Inter-laboratory Consistency and Variability in the Buccal Micronucleus Cytome Assay depends on biomarker scored and laboratory experience: Results from the HUMNxl International Inter-laboratory Scoring Exercise.

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Inter-laboratory Consistency and Variability in the Buccal Micronucleus Cytome Assay depends on biomarker scored and laboratory experience: Results from the HUMNxl International Inter-laboratory Scoring Exercise.

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Running title: Inter-laboratory variability in Buccal MN Assay
Abstract

The buccal micronucleus cytome (BMNcyt) assay in uncultured exfoliated epithelial cells from oral mucosa is widely applied in biomonitoring the exposure to genotoxic agents and is also proposed as a suitable test for prescreening and follow up of precancerous oral lesions. The main limitation of the assay is the large variability observed in the baseline values of micronuclei (MNi) and other nuclear anomalies mainly related to different scoring criteria. The aim of this international collaborative study, involving laboratories with different level of experience, was to evaluate the inter- and intra-laboratory variations in the BMNcyt parameters, using recently implemented guidelines, in scoring cells from the same pooled samples obtained from healthy subjects (control group) and from cancer patients undergoing radiotherapy (treated group). The results indicate that all laboratories correctly discriminated samples from two groups by a significant increase of MN and NBUD frequencies and differentiated binucleated (BN) cells, associated with the exposure to ionizing radiation. The experience of the laboratories was shown to play an important role in the identification of the different cell types and nuclear anomalies. MN frequency in differentiated mono- and BN cells showed the greatest consistency among the laboratories and low variability was also detected in the frequencies of mono and BN cells. A larger variability was observed in classifying the different cell types, indicating the subjectivity in the interpretation of some of the scoring criteria while reproducibility of the results between scoring sessions was very good. An inter-calibration exercise is strongly recommended before starting studies with BMNcyt assay involving multiple research centers.
Introduction

The buccal micronucleus cytome (BMNcyt) assay in uncultured exfoliated epithelial cells from oral mucosa is a minimally invasive approach for evaluating genomic damage, chromosomal instability and cell death in human aerodigestive tract (1-5). The assay was widely applied in biomonitoring studies to evaluate occupational or environmental exposures to genotoxins (1, 3), medical procedures (5-7), micronutrient deficiencies (8,9), lifestyle factors (10-12) and genetic susceptibility (13-16). Moreover, increased micronucleus (MN) frequencies in exfoliated buccal cells were associated with the risk of developing cancer and degenerative diseases (17). Furthermore, the test seems to be a promising approach for prescreening and follow up of precancerous oral lesions (17).

The large majority of MN studies in buccal cells are focused on the evaluation of the MN frequency(1-3). MNi are a biomarker for structural and/or numerical chromosome aberrations because MNi are a consequence of the formation of acentric chromosome fragments or lagging whole chromosomes, not properly segregated during mitotic anaphase and excluded from the main daughter nuclei (18). However, cell death biomarkers such as pyknosis, karyolysis and karyorrhexis were also evaluated in some studies, because their inclusion in assessment provides a more comprehensive evaluation of cytotoxicity and could increase the sensitivity and scope of biomonitoring studies (19-23). It is for this reason that the buccal MN assay involves the novel cytome approach now adopted almost universally amongst MN tests including the most widely used method, the lymphocyte cytokinesis-block MN cytome assay (18).

The main limitation in the practical application of the MN cytome assays in clinics as well as for monitoring occupational and environmental exposures is the variability among the studies. Host factors, such as interindividual variability in metabolism, DNA repair capacity, cell proliferation, multiple exposures to exogenous and endogenous agents alter the cell differentiation and could interfere with MN expression and susceptibility to cell death. Moreover, methodological differences in collection of cells, fixation,
staining procedures, number of cells counted and scoring criteria has been shown to affect the results (1, 24, 25).

