

## REVIEW

## The HUMN and HUMN<sub>XL</sub> international collaboration projects on human micronucleus assays in lymphocytes and buccal cells—past, present and future

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**The International Human Micronucleus (HUMN) Project ([www.humn.org](http://www.humn.org)) was founded in 1997 to coordinate worldwide research efforts aimed at using micronucleus (MN) assays to study DNA damage in human populations. The central aims were to (i) collect databases on baseline MN frequencies and associated methodological, demographic, genetic and exposure variables, (ii) determine those variables that affect MN frequency, (iii) establish standardised protocols for performing assays so that data comparisons can be made more reliably across laboratories and countries and (iv) evaluate the association of MN frequency with disease outcomes both cross-sectionally and prospectively. In the first 10 years of the HUMN project, all of these objectives were achieved successfully for the MN assay using the cytokinesis-block micronucleus (CBMN) assay in human peripheral blood lymphocytes and the findings were published in a series of papers that are among the most highly cited in the field. The CBMN protocol and scoring criteria are now standardised; the effect of age, gender and smoking status have been defined, and it was shown prospectively using a database of almost 7000 subjects that an increased MN frequency in lymphocytes predicts cancer risk. More recently in 2007, the HUMN coordinating group decided to launch an equivalent project focussed on the human MN assay in buccal epithelial cells because it provides a complementary method for measuring MN in a tissue that is easily accessible and does not require tissue culture. This new international project is now known as the human MN assay in exfoliated cells (HUMN<sub>XL</sub>). At present, a database**

**for >5000 subjects worldwide has been established for the HUMN<sub>XL</sub> project. The inter-laboratory slide-scoring exercise for the HUMN<sub>XL</sub> project is at an advanced stage of planning and the analyses of data for methodological, demographic, genetic, lifestyle and exposure variables are at a final stage of completion. Future activities will be aimed at (i) defining the genetic variables that affect MN frequencies, (ii) validation of the various automated scoring systems based on image analysis, flow cytometry and laser scanning cytometry, (iii) standardisation of protocols for scoring micronuclei (MNI) in cells from other tissues, e.g. erythrocyte and nasal cells and (iv) prospective association studies with pregnancy complications, developmental defects, childhood cancers, cardiovascular disease and neurodegenerative diseases.**

### The origins of the HUMN project

In the 1980s, several exciting developments with regards to MN assays in humans were starting to emerge, which included the development of improved methods for scoring MNI in lymphocytes, buccal cells and erythrocytes. The cytokinesis-block micronucleus (CBMN) technique, which was first described in the mid-1980s (1,2), significantly improved the reliability of micronucleus (MN) measurements in lymphocytes and led to its exponential adoption by numerous laboratories worldwide and has since been the most widely used method for studying micronuclei (MNI) in human populations. For this reason, it became increasingly evident that the possibility of measuring DNA damage in human populations worldwide was feasible and that there was, therefore, a need for an internationally coordinated effort to achieve this goal reliably and sustainably. The precise point in time of the origin of the HUMN project is difficult to define but the idea crystallised during a chance meeting and discussion about these ideas between Angelo Abbondandolo and M.F. during the international conference 'Chromosome Segregation and Aneuploidy' in Sorrento, Italy, April 24–29, 1995. During the discussion, Angelo recommended that S.B. would be an ideal partner to explore this project concept. After a year of email correspondence, S.B. and M.F. decided to launch the HUMN project at the Seventh International Conference of Environmental Mutagenesis in Toulouse, France, September 7–12, 1997. They were overwhelmed by the great interest in the HUMN project, which had as its primary aims the collection of data worldwide to determine the main variables affecting lymphocyte MN frequencies, the establishment of scoring criteria for this assay, the performance of an inter- and intra-laboratory slide-scoring exercise to validate established scoring criteria and determine reproducibility of scoring across and within laboratories and a prospective study to test the hypothesis that MN frequency in lymphocytes predicts cancer risk. A coordinating group of

founding members was set up, which consisted of M.F., S.B., N.H., W.P.C. and E.Z. to execute and oversee the various aspects of the project [the HUMN coordinating group in 2007 consisted of, in alphabetical order, S.B., W.P.C., M.F.(Chair), N.H., M.K.-V. and E.Z.]. Because of their expertise with the buccal MN assays, S.K., S.B., C.B. and P.T. were included in 2009 to form the HUMN<sub>xL</sub> coordinating group.

