹ has reviewed the evidence for effects of genotype on background levels of chromosomal aberrations in PBLs and concluded there was evidence for an increase in baseline frequency among *GSTM1*-positive subjects, *CYP1A1 msp1* heterozygotes (in newborns)⁵², *CYP2E1 wt/*5B* heterozygotes and *EPHX* 'low activity' genotype. Norppa¹ (annex2) concludes that these findings were based on few individuals and need to be examined in further studies. Members may also be aware that the COC reviewed the association between genotype and cancer risk a few years ago and made recommendations regarding the need to avoid selective reporting of statistically significant results occurring through multiple statistical analyses. There does not appear to be any studies available which investigated genotype associated with folate metabolism.

Conclusion: Individual variation

37. Overall it is suggested that no definite conclusions can be reached regarding the effect of genotype on background frequency of chromosomal damage in PBLs. The available evidence regarding slow *NAT2* acetylation may reflect exposure to tobacco smoke.

Background variation in chromosomal damage in PBLs due to assay

Metaphase Analysis

38. The Results of interlaboratory trial (n=9, 18 scorers), involving assessment of a set of pre-coordinated metaphase chromosome spreads (2 slides/laboratory, 118 spreads.) and scoring of 32 slides of metaphase chromosomes (4 slides per dose level of X-rays (5-400 rads), 8 dose levels) were reported.⁹ For pre-coordinated study, data from 13 scorers from 8 laboratories available. There was considerable variation in the proportion of cells selected and scored and

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consequently the number and types of chromosomal aberrations reported (particularly for deletions, acentric rings and minutes). There was no unanimous agreement among scorers even for one damaged spread. The authors reported a larger variance between scorers from different laboratories compared to scorers in the same laboratory. The authors documented a large variance in the exchange frequency/cell. The variance for the dose-response in this study was 1.5 (except for one laboratory). A previous study reported a variance in exchanges/cell of 5. A significant variance in dose response for exchanges was reported between laboratories. It was noted that the large differences in scoring aberrations were resolved when exchanges, minutes and acentrics were combined.

39. A tabulation of control data for dicentrics and acentrics was undertaken using data from 65 reference studies and the NRP laboratories at Harwell. This comprised a total of 2000 individuals (211,611 lymphocytes).¹² There was considerable variance in the reporting of dicentrics. The authors noted that scorer variance was one possible explanation for the results, but that variance in the proportion of 2nd metaphase cells was also possible. The authors examined the differences between studies where less than or more than 2000 cells had been analysed and reported that the smaller studies documented statistically significant lower dicentric but higher acentric rates.
40. An interlaboratory trial of variance in chromosome aberration analysis of 10 slide (2 each from five laboratories). There were between 1-3 scorers at each laboratory. In a second part of the study, chromosome aberrations were identified from a set of 50 photomicrographs (10 provided by each laboratory). There was a wide degree of interlaboratory variance in the reported level of chromosome damage, with the assessment of gaps being largely responsible for most variance. After exclusion of gaps there was still some variance due to recognition and assessment of different chromosomal changes. The authors recommended that results should only be compared within laboratories. Interlaboratory assessment of the photomicrographs, was undertaken since the results from the first part of the study could in part be due to selection and scoring of different metaphases. The authors concluded that overall there was an approximate 55% agreement on metaphases. Total agreement was reached for chromatid exchanges, and chromatid breaks and chromosome breaks. Agreement was low for dicentrics abnormal monocentrics (translocations, inversions, markers) ring chromosomes and for the two types of gaps. Stephan and Pressl concluded that there are interlaboratory differences in the reporting of dicentrics (A comparison for interlaboratory data using 1st division metaphases was undertaken). However the dicentrics rate was relatively constant when heavy

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smokers were excluded with age.⁴⁷ It was concluded that each laboratory should establish its own base-line data.

41. Bender et al²⁰ The authors also reported that for 25 subjects were two or more samples were drawn that there was no significant temporal variance reported.

G-Banding studies

42. Galloway reported that variance in the assessment of hypoploidy was predominantly due to selection of metaphases and the low frequency of aneuploid events.¹¹

FISH studies

43. Tucker et al reported a significant variance in the assessment of unstable chromosomal aberrations using FISH.³⁵ FISH analysis revealed that more translocations were routinely scored at the Lawrence Livermore National Laboratory LLNL compared to NRPB. When one and two way translocations were combined the totals were 126/83 in miners at LLNL and NRPB respectively. In controls there were 176/65 at LLNL and NRPB respectively. LLNL should exceed NRPB by 6% using the differences in chromosome sizes examined. Scored slides were exchanged and scored using in house selection criteria. If metaphases where some painted material was absent were included among the scorable cells, the overall translocation yield was higher. There was a good agreement between laboratories with respect to the evaluation of dicentrics and acentrics using FISH (after allowing for the use of different chromosome probes between laboratories). Edwards reported similar findings with respect to criteria used to select cells for scoring translocations in a separate study.⁵⁹
44. An interscorer evaluation of selection of metaphases and scoring for chromosome 5 breakage using a FISH-based assay (72h culture).⁶³ The highest coefficient of variation was found for cells with chromosome breaks (6.2-13.4%). A larger variance in observer exclusion frequency was noted 5.0-40.0%.
45. An interlaboratory study of variability in FISH analysis of interphase cells. The study was based on bladder washes from five patients with concurrent tumours and five bladder tumour specimens.⁴⁰ Replicate hybridisations done in two separate laboratories reported statistically significant variability. The study was focused on counting the number of copies of each chromosome identified.

