Review

The micronucleus assay in human buccal cells as a tool for biomonitoring DNA damage: The HUMN project perspective on current status and knowledge gaps

Nina Holland a,1,*, Claudia Bolognesi b,1,**, Micheline Kirsch-Volders c, Stefano Bonassi d, Errol Zeiger e, Siegfried Knasmueller f, Michael Fenech g

a School of Public Health, University of California, Berkeley, CA, USA
b Unit of Environmental Carcinogenesis, National Cancer Research Institute, Genoa, Italy
c Laboratory for Cell Genetics, Vrije Universiteit, Brussel, Belgium
d Unit of Molecular Epidemiology, National Cancer Research Institute, Genoa, Italy
e Errol Zeiger Consulting, Chapel Hill, NC, USA
f Institute of Cancer Research, Medical University, Vienna, Austria
g CSIRO Human Nutrition, Adelaide, Australia

ABSTRACT

The micronucleus (MN) assay in exfoliated buccal cells is a useful and minimally invasive method for monitoring genetic damage in humans. This overview has concluded that although MN assay in buccal cells 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 micronuclei 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. To address these uncertainties, the human micronucleus project (HUMN; see http://www.humn.org) has initiated a new international validation project for the buccal cell MN assay similar to that previously performed using human lymphocytes. Future research should 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 staining, optimal criteria for classification of normal and degenerated cells and for scoring micronuclei and other abnormalities. The harmonization and standardization of the buccal MN assay will allow more reliable comparison of the data among human populations and laboratories, evaluation of the assay’s performance, and consolidation of its world-wide use for biomonitoring of DNA damage.

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* Corresponding author at: 733 University Hall, Berkeley, CA 94720-7460, USA. Tel.: +1 510 455 0561; fax: +1 510 643 5426.
** Corresponding author.
E-mail addresses: ninah@berkeley.edu, clc9879@berkeley.edu (N. Holland), claudia.bolognesi@istge.it (C. Bolognesi).
1 These authors contributed equally to the work.
1. Introduction

The human micronucleus (HUMN) project (http://www.humn.org), established in 1997, is an international collaborative program aimed at studying the micronucleus (MN) frequency in human populations, and assessing the effects of protocol and scoring criteria on the values obtained. The initial focus of the project was the analysis of MN in peripheral lymphocytes from unexposed and exposed individuals, primarily because this was a well-established human test system at the time the project began. The ultimate goals of the HUMN project are to standardize methodologies, define baseline MN frequency rates, characterize the effects of demographic, genetic, and methodological factors on these frequencies, and to determine whether the MN frequencies in different tissues are predictive of cancer risk [1]. These objectives have been achieved for lymphocytes by providing a detailed description of the scoring criteria [2] and by assessing sources of variability for the cytokinesis-block MN assay through a validation effort undertaken by 34 laboratories from 21 countries [3]. More recently, results from the prospective analysis of the database of more than 6700 subjects from 20 laboratories representing 10 countries, confirmed that an elevated MN frequency in human lymphocytes is predictive of an increased risk of cancer [4].

Genomic damage is probably the most important fundamental cause of developmental and degenerative disease. It is also well established that genomic damage is produced by environmental exposure to genotoxins, medical procedures (e.g. radiation and chemicals), micronutrient deficiency (e.g. folate), lifestyle factors (e.g. alcohol, smoking, drugs, and stress), and genetic factors such as inherited defects in DNA metabolism and/or repair [5–23]. It is essential to have reliable and relevant minimally invasive biomarkers to improve the implementation of biomonitoring, diagnostics, and treatment of diseases caused by, or associated with, genetic damage. The MN assay in exfoliated buccal cells is potentially an excellent candidate to serve as such a biomarker.

The collection of buccal cells is arguably the least invasive method available for measuring DNA damage in humans, especially in comparison to obtaining blood samples for lymphocyte and erythrocyte assays, or tissue biopsies. The buccal cell MN assay was first proposed in 1983 [6] and continues to gain popularity as a biomarker of genetic damage in numerous applications (Fig. 1). More than 40 laboratories from many countries either have used, or are currently using this assay, and the number of articles published annually is steadily increasing. Different issues related to the buccal cell MN assay were reviewed in several publications over the last decade [18,19,23–25]. However, most of these articles focused primarily on either broad evaluation of non-invasive methods for biomonitoring [24,26], the associations of micronuclei (MNI) and other markers with cancer in various types of exfoliated cells [19], or are limited to effects of a specific type of exposure, such as smoking and smokeless tobacco [25] or formaldehyde [23]. More importantly, the identification and relative importance of confounding variables affecting the MN frequency in buccal cells has not been adequately addressed and quantified. The predictive value of the buccal MN frequency for cancer risk has also not been studied in a comprehensive manner. Finally, the assay protocol has not been standardized in such a way that results from different laboratories can be easily compared or combined.

The HUMN project is an effective vehicle for the development and implementation of an international collaborative validation effort to bring together the various buccal MN databases, and to identify and quantify the key variables affecting this biomarker. In addition, an inter-laboratory slide-scoring exercise could be undertaken to evaluate the intra- and inter-laboratory variability in the scoring of buccal cell MN, similar to the approach successfully used by the HUMN project for the MN assay in lymphocytes [2,3].

![Fig. 1. Increase in the number of laboratories and publications reporting the use of the MN assay in buccal cells.](image-url)
The aims of this paper are: (a) to provide an overview of the current status of the MN assay in buccal cells, (b) to identify the most important knowledge gaps in the basic biology that affect the expression of MN and other nuclear abnormalities, and (c) to compare the strengths and limitations of the existing techniques for collection, staining, and scoring of buccal cells. This overview is designed to help to focus the HUMN buccal cell project on the key areas of concern, and those in need of further development.

2. Measurement of MN in exfoliated buccal cells: a brief history and rationale

Micronuclei originate from chromosome fragments or whole chromosomes that lag behind at anaphase during nuclear division [1,27]. The MN index in rodent and/or human cells has become one of the standard cytogenetic endpoints and biomarkers used in genetic toxicology in vivo or ex vivo. In humans, MN can be easily assessed in erythrocytes, lymphocytes, and exfoliated epithelial cells (e.g. oral, urothelial, nasal) to obtain a measure of genome damage induced in vivo. The MN assay can be performed in buccal and other exfoliated cells originating from rapidly dividing epithelial tissue without the need for ex vivo nuclear division, so that the cell cultures required for cytogenetic assays based on analysis of metaphase chromosomes, such as chromosome aberrations and sister chromatid exchanges, are not needed. There is precedence for this because, while the majority of MN studies with lymphocytes in the HUMN database used cytochalasin-blocked proliferating lymphocytes [1], some investigators assessed MNI in non-proliferating lymphocytes during interphase to measure MN expressed during in vivo nuclear division of their parent cells [28]. The general genotoxicity results by both methods of MN analysis are comparable.

Buccal cells are the first barrier for the inhalation or ingestion route and are capable of metabolizing proximate carcinogens to reactive products [29–32]. Approximately 90% of human cancers originate from epithelial cells [33]. Therefore, it could be argued that oral epithelial cells represent a preferred target site for early genotoxic events induced by carcinogenic agents entering the body via inhalation and ingestion.