Through the efforts of the coordinating group and numerous international collaborators, all the objectives were met successfully within the following 10 years and the resulting publications are among the most cited in the MN field (3–8). The results of these studies showed that inter- and intra-laboratory variation in slide scoring, age, gender and smoking status are important factors affecting observed MN frequency in human lymphocytes. Furthermore, the prospective study did in fact show that a mid- or high-tertile level of MN frequency predicted an increased cancer risk several years later (8). The findings from these studies are explained in more detail below.

### The HUMN project manifesto

The first publication from the HUMN project was a ‘manifesto’ that explained the rationale and objectives of the project (3). The HUMN project was organised to collect data on MN frequencies in different human populations and different cell types. The test procedures considered by this project were assays using human lymphocytes (cytokinesis-block method), exfoliated epithelial cells, as well as other cell types. Data (including descriptions of the populations monitored, detailed test protocols and test results) were voluntarily submitted by a large number of laboratories throughout the world and entered into an unified database. The information was used to: (i) determine the extent of variation of ‘normal’ values for different laboratories and the influence of other factors potentially affecting baseline MN frequency, e.g. age, gender and lifestyle, (ii) provide information on the effect of experimental protocol variations on MN frequency measurements, (iii) design and test optimal protocols for the different cell types and (iv) determine the extent to which MN frequency is a valid biomarker of ageing and risk for diseases such as cancer. It was evident at the time that the lymphocyte CBMN assay was the most widely used and robust method for measuring MN in human populations; for this reason, the initial HUMN project focus during the first 10 years was on this assay.

### International lymphocyte CBMN assay database and analysis of methodological and demographic variables affecting MN frequency

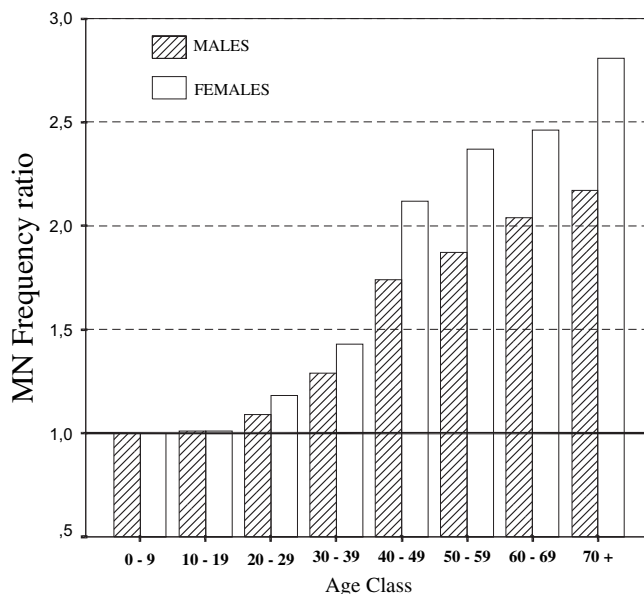
In early 1997, an invitation letter was sent by the HUMN project steering committee to 130 laboratories that had published studies on the MN test in human lymphocytes using the CBMN assay. The letter included a questionnaire for some basic information concerning the data available in each laboratory. The committee received 42 responses and based on that information, a so-called ‘information package’ was prepared and sent to the interested laboratories. All of these laboratories had used the lymphocyte CBMN assay in their investigations. A more sophisticated questionnaire requesting detailed information about the laboratory protocol, scoring criteria, individual data of subjects in the study and references of the published papers was sent with this information package. The identities of the individual participants were not identified by the laboratories. An Excel file, to be used as a template for

submitting original data to the coordinating center (Italian National Research Centre on Cancer, Genoa), was also sent. A total of 25 databases were received from laboratories in 16 countries, mostly in Europe but also in Asia, America, Australia and New Zealand.