In 2007 the international HUMN (Human Micronucleus) project (www.humn.org) coordinating group started a validation project of the MN assay in exfoliated buccal cells (26, 27). A detailed standardized protocol for buccal cell collection, slide preparation and scoring was established by taking into account the available procedures, confounding factors and staining artifacts (28, 29).

An analysis of data obtained in 58 worldwide laboratories that reported the use of the assay in international peer-reviewed journals was carried out. The most important and statistically significant source of variability for the MN frequency, was shown to be the method used for staining buccal cells with further effects depending on the cell collection method and scoring criteria used (30). A comprehensive set of photomicrographs with multiple examples of each cell type and nuclear anomalies associated with detailed scoring criteria was also recently published by the HUMNxl project, as a basis for a slide scoring exercise to assess the inter-laboratory variability (31).

A first exercise, carried out among three experienced laboratories scoring the same set of slides, showed a good agreement among the laboratories in regard to the main parameters associated with the evaluation of chromosomal damage (MN in mononucleated and binucleated differentiated cells), while some inconsistencies were observed in the identification of cell types and/or nuclear anomalies, such as the frequencies of basal cells, binucleated cells condensed chromatin (CC) and karyorrhectic cells (KHC) (32).

On the basis of these results guidelines for slide scoring were improved by better defining the nucleus: cytoplasm area ratio to characterize basal, transitional and differentiated cells, by combining CC and KHC cells anomalies associated with cell death into a single CC/KHC and by more carefully classifying the different types of nuclear buds (NBUDs) to avoid misclassification as BN cells.

Subsequently, as a follow-up of this initiative, an international inter-laboratory scoring exercise was organized, which included laboratories with different level of experience.

The aims of this expanded collaborative study were:
a) to evaluate the inter-laboratory variations in the buccal cytome assay scores when a common set of improved criteria are used in scoring cells from the same pooled samples

b) to determine the extent of variation in the scores obtained by different scorers from the same lab using the same set of criteria and identical slides

c) to determine the extent of variation in the results obtained by the same scorer using the same set of criteria and the same slides

This paper describes the outcomes of this inter-laboratory scoring exercise conducted by 13 laboratories from ten countries.

Materials and Methods

A preparatory one week training course for laboratories who expressed their interest in participating in this exercise was organized in parallel in Genoa, Environmental Carcinogenesis Unit, IRCCS AOU San Martino IST- Istituto Nazionale per la Ricerca sul Cancro, and in Vienna Institute of Cancer Research- Medical University in September-October 2014. The program of the course was coordinated between the organizing laboratories and involved the application of the standardized BMNcyt protocol and scoring criteria.

The course was available to laboratories with different level of experience, from those with many years and substantial number of publications on the BMcyt to the laboratories that only recently started to apply the assay. Its purpose was to present the improved guidelines for slide scoring developed on the basis of the results of the first inter-calibration exercise among the references laboratories. The course involved sample preparation, staining and scoring of the different cellular types and nuclear alterations.

Subject recruitment

The study was approved by the local Ethical Advisory Board at the IRCCS A.O.U. San Martino-IST, in Genoa. The study involved a total of 13 participants including healthy controls and head and neck cancer patients undergoing radiotherapy. Eight head and neck cancer patients (5 males and 3 females aged 56-77 yrs., including 4 smokers, 2 ex-smokers and 3 nonsmokers) and 4 age-matched healthy controls (3 females and 1
male age 30-60 yrs, all nonsmokers) were recruited at the Environmental Carcinogenesis Unit, IRCCS A.O.U.
San Martino-IST, Genoa, Italy. The range of the cumulative radiation dose received by the head and neck
cancer patients in oral region of the head was between 20 and 22 Gy.

**Buccal cell collection, slide preparation and staining**

Buccal cells were collected from both cheeks using a small-headed tooth brush following the standardized
protocol (28). Samples were transferred from the toothbrush into Saccomanno’s fixative (Histoline
laboratories, Milano, Italy) and stored at 4°C for 1-2 months until processing.

