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**Annex 2 to MUT/06/01**

**Data from CBMN peripheral blood lymphocyte biomonitoring studies:  
Data from the Human micronucleus (HUMN) project.**

<b>Study</b>	<b>Methods/Introduction</b>	<b>Results/Outcomes</b>	<b>Comment</b>
27. Fenech M, Mut Res, 404, 155-165, 1998.	<p>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).</p> <p>Following an announcement there was direct approach to scientists and a workshop held during the ICEM in Toulouse 1997. 45 scientists expressed an interest in participating.</p>	<p>Objectives;</p> <p>i) A comparison of the main culture methods to investigate the effect of such methods on base-line MN frequency.</p> <p>ii) A comparison of results accumulated for base-line MN frequencies from various laboratories. (Information on the normal range for different laboratories/countries).</p> <p>iii) A prospective study linking the accumulated MN index data form each laboratory to provide</p> <p>Initial data reported that the number of MN frequency measurements had risen from 209/annum in 1986 to a maximum of 3161/annum in 1996. During this period a total of 26,469 individuals had MN frequencies measured.</p>	<p>The available data showed that essential data such as date of birth were not always documented.</p> <p>Information on diet and vitamin supplementation were considered to be scanty. There was a need for a standard protocol and collection form for the CBMN assay. It was suggested that to maintain consistency with past data it would be appropriate to use RPMI 1640 medium and it would be important to confirm whether data from whole blood cultures or isolated lymphocytes gave similar results.</p>
28. Fenech M et al, Mutation Research, 428, 271-283, 1999	<p>Further publication outlining approach. Information on HUMN for exfoliated epithelial cells incorporated</p>	<p>An overview of possible approach to identify in-vivo derive MN in PBL and MN derived during in-vitro culture after blood sampling is presented.</p> <p>Factors affecting MN in the CBMN assay in peripheral blood lymphocytes reviewed. (Age/gender, diet (folate), plasma vitamin B<sub>12</sub>, homocysteine levels, and propensity of cells to undergo apoptosis. Authors restated previous study showing MN frequency in a small number of healthy individuals was similar when measured every 3 months over a year.</p> <p>Overview of data for exfoliated epithelial cells. Noted that some authors consider up to 10,000 cells are needed to determine a 50% increase in MN frequency. Background rate considered to be 1-3/1000 for all epithelial sources. Rate no affected by age or gender, but affected by occupational/lifestyle exposures such as tobacco. Authors note the need to evaluate cells with a clearly defined nucleus in order to avoid scoring necrotic cells. High intra individual variance (30-102.9%)</p>	<p>This paper presents similar information to the paper published in 1998 in Mutation Research</p> <p>Objectives of HUMN are as given above.</p> <p>Data could potentially cover 42 laboratories, 16,500 individuals, 78,000 person years (product of number of individuals and time since tests),</p>
29. Bonassi S et al, Env and Mol Mutagenesis,	<p>Comparison data for CBMN assay in human lymphocytes. Effect of laboratory protocol, scoring criteria, and host</p>	<p>All labs used venipuncture (heparin as anticoagulant). Blood kept at room temperature (82%) for shortest time (&lt;7h (80%), &lt;4h (64%)). Volume of culture</p>	<p>The authors noted that the data had been collected from a range of laboratories for</p>