This resulted in a database on information for nearly 7000 subjects for whom MN frequency in lymphocytes was determined and the outcomes of the analyses of these data with respect to the effect of laboratory protocol, scoring criteria and host factors were published in 2001 (4). Substantial differences were present in the methods used by participating laboratories, such as in the type of culture medium, the concentration of cytochalasin-B, the percentage of foetal calf serum and in the culture method. Furthermore, discrepancies in criteria for scoring MNi were also evident. Evaluation of the data showed that the overall median MN frequency in non-exposed (i.e. normal) subjects was 6.5 per thousand and the inter-quartile range was between 3 and 12 per thousand. MN frequencies increases monotonically with age in both genders, with the steepest increase after 30 years of age (Figure 1). A more pronounced effect of age in females was confirmed by different female:male MN frequency ratios in age-classes <40 years of age [1.08; 95% confidence interval (CI): 1.03–1.14] and in those over this age (1.23; 95% CI: 1.18–1.28). On average, females were shown to have a 19% higher level of MN frequency (95% CI: 14–24%) compared to males. The best random-effects model, which included genotoxicant exposure factors, host factors, methods and scoring criteria, explained 75% of the total variance, with the largest contribution attributable to laboratory methods (4).

### Outcomes of a slide-scoring exercise using standardised slide-scoring criteria for MNi and nucleoplasmic bridges in lymphocytes

One of the objectives of the HUMN project is to identify the methodological variables that have an important impact on the scored MN frequencies or micronucleated (MNed) cell



**Fig. 1.** Effect of age and gender on MN frequency in peripheral lymphocytes determined using data from the HUMN project. Note: FRs are adjusted by laboratory and exposure to genotoxic agents. All age-classes after 30 years were significantly higher than the reference class (0–9). For more details refer to (4).

frequencies measured in human lymphocytes using the CBMN assay. In the previous study discussed above (4), we had shown that the scoring criteria used were likely to be an important variable. To determine the extent of residual variation when laboratories scored cells from the same cultures using the same set of standard scoring criteria (5), an inter-laboratory slide-scoring exercise was performed among 34 laboratories from 21 countries with a total of 51 slide scorers involved, including some laboratories with more than one scorer (6). The results of this study showed that even under these optimised conditions, there is a great variation in the MN frequency or MNed cell frequency obtained by individual laboratories and scorers. All laboratories correctly ranked the MNed cell frequencies in cells from cultures that were un-irradiated or exposed to 1 or 2 Gy of gamma rays. The study also showed that the intra-scorer median coefficient of variation for duplicate MNed cell frequency scores is 29% for unexposed cultures and 14 and 11% for cells exposed to 1 and 2 Gy, respectively. These values can be used as a standard for quality or acceptability of data in future studies. Using a Poisson regression model, it was estimated that radiation dose explained 67% of the variance, while the staining method, cell sample, laboratory and covariances explained 0.6, 0.3, 6.5 and 25.6% of the variance, respectively, leaving only 3.1% of the variance unexplained. Nucleoplasmic bridges (NPBs) were also estimated by the laboratories, however, inexperience in the use of this biomarker of chromosome rearrangement was reflected in the much greater heterogeneity in the data and the unexplained variation estimated by the Poisson model. The results of these studies (3–6) clearly indicate that even after standardising culture and scoring conditions, it is necessary to calibrate scorers and laboratories if MN, MNed cell and NPB frequencies are to be reliably compared among laboratories and among populations. The calibration procedure involves using a standard set of slides of known MN, MNed and NPB frequency that each scorer has to score and for whom deviation from the real value is recorded. This variation ratio could then be used to correct data to the real value depending on the scorer. These slides can also be used to qualify laboratories for performing the test.

#### Standard protocol and scoring criteria for the CBMN assay with peripheral blood lymphocytes

The slides for the inter-laboratory slide-scoring exercise were prepared using the standard laboratory protocol for the isolated lymphocyte CBMN assay established by Michael Fenech's laboratory in Australia. This protocol for isolated lymphocytes as well as that for whole blood cultures was described in detail and published in 2007 (9). It also includes the scoring criteria used in the slide scoring comparison exercise and is the recommended method for performing the CBMN assay in human peripheral blood lymphocytes. The detailed scoring criteria used in the slide-scoring exercise study described above were also published separately (5). Criteria for scoring (i) MNi, NPBs and nuclear buds in binucleated cells, (ii) mononucleated cells, binucleated cells and multinucleated cells and (iii) necrotic and apoptotic cells are described in these papers. These different end points and scoring criteria are illustrated by a series of schematic diagrams as well as a comprehensive set of colour photographs that are of practical assistance during the scoring of slides. It is expected that these scoring criteria will assist in the development of a procedure for calibrating scorers and laboratories performing the lymphocyte CBMN assay so

that results from different laboratories for the CBMN assay may be more comparable in the future.