Samples were processed at the Environmental Carcinogenesis Unit, IRCCS AUO San Martino IST- Istituto
Nazionale per la Ricerca sul Cancro, Genoa, Italy following the previously reported standardized protocol
(28). Briefly, exfoliated buccal cells were transferred into centrifugation tubes and spun for 10 min at 580g
at room temperature. The supernatants were removed and replaced with 10 ml of buccal cell buffer (0.01M
Tris–HCl (Sigma T-3253), 0.1M EDTA tetra sodium salt (Sigma E-5391) 0.02 M sodium chloride (Sigma
S5886) at pH 7.0. Cells were re-suspended and spun at 580 g for 10 min, then washed twice in the same
buffer. The suspensions were briefly vortexed and then homogenized for 2-3 min in a hand homogenizer
(Wheaton Scientific 0.1–0.15 mm gauge) at medium intensity, to increase the number of single cells in the
suspensions. Samples from subjects belonging to each group (cancer patients and healthy controls) were
pooled in a 50 ml tube and drawn into a syringe with a 21G gauge needle (Becton Dickinson and Company
Limited, Drogeda, Ireland), then expelled to improve cellular separation. Subsequently the cells were
passed through a 100 μm nylon filter (Millipore, code NYH02500) held in a swinnex holder (Millipore, code
SX0002500) to remove large cell aggregates. Cells were further spun at 580 g for 10 min and the
supernatant removed. They were re-suspended in 10 ml of buccal cell buffer and the cell concentration
determined using a counting chamber (Burker hemocytometer). Cell suspensions were prepared containing
80,000 cells/ml of buccal cell buffer. Fifty μl of dimethyl sulphoxide (Sigma D1435) per milliliter of cell
suspension were added to further improve cell separation. 120 μl of each cell suspension were added to
each cytospin cup and spun at 40.65 g (600 rpm) for 5 min in a cytocentrifuge (Shandon Cytospin 3, Thermo
Electron Corporation). Slides containing one spot of cells were air dried for 10 min and then fixed in ethanol:acetic acid (3:1) for 10 min. Subsequently, slides were air dried for at least 10 min.

Fixed slides were treated for 1 min each in 50% ethanol, and then 20% ethanol and subsequently washed with deionized water for 2 min prior to staining. The slides were then treated with 5.0 M hydrochloric acid for 30 min at room temperature followed by washing for 3 min in running tap water. Moist slides were treated with Schiff’s reagent (Sigma 3552016) at room temperature in the dark for 60 min, washed in running tap water for 5 min and rinsed in deionized water. Slides were stained in 0.2% Light Green (Histoline Laboratories, Polyscience Inc code 02753) for 30 sec, rinsed in deionized water and allowed to air dry at room temperature prior to being mounted in DPX mounting medium (a mixture of Distyrene, a Plasticizer and Xilene) (Sigma 44581).

Twenty-five slides were prepared from each pooled sample from the two different groups of subjects, treated cancer patients and controls.

Scoring process

Three laboratories who were involved in the development of the revised BMNcyt scoring methodology (28, 31) and in the first inter-laboratory scoring comparison (32) participated in this exercise as reference laboratories: 1) the Environmental Carcinogenesis Unit, IRCCS AUO San Martino- IST- Istituto Nazionale Ricerca sul Cancro, Genova, Italy; 2) the Institute of Cancer Research, Medical University Vienna, Vienna, Austria, and 3) CSIRO Food and Nutrition, Adelaide, Australia. These reference laboratories are referred to as Lab R1, Lab R2 and Lab R3.

The laboratories participating in the International inter-laboratory scoring exercise are listed Table 1. Each participating lab was provided with: 1) a standard set of scoring criteria (28, 31); and 2) two stained slides, one from controls and 2nd one from treated patients pooled cells. Slides were coded so the scorers were not aware of the identity of the group they belong to.
All laboratories provided information on the level of experience of the scorers with the BMNcyt assay and with the HUMNxl scoring criteria. Some of the laboratories with more than 1 scorer had a different level of experience among the participants, ranging from many years in the field to recently trained scorers.