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<p>37, 31-45, 2001.</p>	<p>factors on MN frequency.</p> <p>Data from 25 databases were submitted to the coordinating centre (INRC, Genoa), mainly from Europe but also including Asia, America, Australia and New Zealand. It is evident that some of the participating laboratories provided data from individuals included in the review of pesticide applicators. A total of 6583 individuals (mean age 44y, 53.3% male) were included from study group sizes ranging from 11-1637 (mean 263), each database was derived from 1-7 individual studies..</p> <p>Classification as exposed was originally undertaken by the submitting researchers. The mean MN frequency for some databases were markedly different and contained individuals who were heavily exposed to genotoxic agents. The authors used a panel of occupational hygienists, epidemiologists, cytogeneticists from the European Study Group on Cytogenetic Biomarkers and Health (ESCH) to reclassify all individuals. A group of 665 individuals exposed to known genotoxic agents were removed from the base line data (but were included in multiple regression models). In a similar way individuals with exposure to radiation were not included in the base line data (numbers not given). There were 26 variables used in the analyses. Data were missing for 108 individuals giving a total of 6475 for which data were available.</p>	<p>0.5-10 ml (most 5 ml). 21/25 whole blood cultures (ratio 4-12% of culture with one lab using 20%). RPMI1640 (15 labs), Ham's F10 (6), McCoy's (2). PHA range 20-240 ug/ml (no value standard), reflecting purity/activity variance from 7 different suppliers. FCS, 2.5% (1), 10% (12), 15% (5), 20-25% (7). Cyt B added at 44 h in majority (&lt;44h (20%), 3ug/ml (44%), 6ug/ml (32%). Cells were harvested at 72 h after PHA in 80% of laboratories, but harvesting at 69,68 and 66 h is described, and hypotonic treatment was widely used (84%) Cells were generally fixed (methanol:acetic acid (64%) before being transferred to slides (16% reported fixing after transferring to slides).</p> <p>There was a very high agreement between labs regarding criteria for scoring for identification of BN cells (&gt;80%), (round/oval nuclei, same condensation state, similar size, tow nuclei can touch/partially overlap, cytoplasm boundary clear) although there was some disagreement over nuclei being attached by a fine nucleoplasmic bridge (adopted by 15 labs), and nuclei must not be in early stage of apoptosis (17 labs). There was also a high degree of agreement for most criteria for scoring MN in BN cells (&gt;80%); morphological identical to nuclei, round/oval, diameter 1/3 to 1/16, nonrefractile, same colour as nucleus, similar staining intensity to nucleus,. There was some disagreement over not linked to main nucleus (73%), and labs were evenly split over may overlap/touch main nucleus (49%)</p> <p>Baseline MN cell frequency (4899 non exposed individuals), median was 6.5/1000 (interquartile range 3-12/1000), 95% below 26.8/1000. Median levels in females were higher (7.0/1000 cf 6.3/1000 in males, aged 40y or more), and (7.6/1000, cf 5.9/1000 in those aged 40 y or less). A median MNC frequency from the median of each laboratory was 7.5/1000 (interquartile range 5.3-12.5/1000).</p> <p>An effect of age was evident in all but two laboratories (base line 0-9 y). The increase was steepest at 30 years of age and more pronounced in females. (Multivariate analysis including 41 covariates)</p> <p>The effect of gender was not so clear with 9 laboratories (representing 18% of subjects) showing a higher frequency in males. Overall females had a MN</p>	<p>different purposes and varied with respect to quality and completeness of information which complicated the statistical analysis of data. Statistical analyses were developed to take account of clustering since many of the variables assessed concerned technical aspects of the assay which were strongly correlated within each laboratory. This was referred to as "overdispersion" A negative binomial regression model was used.</p> <p>Tabulation of data from labs shows that 13 had between 8.3%-1005 exposed individuals, There were 4899 non-exposed individuals.</p> <p>The authors were unable to clearly assess the impact of culture type on MN frequency. No substantial difference was found between using 3ug/ml and 6 ug/ml. (cf 3.9/1000 and 4.4/1000 respectively), although this is limited to those labs where both methods had been used.</p> <p>The authors note that residual confounding attributable to lifestyle factors could be reduced when other data were available. The median was used to report distribution as the MN frequency in all databases was skewed to the right.</p> <p>The authors considered the estimate of effect of gender in this study was lower as effects of exposure to genotoxins had been removed. It was noted that 2 of the</p>
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		<p>frequency 19% higher than males (95% CI 14%-24%).</p> <p>The negative binomial function of probability was used to estimate the proportion of variability of MNC frequency. To reduce strong inter-intra lab collinearity, only four general sources of variability were reported. Host factors (age, gender, continent) accounted for 31% of total variability, exposure to genotoxic agents 45%, methodological parameters accounted for 65% (protocol variables, time before culture, temp of storage, culture method, ratio of whole blood/culture medium, type of medium, percent FCS, time to addition of cytB, conc of cyt B, time after PHA for harvesting, hypotonic treatment, culture/individual (1 24%, ≥2 76%) and criteria for identification of BN cells and scoring MN accounted for 47%. The total amount of variability explained was approximately 75%. (Due to correlation among parameters investigated, the sum variance explained by all individual sources is greater than this value).</p> <p>The authors reported some methodological factors significantly increased MN frequency, blood standing for &gt;7h, blood kept in refrigerator before culture, adoption of scoring criterion (may overlap or touch nucleus), whereas other variables significantly decreased MN frequency, hypotonic treatment, ≥2 cultures/individual, and adoption of scoring criteria, non refractile, or same colour as nucleus.</p>	<p>labs which reported higher MN in males, had focused their studies on children where the effect of gender is not so apparent.</p> <p>The authors accepted that the study had not evaluated inter-scorer variance.</p> <p>The authors felt that diet may have indirectly been incorporated in part into the models (e.g use of continent)</p> <p>Future analyses of the HUMN database would focus on homogenous subgroups of the database to further evaluate the contribution of factors such as diet and scoring variables.</p>
<p>30. Fenech M et al, Mutation Research, 534, 45-64, 2003.</p>	<p>An interlaboratory study was designed to i) determine the extent of inter-laboratory variation in the MN assay scores when a common set of criteria is used to score cells sampled from the same culture, ii) to determine the extent of intra-laboratory and intra-scorer variation in the CBMN assay, iii) to determine the effect of different staining methods, iv) to evaluate the contribution of the parameters measured to the total variability of the assay.</p> <p>A blood sample was taken from a health 30 y old male. Isolated lymphocytes were used for three culture in RPMI 1640 (10% FCS) at a cell concentration of <math>1 \times 10^6</math> /ml. Two cultures were exposed to gamma rays (1 or 2 Gy at a dose rate of 5gy/min) PHA</p>	<p>The MN frequency in controls was <math>8.8 \pm 4.3/1000</math> (2-28), and in unstained slides was <math>7.4 \pm 3.3</math> (1-19). (There were no difference between NDI in stained/unstained slides).</p> <p>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 &lt;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. The scorer CV for NPB was 82%, 26% and 23% at 0, 1 Gy, and 2 Gy respectively. All labs found an increasing MN frequency by dose irradiation. The effect for 1 Gy varied from 7.4-29.2 fold, (50%</p>	<p>Several labs used multiple scorers. Each lab was instructed to estimate MN in the two spots/slide, on separate days (500 cells/spot). Scorers were instructed to omit necrotic/apoptotic cells.</p> <p>The authors concluded the estimated intra scorer median coefficient of variation could be used as standard for quality acceptance criteria for future studies.</p> <p>The results suggested that even after standardising culture and scoring conditions it would be necessary to calibrate scorers and</p>