It is also important to note that this detailed CBMN assay protocol in lymphocytes, which was originally developed for *in vivo* biomonitoring in humans, also served as the basis for the validation of the lymphocyte CBMN assay for genotoxicity testing *in vitro*, which is now incorporated in the OECD guidelines that were established for this purpose (10). As a consequence, we have for the first time the possibility to use the same lymphocyte CBMN assay standard to measure the genetic impacts of a physical or chemical agent both *in vitro* and *in vivo*. This will allow cross-validation and testing of the predictivity of the lymphocyte CBMN *in vitro* test with respect to *ex vivo* MN induction in lymphocytes of human populations exposed to physical or chemical agents that were tested *in vitro* with the same test.

#### A definitive study on the effect of smoking on MN in peripheral blood lymphocytes

The effect of tobacco smoking on the frequency of MNi in human lymphocytes has been the object of many population studies. In most reports, the results were unexpectedly negative, and in some instances, smokers had lower frequencies of MN than non-smokers. An analysis of the pooled 24 databases from the HUMN international collaborative project was performed with the aim of understanding the impact of smoking habits on MN frequency (7). The complete database included 5710 subjects, with 3501 non-smokers, 1409 current-smokers and 800 former-smokers, in studies of exposure to occupational and environmental genotoxicants. The analysis of the combined databases confirmed the small decrease of MN frequencies in current-smokers [frequency ratio (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 smoked per day among subjects occupationally exposed to genotoxic agents. However, a typical J-shaped curve was observed for the smokers who were not exposed to genotoxic agents with a significant increase in MN frequency occurring in individuals smoking  $\geq 30$  cigarettes/day (FR = 1.59, 95% CI:1.35–1.88). This analysis confirmed that smokers generally do not experience an overall increase in MN frequency. However, when the interaction with occupational exposure was taken into account, heavy smokers were the only group showing a significant increase in genotoxic damage as measured by the CBMN assay in lymphocytes. Based on these results, some general recommendations for the design of biomonitoring studies involving smokers can be formulated. Quantitative data about smoking habit should always be collected because, in the absence of such data, the simple comparison of smokers versus non-smokers could be misleading. The subgroup of heavy smokers ( $\geq 30$  cigarettes/day) should be specifically evaluated whenever it is large enough to satisfy statistical requirements. The presence of an interaction between smoking habit and occupational exposure to genotoxic agents should always be tested.

Since this study was published, it was shown that spontaneous and cigarette smoke nitrosamine-induced frequencies of MNi, NPBs and nuclear buds in lymphocytes of smokers who develop lung cancer are higher than those of matched smoker controls who do not develop lung cancer (11,12). These results suggest that DNA damage biomarkers in the CBMN assay are only likely

to be substantially elevated as a result of smoking in those with abnormally high susceptibility to carcinogenic effects of genotoxicants contained in cigarette smoke. Whether the elevated MN frequency in smokers with lung cancer is only due to increased susceptibility to cigarette smoke genotoxicants or also due to the presence of cancer itself will not be known until comparisons are also made for MN frequency between non-smoker and smoker lung cancer cases.