Coded slides were scored using transmitted light at x 1,000 magnification. Mononucleated and binucleated cells containing MNi or NBUDs on bright field were confirmed as being positive by also examining the cells under far red fluorescence as previously described (28, 31). The scorer(s) from each laboratory evaluated the slides twice. A stepwise approach was applied to score the slides. First, the frequencies of various cell types were evaluated in 1000 cells. The cell types scored included: [i] Differentiated Mononuclear (DIFF_MONO); [ii] Differentiated Binucleated (DIFF_BN); [iii] Basal (BASAL); [iv] Condensed Chromatin (CC); [v] Karyorrhectic (KHC); [vi] Karyolytic (KYL) and [vii] Pycnotic (PYC) cells were identified following the detailed criteria as previously described (28, 31). In a second step of analysis, 2,000 DIFF_MONO cells were scored for nuclear abnormalities indicative of chromosomal instability or DNA damage, specifically [viii] mononucleated cells with MNi (MNC_MONO) and [ix] mononucleated cells with NBUDs (NBUDC_MONO) as well as [x] Micronuclei (MNI_MONO) and [xi] Nuclear Buds (NBUDS_MONO) in the same type of cells. In a third step, nuclear abnormalities (MNI and NBUDs) were scored in 100 binucleated differentiated (DIFF_BN) cells representing by Binucleated: [xii] Micronucleus Cells (MNC_BN), [xiii] Micronuclei (MNI_BN), [xiv] Nuclear Bud Cells (NBUDC_BN) and [xv] Nuclear, Buds (NBUDS_BN).

**Statistical methods**

Description of results was made by reporting means and standard deviation of every parameter from each laboratory, session and scorer. For each biomarker the ratio between the means of treated and controls (i.e. Mean Ratio) (17), stratified by laboratory, and its 95% confidence interval were computed and the results were visualized in forest-plots using the logarithmic scale. To study differences between laboratories the results from participating laboratories were compared with the pooled data of the three reference laboratories (Ref1, Ref2, Ref3, see Table 1). The analysis was performed using the Poisson regression model adjusting by the effect of treatment. We verified the suitability of the Poisson model with a goodness of fit test (33). If the test reveals the occurrence of extra-Poisson variability, i.e. overdispersion,
the negative binomial regression model was applied for fitting data (33). Finally, we applied to the data the multilevel mixed-effects Poisson regression models (34). This model allows to estimate two types of parameters: the fixed and random effect. Roughly speaking fixed effect is the difference between the means of 2 levels of a covariate while the random effect estimates the variance around the mean of a cluster in which data are organized (the laboratories in our case), allowing us to estimate the heterogeneity between laboratories and between scorers within laboratories. In this model we included as fixed effects the treatment (i.e. whether the result was from the healthy controls or the cancer patients treated with radiotherapy), and the laboratory training, encoded by a binary variable (yes/no), i.e. if its participating staff members were experienced in the use of the latest scoring procedure (31). The aim of this exercise was to estimate the residual variability between laboratories and scorers after adjusting for the level of experience which is the most noticeable variable among the laboratories. P values <0.05 were considered to be statistically significant. All analyses were performed using Stata 13 Software (35).

**Results**

Table 2 contains data for each BMNcyt biomarker for all participating laboratories. The means appear more homogeneous for DIFF_MONO with a coefficient of variation (CV) of 9% while PYC is the marker that exhibits the higher heterogeneity between the laboratories (CV 146%). Most agreement in data is found for MNC_BN/MNI_BN (CV 34% / 39%) in comparison to MNC_MONO/MNI_MONO (CV 59% / 60%). The same is true for NBUDs that were assessed by the laboratories more evenly in binucleated cells than in mononucleated cells.