The oral epithelium is composed of four strata of structural, progenitor, and maturing cell populations, including the lamina propria (connective tissue), the basal cell layer (stratum basale), prickle cell layer (stratum spinosum), and the keratinized layer at the surface (Fig. 2). A series of finger-like structures called “rete pegs” project up from the lamina propria into the epidermal layer producing an undulating basal cell layer effect. The oral epithelium maintains itself by continuous cell renewal whereby new cells produced in the basal layer by mitosis migrate to the surface replacing those that are shed. The basal layer contains the stem cells that may express genetic damage (chromosome breakage or loss) as MNi during nuclear division. The daughter cells, which may or may not contain MN, eventually differentiate into the prickle cell layer and the keratinized superficial layer, and then exfoliate into the buccal cavity. Some of these cells may degenerate into cells with condensed chromatin, fragmented nuclei (karyorrhectic cells), pycnotic nuclei, or completely lose their nuclear material (karyolytic or “ghost” cells) [5]. In rare cases, some cells may be blocked in a binucleated stage or may exhibit nuclear buds (also known as “broken eggs” in buccal cells [5]), a biomarker of gene amplification (Fig. 3). These biomarkers of genome damage (e.g. MNi, nuclear buds) and cell death (e.g. apoptosis, karyolysis) can be observed in both the lymphocyte and buccal cell systems, and thus provide a more comprehensive assessment of genome damage than only MNi in the context of cytotoxicity and cytostatic effects [5,27].

In the early studies from the 1980s, exfoliated buccal mucosa cells were used to evaluate the genotoxic effects of betel nuts and quids and of chewing tobacco [34–39]. Most studies showed higher MN frequencies at the site within the oral cavity where the quid or tobacco mixture was kept compared to the opposite, control, site. The MN assay in buccal cells was also used to study cancerous and precancerous lesions and to monitor the effects of a number of chemopreventive agents [40–42]. It is notable that the first studies of Stich and Rosin conducted between 1983 and 1984 had higher baseline MN frequencies than subsequent studies. This may have been due to a lack of defined scoring criteria and a relatively small number of scored cells (in some cases less than 500). Since then, published biomonitoring studies using the MN assay in buccal mucosa cells have investigated the effects of multiple factors...
including environmental and occupational exposures, radiotherapy, chemoprevention, vitamin supplementation trials, lifestyle habits, cancer, and other diseases.

3. Biomonitoring

3.1. Occupational and environmental exposures

In the last 15–20 years the MN assay has been applied to evaluate chromosomal damage for biological monitoring of human populations exposed to a variety of mutagenic and carcinogenic chemical or physical agents (Table 1). A broad range of baseline MN frequencies has been reported (0.05–11.5 MN/1000 cells) with the majority of values between 0.5 and 2.5 MN/1000 cells. There is no clear pattern of the variations among laboratories from different countries. Many studies report a statistically significant elevation of MN levels in exposed individuals compared to control groups, although the observed effects are relatively small, ranging between 1.1- and 4-fold [43–47], and many other studies report changes that were not statistically significant [48–50]. A 12-fold increase in MN frequency was observed in mortician students after a 3-month embalming course compared to pre-exposure levels [51]. This increase is unusually large, but has been confirmed by an independent analyses using a different staining procedure,

Table 1

Micronucleus frequency in exfoliated oral mucosa cells

<table>
<thead>
<tr>
<th>Exposure/lifestyle factors</th>
<th>Study subjects/ control</th>
<th>Baseline, MN cells/ 1000 cells</th>
<th>Fold difference vs. control</th>
<th>Cells scored/ subject</th>
<th>Staining method</th>
<th>Scoring criteria</th>
<th>Year</th>
<th>Country</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antineoplastic drugs</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Hospital staff</td>
<td>25/25</td>
<td>1.68</td>
<td>2.1</td>
<td>1000</td>
<td>1</td>
<td>3/4</td>
<td>1994</td>
<td>Brazil</td>
<td>[43]</td>
</tr>
<tr>
<td>Hospital staff</td>
<td>25/14</td>
<td>0.80</td>
<td>2.0</td>
<td>1000</td>
<td>1</td>
<td>3/4</td>
<td>1999</td>
<td>Turkey</td>
<td>[44]</td>
</tr>
<tr>
<td>Day-care hospital nurses</td>
<td>12/30</td>
<td>0.46</td>
<td>1.9</td>
<td>2000</td>
<td>4</td>
<td>4</td>
<td>2005</td>
<td>Italy</td>
<td>[137]</td>
</tr>
<tr>
<td>Pharmacy technicians</td>
<td>5/30</td>
<td>0.46</td>
<td>1.1</td>
<td>2000</td>
<td>4</td>
<td>4</td>
<td>2005</td>
<td>Italy</td>
<td>[137]</td>
</tr>
<tr>
<td>Ward nurses</td>
<td>13/30</td>
<td>0.46</td>
<td>2.0</td>
<td>2000</td>
<td>4</td>
<td>4</td>
<td>2005</td>
<td>Italy</td>
<td>[137]</td>
</tr>
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<td>Areca nut only</td>
<td>Chewers</td>
<td>15/10</td>
<td>1.93</td>
<td>1000</td>
<td>1</td>
<td>3</td>
<td>1992</td>
<td>India</td>
<td>[138]</td>
</tr>
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</table>