### The risk for cancer in the HUMN project cohorts is predicted by MN frequency in peripheral blood.

The ultimate objective of the HUMN project studies was to test the hypothesis that an elevated MN frequency in human tissues is predictive of cancer risk (8). This critical validation step is essential to justify the use of such techniques in human biomonitoring studies in populations, which are suspected to be at a higher cancer risk due to inappropriate environmental or diet and lifestyle exposures that may be genotoxic and to test whether preventive strategies could actually reduce DNA damage. Therefore, although much theoretical evidence has been accumulated supporting the causal role of MN induction in cancer development, prospective cohort studies are needed to validate MN as a cancer risk biomarker. A total of 6718 subjects from 10 countries, screened in 20 laboratories for MN frequency between 1980 and 2002 in *ad hoc* studies or routine cytogenetic surveillance, were selected from the database of the HUMN international collaborative project and followed up for cancer incidence or mortality. To standardise for the inter-laboratory variability, the subjects were classified according to the percentiles of MN distribution within each laboratory as low, medium or high frequency. A significant increase of all cancers incidence was found for subjects in the groups with medium (RR = 1.84; 95% CI: 1.28–2.66) and high MN frequency (RR = 1.53; 95% CI: 1.04–2.25) (8). The same groups also showed a decreased cancer-free survival, i.e.  $P = 0.001$  and  $P = 0.025$ , respectively. This association was present in all national cohorts and for all major cancer sites, especially urogenital (RR = 2.80; 95% CI: 1.17–6.73) and gastrointestinal cancers (RR = 1.74; 95% CI: 1.01–4.71). The results from this study provide evidence that MN frequency in peripheral blood lymphocytes is a predictive biomarker of cancer risk within a population of healthy subjects. Other studies have since been published showing that a higher MN frequency in lymphocytes measured using the CBMN assay is associated prospectively with increased pregnancy complications (13) and cardiovascular disease mortality (14,15).

The success of the HUMN project with the peripheral blood lymphocyte CBMN assay resulted in further collaborations on pooled analyses of related databases to test the impact of genotype (16–18) although this is not discussed here as it is the subject of another review in this special issue (19). Furthermore, it brought to attention the need to achieve similar advances with the MN assay in buccal cells in which there is an increasing interest because of their ease of collection, their proximity to food and air-borne genotoxicants and relevance to carcinogenesis in epithelial tissues.

### The HUMN<sub>xL</sub> project on MN frequencies in human buccal cells

The MN assay in exfoliated buccal cells is a minimally invasive and potentially useful method for monitoring genetic

damage in humans. Although this assay has been used since the 1980s to demonstrate cytogenetic effects of environmental and occupational exposures, lifestyle factors, dietary deficiencies and different diseases, important knowledge gaps remain about the characteristics of MNi and other nuclear abnormalities, the basic biology explaining the appearance of various cell types in buccal mucosa samples and effects of diverse staining procedures and scoring criteria in laboratories around the world and their relationship to disease states and outcomes (20). To address these uncertainties, the HUMN project coordinating group initiated a new international validation project for the buccal cell MN assay similar to the project previously performed using human lymphocytes (as described above) (21–23). To distinguish it from the lymphocyte project, this project was given the acronym HUMN<sub>xL</sub>, i.e. human MN assay in exfoliated buccal cells. The planned research will explore sources of variability in the assay (e.g. between laboratories and scorers, as well as inter- and intra-individual differences in subjects) and resolve key technical issues, such as the method of buccal cell collection and staining, optimal criteria for classification of normal and degenerated cells and for scoring MNi and other abnormalities. The harmonisation and standardisation of the buccal MN assay will allow a more reliable comparison of the data among human populations and laboratories, evaluation of the assay's performance and consolidation of its worldwide use for biomonitoring of DNA damage.

To further consolidate this initiative, the first HUMN<sub>xL</sub> project workshop on the Buccal MN Assay was held at the International Conference on Environmental Mutagens in Human Populations in Antalya, Turkey in 2007 (22). It was attended by 70 representatives from various laboratories, universities, private companies and government departments from around the world. The aims of the workshop were to (i) discuss current state of knowledge on the buccal MN assay, (ii) identify important gaps of knowledge regarding theory, biology and methods, (iii) decide on a plan of action to resolve the key methodological and knowledge gap issues and (iv) explore the possibility of the pooling of databases to determine the most important variables affecting the assay. It was agreed at the workshop that four activities should be initiated as soon as possible, namely (i) a method for collection of databases from different laboratories, (ii) writing of a protocol based on the most commonly used and best-validated methods, (iii) development of slide scoring procedures and (iv) an inter-laboratory slide-scoring exercise, in this order. A follow-up workshop was held at the 10th International Conference on Environmental Mutagens in Florence in 2009 in which progress on these activities was reported. The following has been achieved so far.

1. A systematic review on the current status and knowledge gaps regarding the buccal MN assay was completed and published (20). The review concluded that although many studies have consistently shown a statistically significant increases in the buccal cell MN frequency in human populations exposed to genotoxic agents or a decrease as a result of vitamin supplementation or chemopreventive measures, the magnitudes of the changes are usually relatively small. Different confounding factors influencing the MN frequency in peripheral lymphocytes, such as gender, age and lifestyle habits have been considered for the buccal cell MN assay. However, the majority of studies failed to demonstrate any influence of age or sex, although only a few studies have

investigated a broad age range. The low baseline MN frequency in buccal cells may amplify the statistical problems in the scoring but at the same time provide a low background level against which genotoxic effects may be observed more readily.