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	<p>treatment and cyB (4.5 ug/ml) were used. Cells harvested by centrifuge and two spots/slides fixed. Half the slides were stained the rest left unstained at 4°C. 47 stained/47 unstained slides prepared from each cutler. Each lab in the study, received a pack with instructions, one set of stained and one unstained slides, details for scoring including photomicrographs (for MN and Nucleoplasmic bridges (NPB). [NPB may reflect chromosome rearrangements missed in the standard CBMN assay). Unstained slides were stained on arrival or kept desiccated or at 4°C. (Some labs had to increase their normal staining time (n=6)). A second batch of unstained slides were sent to 4 labs, of whom 2 successfully stained the slides. Data were forwarded to Dr Stefan Bonassi and were coded for blind statistical analyses. One lab did not follow the scoring criteria, and thus data are for 33 labs. A Poisson regression analysis was undertaken.</p>	<p>most frequent estimates were between 10.7-16.6). At 2 Gy the range was 16.0-60.7 fold with 505 most frequent estimates between 28.3-46.4. (The assessment of response using NPB was poorer. The authors concluded that with appropriate training, the sensitivity/reliability of NPB could improve.) Using the Poisson regression model, 67% of the variance was explained by irradiation, , while staining method, cell sample, laboratory, and covariance explained , 0.65, 0.3%, 6.5% and 25.6%, leaving on 3.1% of the variance unexplained. (For NPB the unexplained variance was 22%, reflecting the inexperience in use of this biomarker).</p>	<p>laboratories if the CBMN assay data are to be compared among laboratories and populations.</p>
<p>31. Fenech M et al, Mutation Research, 534, 65-75, 2003.</p>	<p>A description of the scoring criteria used by the HUMN project.</p>	<p>For BNMN see Bonassi above.</p> <p>For necrotic cells; pale cytoplasm, numerous vacuoles, damaged membrane, intact nucleus (early). Late, damaged/irregular nuclear membrane, partially intact nuclear structure, nuclear material leaking from nuclear boundary, staining intensity less than viable cells.</p> <p>Criteria for apoptotic cells, early, chromatin condensation in nucleus. Late, nuclear fragmentation. (Cytoplasmic membrane intact,).</p> <p>Nucleoplasmic bridges; continuous nucleoplasmic link, width may vary considerably but usually does not exceed one fourth of diameter of nuclei, NBP should have same staining characteristics as nuclei, may occasionally be more than one nucleoplasmic bridge, and some cells will also contain micronuclei.</p>	<p>These have been briefly described in the paper by Bonassi et al above. Additional criteria for assessing necrotic/apoptotic cells and NPB are presented.</p>
<p>32. Bonassi S et al, Mutation Research, 543, 155-166.</p>	<p>A study of 24 databases within the HUMN data set. This comprised 5710 subjects, with 3501 non smokers, 1409 current smokers and 800 former smokers.</p>	<p>The authors suggested the 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 exposed to genotoxic agents. In non exposed</p>	<p>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</p>

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		smokers, there was a significant increase in MN frequency at $\geq 30$ 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.)	heavy smokers.
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