Fig. 3. Buccal MN assay cytome model. Schematic diagram of different types of buccal cells and the possible mechanisms for their origin. The majority of these cells have a large cytoplasm relative to the nucleus, and the shape of the cell is angular rather than spherical; the exception is basal cells. Genomic instability or genotoxic insult in the basal cells leads to chromosome breakage/loss and MN formation. Some cells with genome damage may be eliminated via the apoptotic process. The daughter cells from the basal layer differentiate into “prickle cells” which are eventually differentiated into the flattened and keratinized surface mucosal cells which exfoliate from the surface of the oral lining. Each of these cell types may contain MNi to varying extents. The molecular mechanisms leading to the various cell death events, and their inter-relationships, are unknown.
<table>
<thead>
<tr>
<th>Exposure/lifestyle factors</th>
<th>Study subjects/control</th>
<th>Baseline, MN cells/1000 cells</th>
<th>Fold difference vs. control</th>
<th>Cells scored/subject</th>
<th>Staining method*</th>
<th>Scoring criteria*</th>
<th>Year</th>
<th>Country</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td>Arsine</td>
<td>Drinking water</td>
<td>9/8</td>
<td>0.30</td>
<td>5.7</td>
<td>NR</td>
<td>1</td>
<td>4</td>
<td>1997</td>
<td>Chile [124]</td>
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<tr>
<td></td>
<td>Drinking water</td>
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<td>0.58</td>
<td>4.0</td>
<td>1000</td>
<td>1</td>
<td>3</td>
<td>1997</td>
<td>Mexico [14]</td>
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<td>0.65</td>
<td>3.4</td>
<td>3000</td>
<td>1</td>
<td>4</td>
<td>2001</td>
<td>Mongolia [56]</td>
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<td>6.7</td>
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<td>1</td>
<td>1</td>
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<td>Drinking water</td>
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<td>1.28</td>
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<td></td>
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<td>105/102</td>
<td>2.74</td>
<td>1.2</td>
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<td>2</td>
<td>6</td>
<td>2005</td>
<td>Chile [20]</td>
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<td>Glass factory workers</td>
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<td>2.10</td>
<td>7.2</td>
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<td>3</td>
<td>7</td>
<td>2006</td>
<td>India [55]</td>
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<td>Smelter workers</td>
<td>72/83</td>
<td>0.50</td>
<td>2.0</td>
<td>2000</td>
<td>1</td>
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<td>2007</td>
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<td>5</td>
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<td>2001</td>
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<td>0.46</td>
<td>0.7</td>
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<td>Russia [72]</td>
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<td>0.51</td>
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<td>3000</td>
<td>1</td>
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<td>1990</td>
<td>Italy [8]</td>
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<td>0.36</td>
<td>1.6</td>
<td>3000</td>
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<td>3</td>
<td>1991</td>
<td>Sweden [141]</td>
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<td>2.70</td>
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<td>Formaldehyde</td>
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<td>Lead paints, solvents, benzene</td>
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<td>4/6</td>
<td>2003</td>
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<td>6</td>
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<td>3000</td>
<td>1</td>
<td>3/4</td>
<td>1999</td>
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<td>2000</td>
<td>3</td>
<td>NR</td>
<td>2004</td>
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<td>Pesticide mixture</td>
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<td>2000</td>
<td>2</td>
<td>6</td>
<td>2003</td>
<td>Hungary, Poland, Greece [112]</td>
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<td>3.20</td>
<td>3.9</td>
<td>2000</td>
<td>2</td>
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<td>2006</td>
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<td>2</td>
<td>2000</td>
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<td>Tobacco</td>
<td>Beedi smokers</td>
<td>25/25</td>
<td>1.36</td>
<td>1.3</td>
<td>1500</td>
<td>4</td>
<td>7</td>
<td>2004</td>
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<td>8.40</td>
<td>2.4</td>
<td>1500–3000</td>
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<td>1997</td>
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<td>Tobacco (smokeless)</td>
<td>Chewers of betel quid with tobacco Gudalhu users (for &gt;20 years)</td>
<td>102/56</td>
<td>3.50</td>
<td>5.9</td>
<td>1000</td>
<td>1</td>
<td>3</td>
<td>1992</td>
<td>India [92]</td>
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<td>Chewers of tobacco mixed with lime</td>
<td>20/13</td>
<td>1.30</td>
<td>2.9</td>
<td>1000</td>
<td>1</td>
<td>3</td>
<td>1993</td>
<td>India [93]</td>
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<td>3</td>
<td>1997</td>
<td>Turkey [16]</td>
</tr>
<tr>
<td></td>
<td>Powdered tobacco</td>
<td>15/38</td>
<td>1.58</td>
<td>2.4</td>
<td>3000</td>
<td>1</td>
<td>3</td>
<td>1991</td>
<td>USA [10]</td>
</tr>
<tr>
<td>Toluene, hexane, acetone, methyl-ethyl-ketone</td>
<td>Shoe factory workers (exposed to solvent-based adhesives)</td>
<td>29/25</td>
<td>0.31</td>
<td>1.9</td>
<td>2000</td>
<td>1</td>
<td>4/6</td>
<td>2005</td>
<td>Brazil [74]</td>
</tr>
<tr>
<td></td>
<td>Shoe manufacture</td>
<td>21/18</td>
<td>0.33</td>
<td>1.8</td>
<td>3000</td>
<td>1</td>
<td>3/4</td>
<td>2002</td>
<td>Turkey [59]</td>
</tr>
<tr>
<td>2-Trans-hexenol</td>
<td>Banana growers</td>
<td>7/7</td>
<td>0.70</td>
<td>2.6</td>
<td>5000</td>
<td>2</td>
<td>2</td>
<td>1997</td>
<td>Germany [144]</td>
</tr>
<tr>
<td></td>
<td>Hexanol rinse of 10 ppm</td>
<td>7/7</td>
<td>0.96</td>
<td>2.7</td>
<td>5000</td>
<td>1</td>
<td>2</td>
<td>1997</td>
<td>Germany [144]</td>
</tr>
</tbody>
</table>

Naturally present in fruit (i.e. banana).

* Staining techniques: (1) Feulgen/Fast Green; (2) DAPI; (3) Giemsa; (4) Acridine orange; (5) Acetorsine; (6) FISH; (7) Wrights.

* Scoring criteria: (1) Basic; (2) Stich and Rosen [36]; (3) Sarto et al. [9]; (4) Tolbert et al. [10]; (5) Livingston et al. [128]; (6) Titenko-Holland et al. [95]; (7) Countryman [149].

NR = not reported.

c PAH = polycyclic aromatic hydrocarbons.

* Statistically significant at \( p < 0.05 \) according to the authors.
propidim iodide with pancentromeric FISH labeling [13]. Significantly higher frequencies of MN have also been observed in exfoliated buccal cells from people exposed to organic solvents, antineoplastic agents, diesel derivatives, polycyclic aromatic hydrocarbons, lead–containing paints and solvents, and drinking water contaminated with arsenic [14,43–46,52–67]. Studies on the effects of exposure to pesticide mixtures, however, generally show no increases, with the exception of a study involving a small group of floriculturists in Mexico in whom a 2.6-fold increase in MN frequency was observed [66], and a 3.9-fold increase reported for workers at the pesticide manufacturing plant [65]. The lack of effect can be attributed to the relatively weak genotoxicity of most modern pesticides and the dermal, rather than oral, route of exposure [68–70]. Negative results were also obtained in subjects exposed to chromium, ethylene oxide, nickel, benzene, and other chemicals [8,15,47,61,71–74]. Recent studies have also suggested genotoxicity and cytotoxicity of urban air pollution and ozone during the summer season, particularly in places with high ambient levels [22,75,76]. Overall, although the link between exposures and increases in MN frequencies is strong for many chemicals and exposure conditions, further efforts are warranted to build a reliable database on the effects of common exposures, such as pesticide mixtures and air pollution, on induction of MN in buccal cells.

