Mutagenesis vol. 24 no. 4 pp. 295–302, 2009
Advance Access Publication 28 May 2009
doi:10.1093/mutage/gep019

COMMENTARY

State of the art survey of the buccal micronucleus assay—a first stage in the $HUMN_{XL}$ project initiative

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The study of DNA damage in exfoliated buccal cells is a minimally invasive method for monitoring populations for exposure to genotoxic agents. The presence of micronuclei (MN) and other nuclear anomalies within these cells has been shown to be associated with genetic defects in genome maintenance, accelerated ageing, genotoxic damage and some degenerative diseases. To identify important information gaps regarding these biomarkers, a new initiative was launched within the framework of the HUman MicroNucleus (HUMN) collaborative programme, the $HUMN_{XL}$ project ('XL' designating eXfoLiated cell). An invitation to join the project was sent out together with a questionnaire to all laboratories that have published on the buccal micronucleus assay. Overall, 188 messages were delivered and 58 laboratories from 25 countries agreed to participate (43 contributing 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 provide 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 habit. According to this survey, a potential base of 15 103 subjects can be included in future pooled analyses. A number of protocol discrepancies emerged, implying that method standardization is a major priority. The results of this survey will contribute to (i) identify technical and epidemiological key variables that impact on buccal MN frequency in human populations, (ii) drive the design of future intra- and interlaboratory validation studies and (iii) determine the role of MN frequency and other biomarkers, in monitoring genomic damage and predicting cancer and other degenerative diseases.

Introduction

The study of DNA damage in exfoliated cells collected from the oral cavity holds great promise as a minimally invasive method for monitoring populations exposed to genotoxic agents. The presence of micronuclei (MN) and other nuclear anomalies within these cells has been shown to be associated with genetic defects in genome maintenance, accelerated ageing, exposure to genotoxic agents, oral cancer risk and neurodegenerative diseases and was also used in chemopreventive studies (1). While these results indicate the suitability of this assay for measuring chromosomal damage in human populations, the mechanism linking MN in buccal exfoliated cells to chromosome damage in progenitor cells is difficult to prove given the difficulty in performing chromosome metaphase analysis in this cell type. However, given the extensive evidence from *in vitro* studies of cultured epithelial cells and lymphocytes demonstrating that MN originate from either lagging chromosome fragments or whole chromosomes, it is reasonable to infer that MN in buccal cells originate mainly via these mechanisms.

In May 2007, the HUman MicroNucleus (HUMN) project held its eighth workshop in Antalya, Turkey, to seek the opinions of experts in the field on the current status of the method, focusing on its strengths and limitations and need for improvement (2). As a consequence of the workshop, the steering committee of the HUMN project completed an extensive review of existing work in this field (1), proposed a protocol for the so-called 'cytome assay' in the exfoliated buccal cells (3) and launched a new initiative to identify important knowledge and information gaps regarding the detection and characterization of nuclear anomalies in buccal cells. To distinguish between the exfoliated buccal micronucleus assay programme and the HUMN lymphocytes project, a new acronym was proposed for the new initiative, i.e. $HUMN_{XL}$ project ('XL' designating eXfoLiated cell).

During the workshop held in Antalya, it was decided that the first step of the programme would be to perform a detailed survey of the current methodological and data acquisition status of laboratories that have published papers on the buccal MN assay. The results of this survey are discussed here, together with the basic elements of a research programme aimed at (i) developing and testing a standardized improved protocol and (ii) validating the buccal cell micronucleus test as a non-invasive biomarker of genome damage and disease risk.

Materials and methods

Participants

Potential contributors to the survey were identified (i) by using the list of registrants at the 2007 HUMN workshop in Antalya, Turkey, (ii) by searching the PubMed database (National Library of Medicine, National Institutes of Health, Bethesda, MD, USA—http://www.ncbi.nlm.nih.gov/PubMed); in this

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case, we performed a free-text search in the title or abstract field, using the terms 'Micronucleus test' and 'buccal cells', and (iii) by personal communication.