Figure 1 shows distribution of BMNcyt marker values expressed as Mean Ratio in pooled buccal cells from cancer patients in comparison to healthy controls. The purpose of this analysis was to verify which marker discriminated the two sample groups better and the extent of its consistency between laboratories. It is clear that frequency of MNC_MONO and NBUDC_MONO together with DIFF_BN distinguish the two groups well since either all or almost all the confidence intervals do not include the zero value which, in logarithmic scale, indicates no difference between irradiated cells and controls. KHC appears to be a less sensitive
biomarker in discriminating the two groups. These results are consistent among the laboratories although the effect estimates vary.

The data for some of the laboratories in which statistically significant medium percent changes (MPC) with respect to reference laboratories were detected (i.e. (MR-1)*100, for each biomarker, as estimated from regression models adjusting by treatment) are shown in Figure 2. MNC_MONO and MNI_MONO showed the greatest consistency among the laboratories, followed by MNC_BN and MNI_BN and DIFF-MONO values. No statistically significant difference in MNC_MONO or MNI_MONO frequencies was observed in less experienced laboratories in comparison with the reference laboratories’ data with the exception of one laboratory recording lower counts and two having higher counts. MNC_BN values (MPC range -56%/-44%) and MNI_BN (MPC range -63%/-41%) do not show significant differences among laboratories.

DIFF_MONO results from the participating laboratories had only small variations relative to the reference laboratories with MPC ranging between -18% and +16%; in DIFF_BN the variation was slightly larger compared to DIFF_MONO (MPC range -44%/+54%). The rates for basal cells frequencies deviated significantly from those of the reference laboratories in a few of the less experienced laboratories (MPC range -80%+/433%).

MPCs for CC cell frequency ranged from -89% and +273%. KHC cell frequency differed between the reference and many less experienced laboratories, but the magnitude of the differences was smaller compared to other markers (MPC range -66%/+213%). Combining the CC + KHC values (MPC range -71%/+177%) resulted in a reduction of the heterogeneity among the laboratories for these cell death markers. KYL (MPC range -51%/+108%) revealed only a modest agreement between all laboratories. Even larger differences were seen for PYC cells relative to the reference laboratories (MPC range -96%/+665%). A number of participating laboratories differed from the reference laboratories for the values of all NBUDs to a great extent: NBUDC_MONO (MPC range +93%/+814%), NBUDC_BN (MPC range +329%/+401%), NBUDS_MONO (MPC range +102%/+811%), NBUDS_BN (MPC range +427%/+433%), that indicates that these biomarkers are not as consistently evaluated using the current criteria, and may be less reliable.
Table 3 reports the effect of the scoring session (repeated analysis by the same scorer at a different time) after adjustment for (i) treatment (i.e. treated patients or controls), and (ii) between laboratories heterogeneity. The mean ratios (MR) are almost all close to one, with the exception of BASAL and PYK, and no MR is statistically significant indicating a good agreement between two scoring sessions.

A statistically significant effect of treatment was observed for all biomarkers with the exception of PYK using multilevel mixed-effect Poisson regression model (Table 4). All markers were elevated in the cancer patients with the exceptions of DIFF_MONO, CC, KHC, CC + KHC. MN frequencies in MONO_DIFF and in BN_DIFF cells seem to be the most suitable markers to detect the genotoxic effects of irradiation exposure in buccal cells.

Considering the “experience” factor we observed statistically significant effects for DIFF_MONO, BASAL, CC, CC + KHC and PYK and, with exception of DIFF_MONO, less experience in scoring the slides leads to an overestimation in the counting of these parameters.

After removing the effects of “treatment” and “experience”, the heterogeneity of scoring between laboratories remains statistically significant for DIFF_MONO, BASAL, CC, and KHC while the variability of the scorers within laboratories remains below the significance threshold. MNC and MNI appear as the most robust biomarkers of the BMNcyt assay for which there is the best agreement between laboratories.

Discussion

The practical application of the BMNcyt assay in clinics as well for monitoring occupational exposures implies the identification of the factors responsible for the large variability observed in the baseline values of MNi and other nuclear abnormalities in published studies (26, 30).