3.2. MN and radiation

Ionizing radiation plays an important role in the treatment of many neoplasias, but it also produces genetic damage. As a consequence, secondary tumors may develop years after the primary tumor treatment. Several studies evaluated MNi in buccal cells of patients undergoing radiotherapy in the head and neck region. The most striking increase in cytogenetic damage (150–300 MN/1000 cells) was observed in an early study of three patients exposed to a cumulative dose of 3400–4000 cGy [6]. However, these results may have been influenced by the inclusion of degenerated cells, given that criteria for distinguishing viable and degenerating cells were not yet established at the time. Other authors reported 68 MNi/1000 cells after 2000 cGy [9] and 16 MN/1000 cells after treatment with 1000 cGy for 3 weeks [10]. This range of doses and treatment periods then raises the question of the time-course and dose-response for the induction of MN in buccal cells. This issue was carefully addressed in a study by Moore et al. [77] (Fig. 4) in which a more than 16-fold increase in MN frequency was observed shortly after initiation of radiotherapy, followed by return to baseline 12 weeks later and 3 weeks after cessation of the treatment. An interesting aspect of this study was the use of pancentromeric labeling that allowed differentiation between centromere-positive and -negative MNi. At the peak of the radiation-induced cytogenetic response, approximately 90% of MNi contained acentric fragments [77], which reflect chromosome breakage events. In a more recent study, an increase in MN frequency in a group of head-and-neck cancer patients undergoing radiotherapy was evident both in buccal cells and peripheral lymphocytes [78]. In this study a significant decrease in buccal mucosa cells with MNi was observed beginning 30 days from the last treatment. Cao et al. [79] found that the cytokinesis-blocked MN assay in peripheral blood lymphocytes was more sensitive than the buccal MN assay for monitoring genetic damage in nine nasopharyngeal cancer patients undergoing radiotherapy. It was reported that radiation-sensitive oral tumors showed higher MN levels in exfoliated cells after radiation therapy than the more radiation-resistant ones, and the assay can be used as a predictor of tumor radiosensitivity [80]. However, this approach requires further confirmation. Data from such radiation exposure studies have helped to determine the kinetics of appearance and disappearance of MNi in buccal mucosa and to define the optimal sampling time. Whether the observed kinetics of MN expression following radiotherapy is applicable to other genotoxic exposures that affect nuclear division in the basal cell layer of the buccal mucosa remains unclear.

3.3. MN frequencies in buccal cells of patients with cancer and other diseases

Biomonitoring of the changes in patients with diagnosed diseases or pathological changes that may lead to the development of cancer and other illnesses is becoming increasingly popular, and may be the most rapidly growing area of application of the MN assay to epithelial cells. The MNi in buccal mucosa cells were used to study preneoplastic effects by collecting the cells directly from the affected tissues (Table 2). The MN frequencies in patients with oral lesions such as oral submucosal fibrosis, oral leukoplakia and oral lichen planus were increased relative to healthy subjects, but no significant differences in MN rates were observed among the various patient groups [81]. Another study of untreated cancer patients observed increased genomic instability in somatic cells (blood lymphocytes and exfoliated buccal epithelia) in comparison to healthy control subjects [82]. It was suggested that the MNi in buccal mucosa may predict cancer risk for the upper aerodigestive

![Fig. 4. Dynamics of total MN and centromere-negative (MN−) in buccal cells after radiotherapy. Modified from Moore et al. [77]. Data points show the frequency of MN cells at different times during and after 9 weeks of radiation therapy in the oral region. Columns show percentage of MN cells that were centromere negative by FISH with pancentromeric labeling, indicating chromosomal breakage. Approximately 2000 cells were scored at each time period. The MN+/MN− ratio at 12 weeks was not determined.](image-url)
tract, including premalignant stages such as oral leukoplakia [83]. A significantly higher frequency of buccal cells with MN was found in breast and uterus cancer patients and their first-degree relatives, compared to controls [84,85]. At this time it remains unclear whether an elevated frequency of MN in certain tissue, such as oral epithelia, would be predictive of increased risk of future cancer say only for oral cavity, limited to upper digestive tract epithelia, or may be projected for various cancers in other parts of the body. This gap of knowledge will be an important focus of future HUMN projects.

Several publications reported MN frequencies in patients with DNA repair deficiencies with varied results. For example, patients with Louis–Bar syndrome did not have elevated MN frequency [86] while both Ataxia telangiectasia and Bloom’s syndrome exhibited increased genome instability in patients (homozygous) compared to heterozygous subjects [87,88]. A site-specificity was observed for Xeroderma pigmentosum patients, with a higher MN frequency in cells from the dorsal tip of tongue, possibly due to greater light exposure [89].

Recently, a number of papers applied the MN assay and cytome approach [132] in buccal epithelia. In patients with Alzheimer’s disease there was no significant increase in the MN frequency but the frequencies of basal cells, condensed chromatin cells and karyorrhectic cells were lower than in matched controls [147]. Down syndrome was associated with a 733% increase in MNi in comparison to younger healthy controls, and the MN frequency was 78.5% higher than in older controls [148]. A statistically significant decrease in pycnotic, karyolytic and condensed chromatin cells was also observed. An increase in MN frequency in buccal cells was reported for Diabetes mellitus with the patients having double the level of genetic damage in comparison to matched controls [109] and for treated pediatric patients with ulcerative colitis in comparison with controls or children with Crohn’s disease [108].

### 3.4. Lifestyle and host factors

Fig. 5 summarizes the distribution of positive and negative findings with respect to the effect of age, sex, smoking, and alcohol consumption on buccal cell MN frequency. Although many studies report the age and sex of the study subjects, only a fraction of these studies were able to establish a statistically significant effect by gender [14,90] or by age [16,47,67]. It is interesting that in two studies men had a slightly higher MN frequency in lymphocytes of women and older subjects [3]. In the study of 120 healthy subjects from Poland, neither age or sex were significantly associated with MNi [127].

#### Table 2

Micronucleus frequency in exfoliated oral mucosa cells of patients with cancer and precancerous conditions