An electronic message with the invitation to join the project and a questionnaire were sent to all available addresses in March 2008 and twice more in April and May 2008 to non-respondents. Overall, 188 messages were delivered, 41 (21.8%) researchers acknowledged the receipt of the message without further reply, 6 (3.2%) declared their interest in the project but stated that they could not participate, 83 (44.1%) did not reply and 58 (30.8%) agreed to participate in the project and returned their completed questionnaires [43 of them (27.2%) reporting direct experience with the assay].

Questionnaire

The present questionnaire was developed from the version previously validated for the lymphocyte project (4) and was designed to obtain the following data:

- General information about the experience of the laboratory with the buccal MN assay, including the number of subjects studied.
- Detailed information relating to study subjects' background information, including demographics, genetic constitution, gender, health status, occupation, potential genotoxic exposure, lifestyle and diet.
- List of publications from the laboratory using the buccal MN assay.
- Details of the method used for the collection of buccal cells including specification from which part of the mouth the cells were collected.
- Details of the method used for cell washing, fixation and slide preparation prior to staining.
- Description of the staining method used.
- Description of the scoring method and criteria indicating which of the previously published methods were used, if any.
- Specification of methods for data collection, including biomarkers scored for DNA damage, cell proliferation and cell death and the number of cells scored.

A copy of the questionnaire for those interested in contributing their data to the project database is available on the HUMN project website (www.HUMN.org).

Statistical analysis of questionnaire data

Data from each questionnaire were entered into a Microsoft® Access database developed *ad hoc* for this survey. Statistical analysis and graphical elaborations were performed with the statistical software SPSS Release 15.0 (SPSS, Chicago, IL).

Results

Participants

The questionnaire was completed by scientists from 58 laboratories from 54 different research institutions in 25 different countries representing all continents except Africa. Only 43 (74.1%) of these laboratories stated that they had data resulting from the use of the buccal MN assay to report. Three other laboratories have performed validation exercises in a restricted number of individuals but did not publish any studies using the buccal MN assay (Table I). The remaining participants in the survey (12 laboratories) had never performed this assay but stated that they were planning to use it in future studies. These latter questionnaires were archived for future evaluation and not considered further in this survey.

All statistics describing the frequency of epidemiological covariates in the $HUMN_{XL}$ database refer to 43 questionnaires returned from laboratories that have performed the buccal MN assay in population studies, while figures on laboratory methods include data from 46 questionnaires. The participants provided a list of publications describing their work in peer-reviewed journals using the buccal MN assay (5–70).

Type and amount of data available

A majority (n = 34, 79.1%) of the laboratories that reported using the buccal MN assay had also performed the MN assay in lymphocytes. A smaller proportion used the exfoliated cell MN assay with other cell types such as urothelial cells (24%), nasal cells (11%) and cervical smears (13%). Nineteen laboratories (44.2%) reported MN frequency data from buccal cells and lymphocytes in the same individual, creating the opportunity for comparative studies between these two tissues. The reported number of subjects screened with the buccal MN assay has increased dramatically in the last 20 years, rising from the initial sampling of 27 subjects in 1988 to 2292 subjects in 2007. According to the information collected with this survey, data from 15 103 subjects are potentially available for future pooled analyses.

Information available in participants' databases

Based on the responses to the questionnaire, demographic data have been collected in most databases, particularly name/surname (79%), age (93%), gender (93%) and ethnicity (72%). Unfortunately, the availability of essential data for linkage with cancer registries is limited, i.e. 74% for date of birth and 51% for address. Furthermore, the availability for linkage of individual information could be limited by national regulation concerning the treatment of sensitive data.

Information on medical history and disease status

Family history of cancer is available from 25 laboratories (58.1%), while familial incidence for other diseases has been collected less frequently. Information about current or past diseases (72.0%) or chronic disabilities (67.2%) is also available. Medical records concerning treatment and drug consumption can be used as an important tool for validating medical data and this information was collected in 33 databases (76.7%). The use of recreational drugs was recorded in 20 databases (46.5%).

Information on exposure to tobacco smoke and occupational exposure to genotoxins

The impact of these genotoxic exposures on genetic stability was extensively evaluated in the literature for the MN assay in lymphocytes (71,72). The pooled approach, which allows analyses of much larger populations than single laboratory studies, revealed novel features concerning the association between MN frequency and the number of cigarettes smoked (73). In the $HUMN_{XL}$ database, smoking habit is a commonly available covariate, with data on smoking status recorded in 38 laboratories (88.4%). Additionally, most cases (79.1%) have quantitative data on cigarette consumption. Information on exposure to environmental tobacco smoke was collected by 20 laboratories (46.5%). In most databases, data on occupational exposures (86.1%) and exposure to known genotoxic agents (72.1%) are available.