The present study focused on the analysis of the variability attributable to the scoring process which is the critical step in the buccal micronucleus cytome assay.
A previous scoring exercise produced evidence for good agreement among the three experienced reference laboratories on the main parameters associated with the evaluation of chromosomal damage, such as MNi in mononucleated and binucleated differentiated cells (32).

The aim of this international collaborative study involving laboratories with different level of experience was to evaluate the inter- and intra-laboratory variation in the BMNcyt assay results using a common set of criteria by scoring cells from two pooled samples from treated cancer patients and controls with different frequencies of cell types and nuclear abnormalities.

The key result of this exercise is that all participating laboratories correctly discriminated the irradiated samples from the control cells, as shown by a statistically significant increase in the MN and NBUD frequencies and in the number of differentiated BN cells. However, the scores of the other cells types included in the BMNcyt assay approach such as basal BN, CC and KH cells did not show a consistent effect related to the radiation exposure, and therefore were deemed less reliable using current set of criteria.

The main target of ionizing radiation is the DNA molecule. Double-strand breaks (DSBs) and non-DSB highly clustered DNA lesions, consisting of a combination of single-strand breaks (SSBs), abasic sites and oxidized bases, expressed as chromosomal aberrations are the main effects induced by the exposure to ionizing radiations (35, 36). The dicentric metaphase assay and the cytokinesis-block MN test in peripheral lymphocytes are the best validated methods for bio-dosimetry and diagnostic dose-indicators recommended by the International Atomic Energy Agency (37). The frequency of cell types measuring the differentiation process and cell death, included in the BMNcyt assay approach, are mainly associated with cytotoxic effects or normal morphogenesis in buccal mucosa and only partly linked with radiation damage.

The inter-laboratory variation among participating laboratories was evaluated through the comparison with the data from the reference laboratories. The parameters showing the greatest consistency among the laboratories are MN frequencies in mono and binucleated differentiated cells. Furthermore, the numbers for the frequencies of differentiated mononucleated and binucleated cells had low variability increasing the consistency for the identification of MNI and other nuclear abnormalities in these target cells. Large inter-
laboratory variability was observed in values of different cell types such as CC, KH, KL and pycnotic cells, with greatest variability seen for the latter type. A reduction of variability was obtained after combining CC and KH cells, confirming the recommendation from our previous scoring exercise amongst reference laboratories to combine these two biomarkers into a single score (32).

The possible determinants of the inter-laboratory variability could be identified in a subjective interpretation of the scoring criteria based on the previous experience with the BMNcyt assay and possibly on the quality of the microscopes used in the exercise. The experience of the laboratories was shown to play an important role in the identification of the different cell types and nuclear anomalies. This effect was more relevant in the scoring of BASAL, CC and pycnotic cells with an overestimation in less experienced laboratories.

A good reproducibility of the scoring between sessions was observed for all the parameters included in the BMNcyt assay. No significant difference, after adjustment for the treatment and the inter-laboratory heterogeneity, was detected between the two scoring sessions for any participating laboratories. This result indicates a robustness of the criteria for the majority of parameters of the BMNcyt assay. The difficulty in visually assessing the size of cells and nuclei probably best explains the misclassification of some cell types mainly identified by the nuclear/cytoplasm area ratio such as BASAL and pycnotic cells.

The main conclusion of this study is that the current visual scoring criteria are sufficiently robust to obtain consistent results concerning the identification of differentiated cells and MNi which represent the main biomarkers for the evaluation of chromosomal damage in buccal exfoliated cells. It is important to emphasize the need for proper training by experienced laboratory scientists. The inter-calibration among the laboratories is strongly recommended to allow proper and reliable comparisons of the parameters included in BMNcyt assay approach across laboratories in studies with multiple participating centers. Further studies are needed to identify the relevant host factors responsible for the inter- and intra-individual variability in the MN frequency in order to minimize their impact on the results of biomonitoring.
studies. Next steps in the validation of the MN test in buccal exfoliated cells will involve the analysis of the baseline frequency in healthy subjects for the cell types associated with cell differentiation and cell death included in the BMNcyt assay approach and the identification of biological confounding factors (e.g. genetics, nutrition, genotoxic exposures) relevant for explaining its variability in order to define the role of these parameters in human biomonitoring studies.