<table>
<thead>
<tr>
<th>Type of cancer</th>
<th>Study subjects/ exposure control</th>
<th>Baseline, MN cells/1000 cells</th>
<th>Fold-difference vs. control</th>
<th># cells scored/ subject</th>
<th>Stain a</th>
<th>Scoring criteria b</th>
<th>Country</th>
<th>Year</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper digestive tract</td>
<td>38/37</td>
<td>1.30</td>
<td>2.0</td>
<td>1000</td>
<td>1</td>
<td>2</td>
<td>France</td>
<td>1987</td>
<td>[145]</td>
</tr>
<tr>
<td>Oral submucous fibrosis</td>
<td>68/20</td>
<td>1.90</td>
<td>6.1自媒体</td>
<td>1000</td>
<td>3</td>
<td>3</td>
<td>India</td>
<td>1996</td>
<td>[81]</td>
</tr>
<tr>
<td>Oral leukoplakia</td>
<td>18/20</td>
<td>1.90</td>
<td>5.7自媒体</td>
<td>1000</td>
<td>3</td>
<td>3</td>
<td>India</td>
<td>1996</td>
<td>[81]</td>
</tr>
<tr>
<td>Oral lichen planus</td>
<td>14/20</td>
<td>1.90</td>
<td>6.2自媒体</td>
<td>1000</td>
<td>3</td>
<td>3</td>
<td>India</td>
<td>1996</td>
<td>[81]</td>
</tr>
<tr>
<td>Squamous cell carcinomas, upper aerodigestive tract</td>
<td>44/98</td>
<td>9.00</td>
<td>2.3</td>
<td>1000</td>
<td>3</td>
<td>1</td>
<td>Germany</td>
<td>2000</td>
<td>[83]</td>
</tr>
<tr>
<td>Oral leukoplakia</td>
<td>16/98</td>
<td>9.00</td>
<td>2.2自媒体</td>
<td>1000</td>
<td>3</td>
<td>1</td>
<td>Germany</td>
<td>2000</td>
<td>[83]</td>
</tr>
<tr>
<td>Untreated breast cancer patients</td>
<td>30/46</td>
<td>2.70</td>
<td>12.6自媒体</td>
<td>1000</td>
<td>3</td>
<td>NR</td>
<td>India</td>
<td>2000</td>
<td>[84]</td>
</tr>
<tr>
<td>First degree female relatives of breast cancer patients</td>
<td>60/46</td>
<td>2.70</td>
<td>6.1自媒体</td>
<td>1000</td>
<td>3</td>
<td>NR</td>
<td>India</td>
<td>2000</td>
<td>[84]</td>
</tr>
<tr>
<td>Oral carcinoma</td>
<td>30/30</td>
<td>1.13</td>
<td>4.5自媒体</td>
<td>2000</td>
<td>1</td>
<td>4</td>
<td>Brazil</td>
<td>2002</td>
<td>[98]</td>
</tr>
<tr>
<td>Breast</td>
<td>12</td>
<td>0.93</td>
<td>2.2自媒体自媒体</td>
<td>2000</td>
<td>1</td>
<td>2</td>
<td>Armenia</td>
<td>2004</td>
<td>[85]</td>
</tr>
<tr>
<td>Cervix and corpus uteri</td>
<td>36</td>
<td>0.93</td>
<td>2.2自媒体自媒体</td>
<td>2000</td>
<td>1</td>
<td>2</td>
<td>Armenia</td>
<td>2004</td>
<td>[85]</td>
</tr>
<tr>
<td>Various untreated tumors</td>
<td>37/43</td>
<td>0.60</td>
<td>1.3</td>
<td>2000</td>
<td>5</td>
<td>4</td>
<td>Mexico</td>
<td>2004</td>
<td>[61]</td>
</tr>
<tr>
<td>Precancerous oral lesions</td>
<td>29/60</td>
<td>1.6</td>
<td>1.2</td>
<td>2000</td>
<td>3</td>
<td>NR</td>
<td>India</td>
<td>2007</td>
<td>[146]</td>
</tr>
<tr>
<td>Oral cancer</td>
<td>24/60</td>
<td>1.6</td>
<td>1.6</td>
<td>2000</td>
<td>3</td>
<td>NR</td>
<td>India</td>
<td>2007</td>
<td>[146]</td>
</tr>
</tbody>
</table>

a Staining techniques: (1) Feulgen/Fast Green; (2) DAPI, (3) Giemsa, (4) Acridine Orange, (5) Acetorseine.
b Scoring criteria: (1) Basic, (2) Stich and Rosen [36], (3) Sarto et al. [9], (4) Tolbert et al. [10], NR = not reported.
* Statistically significant at p < 0.05 as defined by the authors.

![Fig. 5. Baseline distribution of MN frequency: role of host and lifestyle factors](image-url)
average had almost triple the MN frequency compared to non-smokers. Yet other publications report no difference between smokers and non-smokers or men and women [82]. Thus, the potential contribution of gender and age in the frequency of MN in buccal cells warrants further investigation.

Lifestyle factors that are associated with genetic damage include smoking, alcohol consumption, and diet, especially vitamin deficiencies and supplementation [1,91]. The majority of the studies reporting a significant increase in MN in buccal mucosa cells related to a risk of oral cancer were performed in subgroups of subjects with specific lifestyle habits, i.e., chewers of betel quids (areca nut, betel leaves, slaked lime and tobacco) from India, Taiwan and Philippines; reverse smokers (who hold the lit end of the cigarette inside their mouths) from India and Philippines; snuff dippers from Canada; users of Khaini tobacco (tobacco mixed with slaked lime) from India, and other similar practices [10,92–94]. Results in these studies may be affected by overestimates of the MN frequency because both smoking and chewing of tobacco mixtures are known to cause nuclear degeneration and appearance of MN-like bodies in exfoliated cells, which may be confused with MNi. The increased frequency of cells with degenerating nuclei may occur due to normal cytotoxic processes and may not necessarily be associated with clastogenic or aneugeneic DNA damage [5,95–97]. It is important for monitoring and risk assessment to distinguish cell death events from genome damage in viable epithelial cells both in terms of biological dosimetry and for evaluating cancer risk.

In several studies of lifestyle factors, however, it was difficult to differentiate the effect of alcohol from that of smoking. For example, neither alcohol or smoking, alone, increased the MN frequency in buccal cells, but a synergistic effect of smoking and alcohol was evident, with up to a 5.5-fold increase relative to non-smoker and non-drinker controls [36]. A prospective study comparing alcoholics with oral or oropharyngeal carcinoma to healthy subjects who did not consume any alcohol found a significant association between MN frequency and the age of the beginning and cessation of drinking, and the duration of alcohol consumption [98]. This apparent synergistic interaction of alcohol consumption and smoking, and the relative contribution of each exposure, is another aspect of buccal cell monitoring that requires further study.

A number of micronutrients, including beta-carotene and other vitamins, have been shown to significantly decrease MN levels (1.4–4-fold) in healthy tobacco users, as well as in individuals with precancerous lesions [7,99–101]. A study of heavy smokers in the Netherlands found a statistically significant decrease in MN frequency when N-acetylcysteine was administered as chemopreventive agent [101]. The effect of chemoprevention on the remission of specific lesions such as leukoplakia, and on the prevention of new lesions, can be observed after treatment, and it is accompanied by a corresponding decrease in MN frequency [102,103]. These results suggest that decreases in MN frequency in established precancerous lesions, which are likely to be genomically unstable, may be indicative of a reversion to a more normal phenotype, but whether these changes are causal or coincidental remains unclear. Other micronutrients, such as retinol, riboflavin, zinc, and selenium, however, failed to reduce the MN frequency in a study carried out in China in areas with a high incidence of oesophageal cancer [104]. There was no effect of drinking water in women from two regions in Turkey with high or low boron [105]. In contrast, a reduced MN frequency was associated with green tea consumption in patients with oral leukoplakia [106]. Declines in MN frequencies were reported in children and women who received controlled folate supplementation [107,108] and in patients with diabetes [109]. It is not clear whether the observed reductions in the MN frequencies in these studies were due to a decrease in chromosomal instability as a result of supplementation, or to a modified basal cell proliferation that altered the kinetics of MN expression.

4. Methodological aspects affecting identification of MN

Methodological factors that can affect the levels of MNi in buccal cells include differences in cell collection (timing and implements used), fixation and staining techniques, selection and number of cells counted, and the scoring criteria for MNi and other nuclear anomalies in normal and degenerated cells. Many of these methodological factors for buccal cells overlap with the MN assay in lymphocytes, but the differences in tissues may contribute to the variability. Thus, the data reported by the HUMN on MN assay in human lymphocytes [3] can be applied to buccal cells with caution, although a special validation study for buccal cells is needed. It is difficult to tease apart the effects of these factors or their combinations based on the available studies in different laboratories. Many laboratories have their preferred protocols and only a handful of attempts have been made to compare modifications of the procedure within the same study [95–97,110]. These are discussed in more detail in Section 4.2. The main sources of variability among the laboratories may lie in the scoring criteria and staining procedures used. The effects of these factors on MN scoring in the buccal assay have not been properly evaluated or quantified.