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46. Ramsey et al undertook a reanalysis of stable aberrations using FISH analysis (chromosomes 1,2,4) in 20 individuals selected as having high or low aberration frequencies for their age. Overall there was no substantial differences between the two sampling times. The variance in the data exceeded a Poisson distribution. This was due to three individuals where the aberration frequency significantly reduced on the second blood sampling (aged 56, 60, and 70 at the first sample. Data for the remaining 17 individuals showed no significant changes.³⁶

Conclusion : Assay variables

47. Thus overall interlaboratory trials using experimental studies and photomicrograph data from metaphase analyses report considerable variance in results due to individual scorer selection of metaphases and scoring of aberrations with a low frequency (in particular unstable aberrations). A variance in metaphase analysis response to radiation exposure was reported which is a similar finding to that reported for MN formation in PBLs. It is noted that the variance in the reporting of dicentric in metaphase analysis may be confounded by heavy smoking. There are relatively few data on variance in G-banding studies, but the available information for hypoploidy is consistent with that reported for metaphase analysis. The available studies on FISH analysis in PBLs suggest this approach may be an appropriate method for identifying dicentric. Variance in FISH studies due to selection of cells and scoring for other aberrations, in particular translocations has been reported. There is also the possibility of variance due to the hybridization techniques adopted. There is quantitative assessment of the overall impact of assay variance in the assessment of cytogenetic damage in PBLs (as reported in HUMN project for MN formation.)

Discussion Impact of factors affecting cytogenetic damage in PBLs.

48. There is no equivalent summary evaluation of data with apportionment of variance as published by the HUMN project for MN formation in PBLs. Members will recall that for MN formation experimental factors such as methodology and scoring probably accounted for most of the variance followed by host factors (age, gender) and the third component was exposure to genotoxic agents. Thus the following discussion is presented for members consideration. Any conclusions drawn are tentative.
49. Milillo CP reported that age accounted for 66% of the variance in metaphase analysis (48h culture, 159 males, 150 females).³⁹ Tucker et al estimated that age accounted for 69% of the variance in a small study of 47 adults (14 males, 33 females, using FISH for chromosomes 1,2,4).³⁵ Ramsey et al reported that age accounted for 70% of the variance in FISH analysis of chromosomes 1,2,4 in 14 newborns and 77 adults (19-70y).³⁶

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50. There is good evidence to conclude that scorer selection of cells for metaphase analysis and FISH and scoring criteria for unstable chromosome aberrations in metaphase analysis and translocations in FISH analysis would contribute to overall variance but there is no quantitative assessment available. It is uncertain whether the pattern identified for MN formation (i.e. greater variance due to experimental factors than host factors) would also be appropriate for cytogenetic damage. Since experimental factors appear to be consistently identified as relevant factors, a prudent assumption would be that they are of similar importance to age.
51. There is evidence that heavy smoking is association with chromosomal damage and in particular the formation of unstable aberrations from metaphase analysis but the incomplete data on level of smoking and the possible confounding by nutritional status of heavy smokers do not allow any definite conclusions to be drawn on overall impact. It is unclear why FISH studies have not documented a similar effect consistently at heavy levels of smoking, but this may relate to the design and size of studies (i.e number of heavy smokers assessed). Overall the impact of smoking can't be assessed but it is suggested that it may be less than age and experimental variation.
52. There are insufficient data to draw any conclusions regarding diet and micronutrient impact on background levels of cytogenetic damage. It is noted that there are an absence of studies specifically designed to investigate folate and vitamin B₁₂.
53. There is evidence to show a gender effect on sex-chromosome aneuploidy. There is good evidence to suggest non disjunction of the X-chromosome and exclusion of X-chromosomes into micronuclei occurs in females particularly with advancing age. (These data explain the high frequency of MN formation in females). The available data did not allow a definite conclusion to be drawn regarding whether overall aneuploidy (autosomal and sex-chromosome related) differed significantly between males and females. The early study by Galloway reports a slight increase in overall aneuploidy in females The difficulty in assessing chromosome numbers from conventional metaphase spreads limits the available information.
54. Members are asked to consider the following questions which are designed to help draft a statement on guidance for consideration in evaluating biomonitoring studies of chromosomal aberrations in PBLs.
 - i) What are the factors which influence the background frequency of chromosomal aberrations (including aneuploidy) in PBLs?

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

iii) Can any conclusions be drawn regarding the appropriateness of the various approaches to measuring cytogenetic damage (e.g. metaphase analysis, G-banding and FISH)?

iv) Is the information adequate to identify all the relevant factors relating to the various assays considered, or is more information required?

Secretariat May 2006

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