Information on dietary habits and alcohol consumption

Most recent population studies include data concerning the diet. The $HUMN_{XL}$ questionnaire also considered this factor, identifying commonly reported food items. Overall, diet was investigated in 29 laboratories (67.4%). Data on fruit and vegetable consumption are available from 22 laboratories (51.2%). Tea- and coffee-drinking patterns have been reported in 46.5 and 51.2% of laboratories, respectively. Another dietary item that has been frequently assessed is alcohol consumption; this information is available in 34 of 43 laboratories that also

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Table I. List of the 46 laboratories (alphabetically by country) performing the buccal MN assay that have contributed questionnaires

Referent	Institution	Country
P. Thomas and M. Fenech	CSIRO Human Nutrition	Australia
A. Nersesyan	Institute of Cancer Research	Austria
S. Haveric	Institute for Genetic Engineering and Biotechnology	Bosnia and Herzegovina
J. Da Silva	Universidade Luterana do Brasil	Brasil
D. F. Salvadori	Botucatu Medical School, UNESP-Sao Paulo State University	Brasil
M. G. Martino-Roth	Universidade Catolica de Pelotas	Brasil
G. J. F. Gattas	Universidade de Sao Paulo	Brasil
D. A. Ribeiro	Federal University of São Paulo	Brasil
V. M. De Andrade	Universidade do Extremo sul Catarinense	Brasil
V. Hadjidekova	National Centre Radiobiology and Radiation Protection	Bulgaria
A. Domínguez Odio	Centro de Toxicología y Biomedicina	Cuba
D. Carnesoltas	National Institute of Oncology and Radiobiology	Cuba
M. Bloching	Helios Klinikum Berlin Buch, Charite	Germany
P. Grover	Indian Institute of Chemical Technology	India
A. K. Giri	Indian Institute of Chemical Biology	India
A. Halder	All India Institute of Medical Sciences	India
A. S. Yadav	Kurukshetra University	India
M. Kamboj	Career Dental College and Hospital	India
D. Cavallo	ISPESL-National Institute for Occupational Safety and Prevention	Italy
C. Bolognesi	National Cancer Institute	Italy
F. D'Agostini	University of Genoa	Italy
K. Kurteshi	University of Prishtina, Faculty of Natural Science	Kosovo
G. M. Zuniga-Gonzalez	Centro de Investigacion Biomedica de Occidente	Mexico
R. Montero	Universidad Nacional Autonoma de Mexico	Mexico
S. Gomez-Arroyo	Universidad Nacional Autónoma de México	Mexico
O. Torres Bugarin	Universidad Autónoma de Guadalajara	Mexico
D. Malgorzata	National Institute of Public Health	Poland
M. J. Silva	Instituto Nacional de Saúde Dr. Ricardo Jorge	Portugal
J. P. Teixeira	National Institute of Health	Portugal
I. Vorobtsova	Russian Research Center for Radiobiology	Russia
V. Druzhinin	Kemorovo State University	Russia
R. Marcos	Universitat Autonoma de Barcelona	Spain
W. A. Hsieh	Tzi Chi University	Taiwan
J. Cao	College of Preventive Medicine, Third Military Medical University	People's Republic of China
A. Celik	Mersin University	Turkey
H. Dönmez-Altuntas	Erciyes University, Faculty of Medicine	Turkey
E. Coskun	Gazi University, Faculty of Pharmacy	Turkey
G. C. Demircigil	Gazi University, Faculty of Pharmacy	Turkey
M. Korkmaz	Yuzuncu Yil University	Turkey
Z. Hamurcu	Erciyes University	Turkey
H. Taylor	Covance Laboratories Europe, Harrogate	UK
I. Klimkina	National Mining University	Ukraine
N. Holland	University of California, Berkeley	USA
C. Piyathilake	University of Alabama	USA
A. Vaglenov	University of Findlay	USA
J. Crott	Tufts University, Human Nutrition Research Center on Aging	USA

collected population data (79.1%). The measures of the body mass index and vitamin supplementation are variables that are directly linked to diet and were reported in 23.3 and 48.8% of all questionnaires, respectively.