4.1. Collection of buccal cells

Exfoliated buccal mucosa cells can be collected using a wooden tongue-depressor, a metal spatula, or a cytobrush moistened with water or buffer to swab or gently scrape the mucosa of the inner lining of one or both cheeks. A few studies have used toothpicks or toothbrushes [17,49,50,56,90,111,112]. In some cases, buccal cells have also been collected from the inner side of the lower lip and from the palate; the variability in MN frequency between these areas was minimal for control subjects as reported in earlier studies [35,113]. In contrast, MN frequency was significantly higher in epithelia of the pharynx of adolescent girls from Mexico City in comparison to their cheek [114]. Cytobrushes appear to be most effective for collecting large numbers of buccal cells. Casartelli et al. [115] observed that MN frequencies were higher when cells were collected by vigorous, rather than by light, scraping, suggesting a decreasing MN frequency gradient from basal to superficial layers of mucosa.

The turnover rate for the appearance of MN in exfoliated buccal cells in an otherwise normal cell after exposure to an acute genotoxic event, such as ionizing radiation, is estimated to be a minimum of 5–7 days. Inter-individual variation during this time-course of MN expression, however, has been observed so that peak expression of MN may be delayed up to 21 days [6,8,77]. Thus, multiple sample times are required to identify optimal timing between 7 and 21 days after exposure because peak expression may vary depending on the effects of the particular DNA damage or chromosomal exposure on basal cell turnover rate. It is possible that certain genotoxic exposures could cause an inhibition or an enhancement of the basal cell proliferation and thus confound the kinetics of MN expression.

4.2. Slide preparation and staining

In many MN studies, buccal cell smears have been prepared by spreading the cells on a clean slide. In a number of studies the cytobrush used to collect buccal cells was shaken in a centrifuge
tube containing saline solution (Hank's basic or other buffer solution) to release the cells, and the tube is then centrifuged to wash the cells in a buffer solution [17,37,43,47,51,54,60,67, 72,95,111,116–118] or a fixative [56]. This washing procedure helps to remove bacteria and cell debris, which confound the scoring. Cells are then transferred to slides either by careful dropping with pipette or by cytocentrifugation followed by fixation. Commonly used fixatives include 80% methanol, absolute ethanol, or a methanol–glacial acetic acid mixture. The stringency of the fixative and the condition of the cells before fixation (air dried or fresh) may affect cell integrity and the preservation of normal and degenerated cells with MN and other abnormalities.

Several staining methods have been used, although DNA-specific stains are preferred for staining nuclei, MN, and other nuclear anomalies in buccal exfoliated cells. Feulgen-Fast Green (FFG) staining is favored by many investigators because of its DNA specificity and a clear transparent appearance of the cytoplasm which enables easy identification of MNi (Table 1). MNi stained with FGG have been examined under light microscopy in the majority of the studies, although it is possible to examine these slides under fluorescence. One shortcoming of this staining method is that it is relatively lengthy and may lead to the underscoring of MNi [96]. Some studies used fluorescent dyes such as DAPI [104,111,112], acidine orange (AO) [73,119], Hoechst [96], and propidium iodide (PI) [15,77,95,96]. May-Grünwald–Giemsa (Giemsa) stain has been used at different concentrations (2–10%) in several laboratories [22,81,83,84,86,104,120]. Some of the studies reported increased frequencies of MNi with Giemsa staining and suggest the possibility that cellular structures resembling MNi, such as keratohyalin granules or bacteria, can lead to false positive results [96,97]. Keratohyalin granules are reported to occur in cells of the granular layer of interfollicular epidermis of the skin [121] and are predominantly composed of the 400-kDa protein profilagrin and, therefore, possibly distinguishable from MNi using immunohistochemical methods. However, no specific data about the commonality of these granules or their frequency in oral epithelia is currently available.

Another factor that can interfere with MN scoring is contamination by the bacteria that are commonly found in the mouth [95,110]. Bacteria can be differentiated from MNi by their characteristic shape, smaller size, color, staining intensity, and their presence upon and between buccal cells on the slide. Another common confounding issue is the small dye granules that may sometimes resemble MNi but usually have a slightly different refractility and color intensity. These factors need to be taken into consideration when optimizing the staining protocol to minimize the possibility of scoring these artifacts.

A wide range of baseline MN values have been reported, regardless of the staining method used (Table 1). The conclusions differed in five studies that attempted a systematic comparison of two or more staining methods for buccal cells. For example, FFG staining was compared with PI, which also served as a counterstain for pancentromeric fluorescent probes following in situ hybridization (FISH) [95]. In this study the fluorescent dye PI performed equally well as FFG, but provided an additional advantage of identifying the mechanism of MN formation through centromeric FISH labeling. Casartelli et al. evaluated the strengths and limitations of three staining procedures (Hoechst, PI, and Giemsa), and suggested that Hoechst was the easiest and most reliable for identifying buccal cell MN [96]. A more recent paper that assessed four staining procedures (Giemsa, FGG, DAPI, and acidine orange), concluded that Giemsa is more likely to lead to false-positive identifications of MNi and recommended FGG as a preferable staining method [97]. Another recent study compared Giemsa with the Papanicolaou (Pap) stain for buccal cell analysis under field conditions [110] and concluded that the Pap stain was the preferred method of detecting MN in oral epithelia. FFG was compared with acidine orange in the study of oral exfoliated subjects with leukoplakia and squamous cell carcinoma [122], and the authors reported that in their hands fluorescent staining (acidine orange) was more sensitive for MN detection than FFG. The possibility that Giemsa staining could lead to the misidentifications of artifacts as MNi, because it may not distinguish true MNi from profilagrin or keratohyalin granules also needs to be investigated. Because these studies came to different conclusions, it is apparent that the stain, staining procedure, and laboratory scoring procedures, may all contribute to the variability seen. Studies are needed to determine whether some MNi and nuclei may lose DNA through karyolysis while maintaining the protein structure of chromatin and the nucleus envelope, so that they would still be detectable by stains that are not DNA-specific. Therefore, a systematic study investigating the performance characteristics and the extent of correlations among the MN frequencies determined with various staining methods currently in use is urgently required so that an optimal staining protocol can be determined. Because FFG has been shown to be the most consistent, and is DNA-specific, it would be advisable to suggest this staining method as the interim standard against which other methods should be validated.

### 4.3. Scoring criteria

Another important aspect of the MN assay in buccal mucosa cells is the criteria for identifying and scoring the cells. The first publications of Stich and Rosin [7,34,35,40,99] referred to the well-established basic criteria for MN that were initially described by Heddle [123]. However, criteria for identifying cells for inclusion into the MN frequency count were not provided. Other authors refer to the Heddle criteria as such, or with minor modifications (Table 1). The criteria developed by Tolbert et al. [5,10] for choosing the cells are the most widely used. They consist of the following parameters for cell inclusion in the cells to be scored: (a) intact cytoplasm and relatively flat cell position on the slide; (b) little or no overlap with adjacent cells; (c) little or no debris; and (d) nucleus normal and intact, nuclear perimeter smooth and distinct. The suggested criteria for identifying MN are: (a) rounded smooth perimeter suggestive of a membrane; (b) less than a third the diameter of the associated nucleus, but large enough to discern shape and color; (c) Feulgen positive, i.e. pink in bright field illumination; (d) staining intensity similar to that of the nucleus; (e) texture similar to that of nucleus; (f) same focal plane as nucleus; and (g) absence of overlap with, or bridge to, the nucleus.