In this context, the review considered that it was mandatory to standardise the buccal cell MN assay protocol, including the scoring criteria and that an inter-laboratory calibration exercise be organised to validate the protocol. This standardised method would enable collection of data from different laboratories and different countries, which are more comparable and minimise uncertainty due to methodological variables and scoring criteria. In addition, the development of automated scoring systems is encouraged as a critical advancement for high-throughput and statistically powerful analysis.

2. A detailed protocol for buccal cell collection, slide preparation and scoring of cells based on the best available methods was in fact prepared and published (24). This protocol describes one of the current established methods for buccal cell collection using a small-headed toothbrush, the generation of a single-cell suspension, slide preparation using cytocentrifugation, fixation and staining using Feulgen and Light Green for both bright field and fluorescence microscopic analysis. Also, the scoring criteria for MNi and other nuclear anomalies are described in detail. The protocol in its current form takes ~4 h to complete from the time of buccal cell collection to the generation of stained slides for microscopic analysis.

3. A survey of the current use and practice of the buccal MN assay was completed as an initial step in the HUMN<sub>xL</sub> project initiative (23). An invitation to join the HUMN<sub>xL</sub> project was sent out together with a questionnaire to all laboratories that have published on the buccal MN assay. Overall, 188 messages were delivered and 58 laboratories from 25 countries agreed to participate (43 indicating intention to contribute buccal MN data). The questionnaire was designed to collect methodological information regarding the laboratory's performance of the assay and to assess the extent and type of epidemiological data that are routinely collected. The results provided an overview of the most commonly used methods for buccal cell collection and preparation, slide preparation, staining, scoring criteria and an evaluation of epidemiological data, including demographics, genetic background, gender, health status, occupation, exposure, lifestyle and dietary habits. According to this survey, a potential database of up to 15 000 subjects may be available for future pooled analyses. A number of protocol discrepancies emerged, implying that standardisation of the method is a major priority. The results of this survey will (i) identify technical and epidemiological key variables that impact on buccal MN frequency in human populations, (ii) drive the design of future intra- and inter-laboratory validation studies and (iii) determine the role of buccal MN frequency and other biomarkers, in monitoring genomic damage and predicting cancer and other degenerative diseases.

4. At the time of writing this paper, 29 databases were received from laboratories located in 16 countries and the combined HUMN<sub>xL</sub> database currently contains results for 5157 subjects. This database is being analysed to determine the association of buccal MN frequency with methodological, exposure, diet, lifestyle and demographic variables.

5. A systematic review of recent literature on the buccal MN assay was carried out to provide a state-of-the-art evaluation of how critical topics such as control for confounding, sample size and statistical power, number of cells scored, end point selection and statistical modelling are considered (25). In

addition, a meta-analysis was performed on the combined database to estimate the impact of most common confounders on MN frequency and to provide a baseline value and range of MN frequency in the control population. (23,25). Age, gender and smoking habit were the most commonly studied confounders being reported in 98.4, 85.7 and 90.5% of databases, respectively. Univariate statistics were estimated in most studies while multivariate statistical analysis was applied only in the 47.6% of studies. The mean baseline MN frequency in controls was 1.10/1000 cells (95% CI: 0.70–1.72), and the relative increment in subjects exposed to genotoxic agents or affected by disease correlated with similar observations in lymphocytes ( $R^2 = 0.74$ ). A minimum sample number of 4000 cells is recommended to reduce the variability of the MN mean estimates, in contrast to the current practice of scoring only 2000 cells (in 81% of submitted databases). The authors of this review recommended the use of Poisson or Negative Binomial statistical models when >2000 cells are scored and that studies scoring smaller numbers of cells should consider the use of statistical models taking into account the excess of zeros, e.g. the Zero Inflated Poisson (ZIP) models. Furthermore, the review (25) concluded that the quality of papers published on the buccal MN assay can be substantially improved, with better consideration of basic issues such as power analysis, control for confounding, choice of the statistical model and the number of cells to be scored.