Genetic data

The evaluation of individual genetic and epigenetic profiles is a feature increasingly considered in human population studies. The most frequently acquired parameter is the presence of genetic polymorphisms (nine laboratories corresponding to 20.9 %), while data on biomarkers of genomic damage are available from 18 (41.9%) laboratories.

Information on laboratory methods

Buccal cell collection. Several methods for collecting buccal cells have been reported (Figure 1). The most common method involved a wooden tongue depressor (54%), followed by the use of a toothbrush (20%) and a cytobrush moistened with buffer (11%). All but two laboratories (95.6%) reported sampling buccal cells from the insides of both the left and right cheeks, with only a small proportion (6.5%) reporting further sampling from the sublingual, palatal and pharyngeal areas. Procedures for collecting buccal cells, e.g. how the collection devices were applied to the inside of the cheek, including the number of times the device was rotated against the mucosal surface before transferring cells into a solution and the number of repetitions of the collection procedure within a specified timeframe, were described in more detail in 43% of the questionnaires. Most laboratories (61%) collected only a single sample per individual on a single day and only 37% of the laboratories that collected from both cheeks separately analysed the samples from the left and right cheeks.

Buccal cell and slide preparation. Forty-eight per cent of laboratories reported that collected cells were re-suspended in a buffer and washed to remove debris and bacteria. The washing

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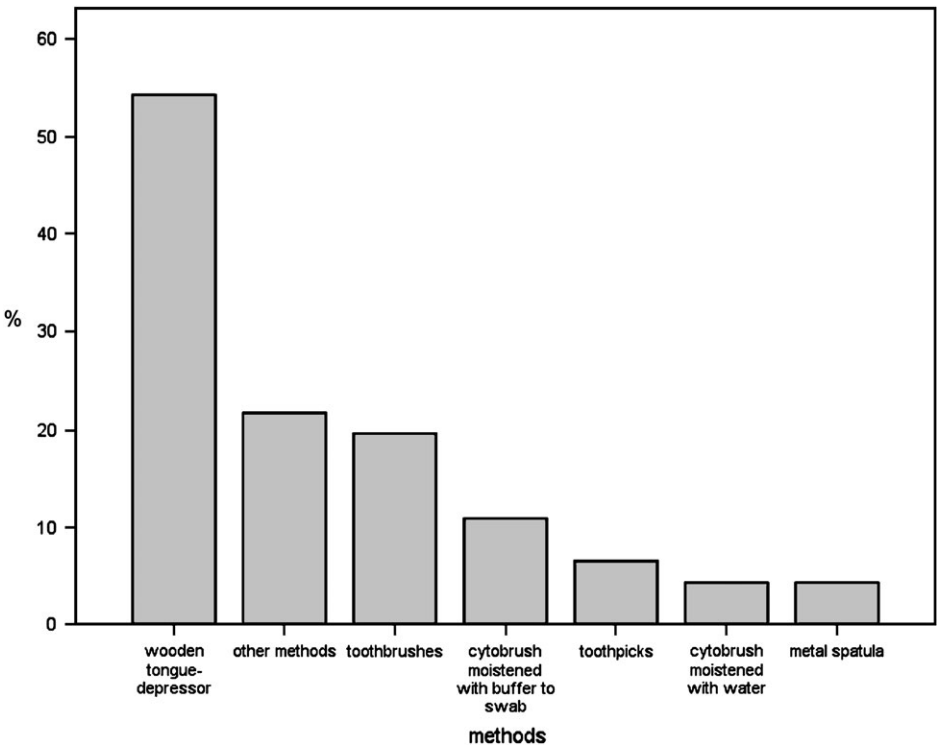


Fig. 1. Most commonly adopted buccal cell collection methods in 46 laboratories participating in the $HUMN_{XL}$ survey (some laboratories reported more than one method). *x*-axis legend: methods, *y*-axis legend: %.

solutions, Tris–ethylenediaminetetraacetic acid (EDTA) (0.01 M Tris–HCL, 0.1 M EDTA, 0.02 M NaCl at pH 7.0) and 0.9% saline, were the most frequently used (both were 31.8%), followed by phosphate-buffered saline (22.7%). Other laboratories (13.6%) suspended cells in fixative. Cells are directly transferred to slides in 52% of laboratories, 37% by dropping, 7% by cytocentrifuge and 7% by other methods. In the majority of cases (76%), cells were fixed after transfer to slides, only 22% of laboratories fixed cells before transferring to slides. A wide range of fixatives was used; most solutions included methanol (>40%).