Tolbert et al. [10] outlined all the specific nuclear alterations that should be taken into account when deciding to include or exclude cells for determining the frequency of MN cells, and provided evidence that other nuclear anomalies are at least as common as micronucleation and, therefore, have the potential for misclassification. An updated diagram, based on the original scheme published by Tolbert et al. [5] of the various cell types and nuclear anomalies that can be observed as the cells migrate to the surface of the buccal mucosa is shown in Fig. 3. Common cell types associated with the differentiation process are shown, including cells with pyknotic, condensed chromatin, karyorrhectic, karyolytic (“ghost” cells), nuclear bud (or “broken egg”) and binucleated cells. Alternately, these cells could also represent responses to cytotoxicity or be secondary to genotoxic events. The processes underlying the frequency and appearance of these structures are not yet fully understood. Despite these drawbacks, a large number of publications refer to these criteria [16,43–46,54,67, 71,72,75,81,120,124–127]. Less detailed criteria, similar to those
of Tolbert’s but without dividing the cells into three categories of certainty of identification, have also been used [9,102,104,107,117,128,129]. Results from studies that do not report scoring certainty of identification, have also been used [9,102,104,107,117,128,129].

4.4. Number of cells scored; power of the test to detect an increase

The number of buccal mucosa cells to be scored in order to obtain statistically significant results needs to be addressed. The first studies from the 1980s [6,34,35] evaluated a relatively low number of cells (∼500). Later, Tolbert et al. [10] recommended the scoring of at least 1000 cells, with an increase to 2000–3000 if fewer than 5 micronucleated cells were observed after counting 1000 cells. Most published studies have scored between 1000 and 3000 cells (Table 1), although it has been suggested that 10,000 cells may be needed to observe a statistically significant, 50% increase, in MN frequency [130]. This approach would require automation, especially in biomonitoring studies that include a large number of subjects. However, the power to detect statistically significant results also depends on the baseline MN level in the study population. The factors that may modulate baseline MN levels in buccal cells are discussed elsewhere in this review. The frequencies of the various cell types illustrated in Fig. 3 should be determined independently and concurrently during the scoring as these markers may also be correlated with exposure to genotoxins. Further research is needed to quantify these various cell types and nuclear anomalies in buccal cells, and to establish their relationship with MN frequency and exposure to genotoxins.

5. A proposed validation study to examine slide preparation and scoring variables

The HUMN project’s experience with the assessment of the intra- and inter-individual variability of the lymphocyte MN assay using an international validation project and slide–scoring exercise [2,3] can serve as an example for developing a strategy for understanding and validating the buccal cell MN procedures. Fig. 6 shows the different factors contributing to the variability of the human lymphocyte MN assay that was developed by the HUMN validation project; it is evident that, in this assay, there is only a very small fraction of unexplained variance (3.1%) in the MN frequency [3]. These values were obtained through the use of a careful study design which took the main explanatory variables into consideration, and also because of prior agreement and standardization of the scoring procedures among participating laboratories. It should be noted that the participating laboratories represented a wide range of prior experience with the assay. This inter-laboratory agreement was possible because scoring criteria with detailed photos illustrating each endpoint were prepared for the participants before the exercise [2] and later published along with its results [3]. The main response variable, i.e. dose of genotoxin (in this case, irradiation, which would be expected to produce less inter-laboratory variability than chemical treatment) accounts for the largest portion of the variance (65%). The contribution of the laboratory to the MN frequency variance was much smaller (4.6%), and the intra-scorer variability and effect of staining method each contributed less than 1% of the total variance. The situation with the buccal cell MN assay may be somewhat different because a wider range of cell collection, slide preparation, and staining methods are currently being used, and there is a larger variability in scoring procedures among the different laboratories.

HUMN committee recently sent out an invitation to the prospective collaborators to contribute to HUMNXL project (XL subscript was designed to emphasize the focus of this HUMN initiative on the MN assay in exfoliated buccal cells). A detailed questionnaire was mailed to over 200 researchers who have published papers using the buccal cell MN assay, participated in the HUMN Workshops over last 10 years, or contacted HUMN committee through the HUMN website or personal communications. We anticipate that MN data for several thousands subjects will be incorporated in the database to be used for analysis of factors of variability, and later for establishing predictive value of the MN frequency in buccal cells for tissue-specific or generalized cancer risk. These documents are available on the HUMN website (http://www.humn.org).

5.1. Study design

The next step of the HUMNXL will be to conduct a specially designed validation exercise focused primarily on the effects of staining and scoring procedures. The process of the initial slide generation for HUMNXL buccal MN project may be more complicated than it was for the lymphocyte MN validation exercise. In the latter case, blood cells from the same donor were used for control and irradiated cultures, and the half of the slides were stained in a single laboratory by a standard Giemsa method [2]. The remainder of the fixed slides were stained in each of the participating laboratories by their method of choice. Because most of the laboratories had one or two scorers involved in the exercise and only few research groups had four participants, the scope of the intra-laboratory scoring analysis remained manageable. Each scorer spent approximately 2 days to analyze six slides using two separate, delineated areas (spots) on each slide. However, for the buccal cell study, it may be necessary to collect cells from a number of subjects, because only a limited number of cells can be obtained from each subject at one time without introducing a possible variability of the depth of exfoliation or the timing of collection. In
order to standardize the slides for the scoring exercise, cells from
these several subjects can be pooled together and thoroughly
mixed before placing them randomly on a sufficient number of
slides for the distribution to the participating laboratories. Initially,
the slides could be generated and stained by a standard method
(e.g. FFG) in one laboratory, with the provision that a portion of the
slides will be available for staining in each of the participating
laboratories using their preferred method. All slides would be
coded before distribution to the scorers in different laboratories.
The background MN frequency in buccal cells is relatively low
(∼0.1%); therefore the large number of cells that will have to be
scored for sufficient statistical power of analysis may impose a
significant demand on time and resources of participating
laboratories. However, if the assay is to have any validity as a
biomarker of exposure or effect, sufficient cells would have to be
scored to allow for a stringent statistical analysis of the data.

This initial stage of assay validation will focus on (a) addressing
the effect of different staining methods on the results, and (b)
determining the extent of variability in slide scoring among scorers
in the same laboratories, and among laboratories. This latter
exercise would only be performed after detailed scoring criteria are
established and distributed, as described in Section 5.5. A
subsequent phase of the validation study may include a
simultaneous collection of samples in different locations from a
designated number of subjects in order to perform a sufficiently
powered study aimed at determining the effect of geographic,
ethnic, and lifestyle variability, as well as sampling variability. The
slides used in such a study would be prepared, stained and scored
according to a previously standardized protocol developed and
endorsed by the HUMNXL project participants.