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Legends to figures

Figure 1.
Mean Ratio of BMNcyt results for pooled buccal samples from the head and neck cancer patients undergoing radiotherapy (treated group) relative to results for pooled samples from healthy controls.

Figure 2.
Differences among participating laboratories for BMNcyt biomarkers in respect to reference levels (R1+R2+R3)
Table 1. List of laboratories participating in the inter-calibration exercise

<table>
<thead>
<tr>
<th>ID</th>
<th>Responsible</th>
<th>Department/Unit</th>
<th>Institute</th>
<th>Country</th>
<th>Number of Scorers</th>
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<td>Ref1</td>
<td>Claudia Bolognesi</td>
<td>Environmental Carcinogenesis</td>
<td>IRCCS AOU San Martino-IST, Genoa</td>
<td>Italy</td>
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</tr>
<tr>
<td>Ref2</td>
<td>Siegfried Knasmueller</td>
<td>Dept. of Chemical Safety and Cancer Prevention</td>
<td>Institute of Cancer Research, Medical University of Vienna</td>
<td>Austria</td>
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</tr>
<tr>
<td>Ref3</td>
<td>Michael Fenech and Philip Thomas</td>
<td>Genome Health and Personalized Nutrition Laboratory</td>
<td>CSIRO Food and Nutrition, Adelaide</td>
<td>Australia</td>
<td>2</td>
</tr>
<tr>
<td>Lab1</td>
<td>Ewa Błaszczyk</td>
<td>Department of Environmental Risk Analysis</td>
<td>Institute for Ecology of Industrial Areas, Katowice</td>
<td>Poland</td>
<td>1</td>
</tr>
<tr>
<td>Lab2</td>
<td>Stefano Bonassi</td>
<td>Clinical and Molecular Epidemiology</td>
<td>IRCCS &quot;San Raffaele Pisana&quot;, Rome</td>
<td>Italy</td>
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<td>Lab3</td>
<td>Daisy Maria Fávero Salvadori</td>
<td>Laboratory of Toxicogenomics and Nutrigenomics</td>
<td>Botucatu Medical School, UNESP – São Paulo State University, São Paulo</td>
<td>Brasil</td>
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<td>Lab4</td>
<td>Juliana Da Silva</td>
<td>Laboratory of Genetic Toxicology</td>
<td>Lutheran University of Brazil Porto Alegre</td>
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<td>Lab5</td>
<td>Helena Groot</td>
<td>Laboratorio de Genética Humana (LGH)</td>
<td>Universidad de los Andes, Bogota</td>
<td>Colombia</td>
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<td>Lab6</td>
<td>Sema Burgaz</td>
<td>Department of Toxicology</td>
<td>Gazi University, Ankara</td>
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<td>Lab7</td>
<td>Metka Filipic</td>
<td>Department of Genetic Toxicology and Cancer Biology</td>
<td>National Institute of Biology, Ljubljana</td>
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<td>Lab8</td>
<td>Michael Kundi</td>
<td>Institute of Environmental Health</td>
<td>Medical University of Vienna</td>
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<td>Lab9</td>
<td>Tamara Grummt</td>
<td>Department Toxicology of Drinking Water</td>
<td>Federal Environment Agency</td>
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<tr>
<td>Lab10</td>
<td>Nina Holland</td>
<td>Center for Environmental Research and Children’s Health</td>
<td>School of Public Health, University of California, Berkeley</td>
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### Table 2. Descriptive analysis of BMNCyt biomarkers in 13 participating laboratories.