Staining. A large variety of staining methods are often employed within the same laboratory and in many instances different staining methods were used in different studies. The methods most commonly employed were Feulgen–Fast green (52.2%), May–Grunwald Giemsa (23.9%), Acridine orange (19.6%) and 4′,6-diamidino-2-phenylindole (DAPI) (15.2%) (Table II). Only 13% of laboratories used FISH with pan-centromeric probes to investigate the mechanism of MN formation.

Table II. Distribution of preferred staining methods in 46 laboratories replying to questions about the protocol (some laboratories reported more than one staining method)

Staining method	No. of laboratories (%)
Feulgen[a]	25 (54.3)
DAPI	7 (15.2)
Giemsa[b]	14 (30.4)
Acridine orange	9 (19.6)
Aceto-orceine	4 (8.7)
Haematoxylin–Eosin	1 (2.2)
Other	2 (4.3)

[a]Ninety-six per cent of laboratories using Feulgen counterstained the cytoplasm with Fast green.
[b]Eighty per cent of laboratories using Giemsa reported that they used May–Grunwald Giemsa.

Number of cells scored and scoring criteria

Laboratories participating in the survey generally reported preparing at least two slides per subject studied (60.9%), scoring 1000 cells per slide (26.1%) and a total of 2000 cells per subject (39.1%). The number of cells scored per subject ranged from 500 to >4000 in some studies. The choice of scoring criteria is obviously a critical issue since in lymphocytes this parameter is likely to be among the most important determinants of interlaboratory variability in MN frequency until automated scoring software is fully implemented (73). The scoring criteria described by Tolbert *et al.* (74) were by far the most popular (50.0%), followed by those published by Titenko-Holland *et al.* (75) (17.4%) and the basic methods in which MN were scored in any cell (17.4%) described by Stich *et al.* (76,77) (15.2%) and Sarto *et al.* (78) (13.0%) (Figure 2).

Regarding the cell types used for scoring, most laboratories scored MN in basal cells (16.7%), normal differentiated cells (22.9%) or both (31.3%), while others did not specify the cells. Overall, 70.8% of laboratories that answered this question scored MN only in viable/healthy cells, whereas 29.2% recorded all cell types, including condensed chromatin, karyorrhectic, pyknotic and karyolytic cells. Besides MN, other cell types and nuclear anomalies are recorded by about one-third of the laboratories (Table III). The most commonly scored anomalies and cell types are binucleated cells (45.7%), nuclear buds (broken eggs) (37.0%), karyorrhectic cells (37.0%) and karyolytic cells (37.0%). Slides are generally