### Future challenges

Although the HUMN and HUMN<sub>xL</sub> projects have been largely successful in advancing our knowledge of the use of MN assays to study DNA damage in humans, there are still several challenges to be met. The following is a list of these challenges, the sequence of which is not necessarily related to priority.

(a) MN assays in other tissues such as erythrocytes, nasal epithelium and hair root cells have been shown previously to be of relevance to human studies in specific scenarios (e.g. erythrocytes in micronutrient deficiency and nasal epithelium in air pollution) (26,27). Protocols for these systems have been reported but these have not been standardised or validated with respect to variables that may be expected to affect baseline frequencies and in terms of prospective association with disease outcomes. A focus on MN assays in these other tissues will eventually occur once the HUMN<sub>xL</sub> inter-laboratory slide-scoring exercise is completed. The latter exercise is due to commence late in 2010 and those interested in participating should contact the corresponding author of this paper.

(b) Up to now, only the lymphocyte MN assay has been tested for its sensitivity to the impact of diet and lifestyle factors and the studies reported to date are sparse. A greater effort with large well-defined populations is required to replicate these initial studies and identify those diet and lifestyle patterns that associate with reduced or increased MN frequencies.

(c) Although several studies have reported on the impact on MN frequency of common single-nucleotide polymorphisms (SNPs) in candidate genes involved in metabolic pathways that may directly or indirectly affect genome stability, most of these studies were underpowered (19). Furthermore, it is important that genome-wide association studies using random SNP analysis be performed with large cohorts to identify unknown genes that may strongly affect MN frequency or to verify those that have been identified as being determinants of this biomarker when mutated.

(d) An important development in MN assays is the adoption of the cytochrome approach that not only scores MN but also captures other nuclear abnormalities such as nuclear buds and NPBs as well as capturing frequencies of necrotic and apoptotic cells as well as the proportion of cells undergoing cell division (9,24). In the cytochrome approach, even MN in non-divided cells may need to be considered due to alternative mechanisms of MN formation such as nuclear budding or in the case of lymphocytes due to pre-existing MN expressed in aberrant *in vivo* nuclear divisions (9,28). The comprehensive micronucleus cytochrome approach is increasingly being adopted as it enables all major nuclear anomalies and cytotoxicity events to be captured simultaneously. It will take considerable effort to validate the other biomarkers in the cytochrome system but this will become necessary if the addition of these indices improves association with genotoxicant exposures and/or disease outcomes as has been suggested by a recent study on the association between the CBMN cytochrome assay biomarkers in lymphocytes and lung cancer (11,12).

(e) During the past decade, great advances have occurred that have enabled automated scoring of MNi as well as high-content analysis of MNi and nuclei using molecular probes that provide additional information on DNA damage mechanism (e.g. centromere and telomere detection in MNi and nuclei). Procedures and reagents for these methods require standardisation particularly if they are to be used on a routine basis for MN analyses.

(f) Larger and/or longer studies are required to verify the results of previous studies concerning the association of MN frequency with pregnancy complications, cancer and cardiovascular disease (8,13–15). Prospective studies of MN in cells from the umbilical cord (at birth) with respect to association with cancers in childhood and later in life are as yet uninvestigated and deserve attention given that the carcinogenic risk from DNA damage early in life may be of great significance. Associations with other diseases such as Alzheimer's disease, Parkinson's disease and diabetes have been reported in cross-sectional studies (29,30) but prospective data are required for ultimate validation of the MN biomarker as a predictor of these degenerative diseases.

(g) Future studies should also explore the relationship of MN expression with changes in DNA methylation and the associated transcriptome, metabolome and proteome profiles to unravel the underlying molecular mechanisms that correlate with this DNA damage biomarker. This 'omic' data could provide valuable information on the likely origin of MN when the exposure profile is unknown or difficult to ascertain.

In conclusion, the activities of the HUMN and HUMN<sub>xL</sub> projects have transformed the approach to MN assay validation, standardised the performance of the assay and scoring procedures and facilitated the wide-spread adoption of this valuable technology as a tool for investigating the most fundamental pathology of the human condition, i.e. damage to the human genome. The ultimate goal is to see the validated MN assays becoming a routine diagnostic in the new disease prevention paradigms and strategies required for this new millennium based on personalised prevention of DNA damage.

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