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<th>Labs (N. of score values)</th>
<th>LabR1 (8)*</th>
<th>LabR2 (4)</th>
<th>LabR3 (8)</th>
<th>Labs R1+R2+R3 (20)</th>
<th>Lab1 (4)</th>
<th>Lab2 (4)</th>
<th>Lab3 (12)</th>
<th>Lab4 (8)</th>
<th>Lab5 (7)</th>
<th>Lab6 (4)</th>
<th>Lab7 (4)</th>
<th>Lab8 (4)</th>
<th>Lab9 (8)</th>
<th>Lab10 (8)</th>
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<td>BMNCyt Parameters</td>
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<td>Mean (SD)</td>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
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<td>Mean (SD)</td>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
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<td>DIFF_MONO (per 1000 cells)</td>
<td>811.9 (31.0)</td>
<td>815.2 (44.7)</td>
<td>833.2 (52.8)</td>
<td>821.1 (42.4)</td>
<td>756.9 (54.1)</td>
<td>789.0 (107.4)</td>
<td>945.6 (49.1)</td>
<td>839.1 (26.4)</td>
<td>667.9 (75.0)</td>
<td>711.0 (42.3)</td>
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<td>890.7 (67.0)</td>
<td>788.1 (109.4)</td>
<td>725.5 (61.2)</td>
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<tr>
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<td>36.2 (26.5)</td>
<td>32.2 (23.3)</td>
<td>33.8 (22.2)</td>
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<td>43.2 (33.8)</td>
<td>50.2 (29.6)</td>
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<td>23.7 (21.7)</td>
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<td>42.6 (34.2)</td>
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* A number of score values for each laboratory included the repeated scoring of two slides (from cancer patients and controls) by the scorer(s).
Table 3. Comparison between sessions adjusted for treatment, and random effects of laboratory and scorer.

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<th>[95% Conf. Interval]</th>
<th>Z p-value</th>
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<td>0.75-1.11</td>
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<td>0.74-1.26</td>
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<td>NBUDS_MONO</td>
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<td>0.64-1.53</td>
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Table 4. Results of multi-level mixed-effects Poisson regression model

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<td>Treatment</td>
<td>Experience</td>
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<td>MR (95%CI)</td>
<td>Z p-value</td>
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<tr>
<td>DIFF_MONO</td>
<td>0.92 (0.91-0.94) &lt;0.001</td>
<td>1.08 (1.02-1.15) 0.008</td>
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<tr>
<td>DIFF_BN</td>
<td>4.01 (3.43-4.70) &lt;0.001</td>
<td>1.01 (0.78-1.30) 0.939</td>
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<td>BASAL</td>
<td>2.10 (1.53-2.87) &lt;0.001</td>
<td>0.27 (0.12-0.57) 0.001</td>
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<td>CC</td>
<td>0.74 (0.60-0.92) 0.006</td>
<td>0.48 (0.25-0.94) 0.032</td>
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<tr>
<td>KHC</td>
<td>0.62 (0.51-0.75) &lt;0.001</td>
<td>0.75 (0.50-1.13) 0.167</td>
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<td>CC+KHC</td>
<td>0.66 (0.56-0.79) &lt;0.001</td>
<td>0.64 (0.41-0.98) 0.041</td>
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<td>KYL</td>
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<td>0.90 (0.65-1.26) 0.556</td>
</tr>
<tr>
<td>PYK</td>
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<td>0.34 (0.14-0.81) 0.015</td>
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<tr>
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<tr>
<td>MNI_MONO</td>
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<tr>
<td>MNC_BN</td>
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<tr>
<td>MNI_BN</td>
<td>26.05 (14.19-47.83) &lt;0.001</td>
<td>1.13 (0.72-1.77) 0.602</td>
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<tr>
<td>NBUDC_MONO</td>
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<td>0.84 (0.31-2.27) 0.724</td>
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<tr>
<td>NBUDS_MONO</td>
<td>4.06 (2.96-5.55) &lt;0.001</td>
<td>0.77 (0.28-2.14) 0.617</td>
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<tr>
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<td>0.47 (0.20-1.12) 0.088</td>
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<td>NBUDS_BN</td>
<td>5.87 (3.53-9.77) &lt;0.001</td>
<td>0.43 (0.18-1.02) 0.056</td>
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