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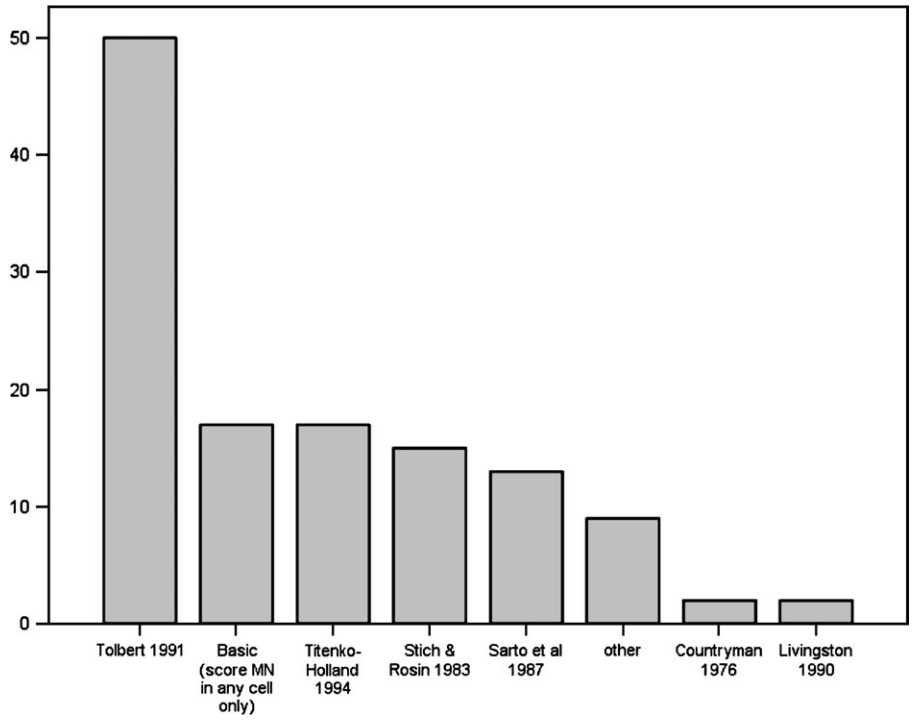


Fig. 2. Most commonly adopted scoring criteria in the buccal exfoliated cell MN assay in 46 laboratories participating in the $HUMN_{XL}$ survey (some laboratories reported more than one criteria). *x*-axis legend: criteria, *y*-axis legend: %.

Table III. Cell types and nuclear anomalies, other than MN, most commonly scored and reported in the buccal MN assay

Cell type or anomaly	No. of laboratories (%)
Basal cells	8 (17.4)
Normal differentiated cells	12 (26.1)
Cells with nuclear buds (broken eggs)	17 (37.0)
Binucleated cells	21 (45.7)
Karyorrhectic cells	17 (37.0)
Fragmented nucleus cells	12 (26.1)
Condensed chromatin cells	13 (28.3)
Pyknotic cells	15 (32.6)
Karyolytic cells (ghost cells)	17 (37.0)

scored by one (53.1%) or two persons (42.9%) and rarely by three scorers (4.1%).

Discussion

This survey has produced a substantial database across a wide range of laboratories and shows that the buccal cell MN assay is far from a standardized procedure, even among those in the same country. In addition, results from published studies suggest that there is a wide range of control values among laboratories and a broad variety of responses in subjects exposed to genotoxic agents (data not shown) (1). This database will allow us to pool the MN and nuclear anomaly data from a wide range of laboratories and study variability among laboratories, which will contribute to the development of standardized procedures. The availability of this rich source of information will also allow the addressing of fundamental questions regarding demographics, potential genotoxic exposure, dietary and lifestyle variables that may significantly impact on MN frequency in buccal cells. This approach, i.e. the assembly of data from a wide geographical range of laboratories, has already been shown to be effective for the standardization of the cytokinesis-block MN assay in lymphocytes and has helped to define the key variables that need to be controlled in biomonitoring studies for MN induction and the technical conditions that explain most of the interlaboratory variability (4). Furthermore, the creation of a network of laboratories is a basic condition for performing systematic investigations that, together with the results of pooled studies contribute to setting qualitative and quantitative standards.

The database assembled in this first phase of the $HUMN_{XL}$ project is highly representative of the current state of the science of the buccal cell MN assay. The geographic distribution of participating laboratories provides a worldwide representation of the techniques used in the various laboratories and the populations that have been screened with the buccal MN assay. The number of subjects available for pooled analyses from this database, i.e. 15 103, is almost twice as high as that included in the previous project on the cytokinesis-block MN assay (4,79) and it will provide adequate statistical power for testing many hypotheses, including the link between MN frequency in buccal cells and disease risk.

A number of protocol discrepancies that may have been responsible for the interlaboratory variability seen in MN frequency emerged from the evaluation of questionnaires. Technical heterogeneity was evident, beginning with the way the buccal cells were collected. Previous studies have shown that the buccal MN frequency, as well as the frequency of other nuclear anomalies, can vary depending on the degree and vigour of the sampling procedure (55,79). This implies that the definition of a standardized approach for buccal cell collection aimed at minimizing variation due to different sampling techniques is a high priority. Ideally, a robotic system that could collect cells uniformly and efficiently by maintaining

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constant tension against the inside wall of the cheek would be desirable.

The observed variation in staining procedures may present a problem because it has been shown that the use of stains that are not DNA specific could produce false-positive or false-negative results (80,81). There is a need to define a reliable and reproducible protocol for staining of buccal cells in a way that minimizes artefacts and is usable under both transmitted light and fluorescence microscopy. The Feulgen–Light green (or Fast green) stain seems to be the most promising given that it is DNA specific and can be used in combination with both common methods of microscopy illumination, i.e. bright field and fluorescence (55,56).

Among the potential sources of variability, a major problem is the use of different criteria for identifying and scoring MN and other nuclear anomalies. There is no current consensus on which cell types should be scored (e.g. basal cells only, normal differentiated cells only, both basal and normal differentiated cells or all types of cells including those that are dead or dying). Because MN require nuclear division, it would seem reasonable that scoring should be restricted to the basal cell layer as this is the regenerative layer where nuclear division occurs. On the other hand, expression of MN in these cells could be affected by altered cell division kinetics. Another option is scoring MN in the normal differentiated cells that are the daughter cells of the basal layer and have therefore completed one nuclear division. The counter argument for normal differentiated cells is that they may lose MN during the differentiation process, although no evidence is available to refute or confirm this assumption.

Inclusion of the other end points, measured using a cytome approach, has proved to be a valuable diagnostic tool as was recently shown in studies on Alzheimer's disease, Down syndrome and normal ageing, in which large differences in the frequencies of basal cells, condensed chromatin cells, karyorrhectic cells, binucleated cells and cells with MN were observed (55,56). These changes showed distinct differences between the cytome profile of normal ageing and the profile of a premature ageing syndrome. The key buccal cell assay challenges are to define clearly the scoring criteria for each nuclear anomaly and cell type with meticulous graphic detail, as has been achieved previously for the cytokinesis-block micronucleus cytome assay (82). One good example is the definition of a karyorrhectic cell, which is usually defined as a cell with a broken nucleus. As a matter of fact, this alteration is seen very rarely and has therefore sometimes been interpreted as meaning a cell with a nucleus containing very densely condensed chromatin relative to a condensed chromatin cell (1,3). More precise definitions of other cell types such as the nuclear density and the diameter that defines the nucleus of a pyknotic cell and the criteria for defining a nuclear bud (or broken egg) in buccal cells require more attention. Resolving these scoring criteria issues, together with a standardized approach for recording, analysing and reporting data will be an important task for collaborative approaches across laboratories in the future and for interpreting data arising from a variety of laboratories. These criteria have been defined more clearly recently in an attempt to resolve these issues (3).

The evaluation of the questionnaires clearly showed that the quality of information that can be used for epidemiological studies has increased greatly in recent years. Most laboratories have realized that the availability of host factors or lifestyle parameters of subjects in study groups enhances the scope of the data analyses and allows adjustment for confounding lifestyle factors.

In conclusion, the results of this survey identified the scope, as well as future challenges, for the $HUMN_{XL}$ project. These include

(i) Defining the technical and epidemiological key variables that impact on buccal MN frequencies in human populations as measured using current, non-standardized protocols.
(ii) Designing the intra- and interlaboratory studies needed to quantify these variables and produce reliable and robust standard protocols for
  (a) Buccal cell collection and storage
  (b) Slide preparation and staining
  (c) Slide scoring and the identification of
    (1) the various cell types and
    (2) the different indices of damage, MN and nuclear anomalies other than MN
  (d) Data recording, analysis and reporting.
(iii) Determining whether MN frequency in buccal cells and other cytome biomarkers can be used for prediction of cancer and other degenerative diseases.

Funding

Associazione Italiana per la Ricerca sul Cancro; Italian Space Agency; Italian Ministry of Health to SB and BB.

Acknowledgements

We are extremely grateful to the many scientists, their laboratory associates and staff, as well as the volunteers who have contributed their buccal cell samples for enabling these important buccal MN assay data to be collected and compiled.

The $HUMN_{XL}$ Project consortium includes the authors of this paper and all contributors of completed questionnaires for this paper listed in Table I.

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Received on March 3, 2009; revised on April 24, 2009; accepted on April 24, 2009

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