5.2. Positive control

The choice of a positive control (comparable to in vitro
irradiation that was employed for lymphocyte project) will present
a challenge for the buccal cell MN validation project. One of the
possibilities is to use cells from patients undergoing X-ray
treatment in the head/neck area during the period most likely
to lead to significant MN induction, as was discussed above.
However, it may be difficult to generate sufficient, identical
positive control slides to provide to all participating laboratories.
Another alternative is to use specimens from patients with a DNA
repair disease, such as Bloom syndrome, which have been shown
to have an elevated buccal cell MN frequency [131]. Before these
positive control slides are used, however, a pilot scoring exercise
will be needed to assure that significantly increased MN levels can
be observed.

5.3. Sampling method

It will be necessary to design a protocol to allow the scoring of
the ratios of basal, differentiated, and degenerated cells, and to
determine whether the buccal cell MN frequency is affected by
these ratios because (a) sampling method and inter-individual
differences may alter ratios of basal to differentiated and
degenerating cells, and (b) MNi in the degenerating cells may be
lost due to lysis or degradation. This potentially confounding factor
could be addressed by alternating the timing of collection after
controlled exposure, and by variation of intensity of “brushing” of
the cheek lining.

5.4. Slide preparation method

The effect of staining method on the MN frequency in various
buccal cell types needs to be determined conclusively. A standard
protocol for slide preparation, fixation, and staining for both light
and fluorescence microscopy will have to be developed and
validated. Alternative methods of staining should be validated
against the better-established FFG method, which allows scoring of
slides under both transmission and fluorescence microscopy. It is
evident that methods that could also stain other structures that
resemble MNi, such as keratohyalin granules, need to be
discouraged or should be required to include a process (e.g.
immunohistochemical stain to profilagrin) that allows these
structures to be distinguished from true MNi.

5.5. Scoring criteria

A standard set of scoring criteria, together with matching
photomicrographs and line diagrams for the buccal MN assay,
similar to those developed for the cytokinesis-block lymphocyte
MN assay [2], is required to standardize scoring and minimize
inter-laboratory differences. These criteria should be designed to
enable (a) easy and unbiased classification of the various cell types,
as outlined in Fig. 3, and (b) identification of those cells that contain
one or more “true” MNi. Because “true” MNi are originally
expressed in basal cells and may be retained in normal
differentiated cells but lost in degenerating cells (i.e. condensed
chromatin, karyorrhectic, pycnotic, and karyolytic cells), and cells
with degenerating (apoptotic) nuclei may generate structures that
look similar to MNi, it is essential that MN frequencies in these
different cell types are reported separately.

5.6. Number of cells to be scored

A statistical assessment of the optimal number of buccal cells to
be scored is required because of the low spontaneous MN
frequency in these cells. Currently, most of the published reports
use 1000–3000 cells. It is anticipated that more precise results
would be obtained with increasing numbers of cells, up to a point
where the additional precision gained would not be worth the
additional effort. Automated scoring of MN in buccal cells could
potentially increase feasibility of scoring of 10,000 cells or more,
thus significantly increasing the power of analysis. An acceptable
minimum number of basal and normal differentiated cells needs to
be established for determining MN frequency, and an adequate
coefficient of variation for repeat measurements needs to be
established.

5.7. Cytome approach

Experience with the cytokinesis-block lymphocyte MN assay
has shown that a “cytome” approach based on scoring not only MN
frequency but other genome damage markers (e.g. nuclear
buds, etc.), dead or degenerated cells, as well as the proliferation
index, assures a more comprehensive measure of cytotoxic and
genotoxic effects and provides important mechanistic insights
[132]. In this regard, the development of strict scoring criteria and
visual examples of various types of exfoliated buccal cells for all
participating scorers becomes essential.

6. Addressing knowledge gaps with the buccal cell assay

Although it would be wonderful to present a detailed plan for all
future stages of the HUMNXL at this time, we view the proposed
overview as a work in progress, to be developed following
discussions among the collaborators, including the prospective
participants. Because there are several different aspects of the
assay to be addressed, the “plan” of attack will evolve as the project
progresses based on information developed from the initial
activities and from new information generated by the scientific community as a consequence of this article. There are many remaining gaps of knowledge related to biomonitoring using the MN assay in buccal cells that will be addressed in next sections, including the effects of cell kinetics, a better understanding of the buccal cells biology and structure, effects of various host and lifestyle factors, correlation of the MN data between buccal and other cell types, and a predictive value of the MN frequency for cancer and other diseases.

6.1. Effect of cell division kinetics on MN frequency

MN expression occurs only in cells that have completed nuclear division following the genotoxic insult, and the proportion of those cells in the overall cell population affects the observed MN frequency. The relationship between the scorable MN frequency and the proportion of dividing cells among the basal cells is not known, but may be altered by division kinetics in response to an environmental challenge or a genetic defect in cell cycle checkpoints. The modeling of the effect of altered cell division kinetics in basal cells on the MN frequency in buccal cells is a necessary area of research.

6.2. Improved knowledge of the biology and structure of buccal cells

A better understanding of the molecular events underlying the cytogenetic and cytological changes in basal and differentiated buccal cells in response to environmental and nutritional stress is needed. This would help to clarify whether other structures in these cells (such as keratohyalin granules) are increased concomitantly with MN formation. Insufficient information is available about metabolic capacity of buccal cells and persistence of MNi in the epithelial stem cells.

6.3. Variables that affect MN frequency in buccal cells

A systematic and adequately powered investigation of key variables such as age, gender, genotype, season, diet, oral hygiene (e.g. hydrogen peroxide in tooth paste) and dental health (e.g. high number of missing teeth, periodontal status, etc.), lifestyle, smoking, alcohol, and other recreational drugs, needs to be performed to identify the variables that have to be controlled.

6.4. Correlation with MN frequency in other cell types

A number of studies used two or three cell types from the same subjects to assess MN frequency [15,51,107,112]. However, it remains unclear whether the MN frequency in buccal cells would be predictive of MN frequency in other cell types such as lymphocytes. Further, in addition to a direct contact exposure in the mouth, a possibility of MN induction in buccal cells resulting from systemic exposure throughout blood stream should be considered. This knowledge is important to determine whether one of these measurements can be a surrogate for the other. Until this is known, it may be necessary to biomonitor both buccal and blood cells, and determine the extent to which one can extrapolate observations between the two cell types and integrate the damage from a variety of concurrent exposures.

6.5. Does the MN frequency in buccal cells predict risk for cancer or other chronic diseases?

It is well-established that chromosome aberrations in human lymphocytes predict a risk of cancer [133–136], and the same association was recently confirmed for MNi in lymphocytes [4]. However, it remains unknown whether an elevated MN frequency in buccal cells is predictive of cancer risk, and whether the relationship is primarily site-specific (oral cavity), or extends to overall cancer risk. A prospective study would help to validate the use of the buccal MN assay as a biomarker of cancer risk in addition to being a biomarker of exposure.

7. Summary and future directions

Although many studies have consistently shown a statistically significant increase in the buccal cell MN frequency in human populations exposed to genotoxic agents, or a decrease as a result of micronutrient supplementation or chemoprevention, the magnitude of changes is 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 few of the studies have included a sufficiently broad age range, and some were underpowered for these effects. Only heavy smoking and other forms of tobacco consumption have been associated with oral malignancies. 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 induced genotoxic effects may be more readily observed. Despite the considerable potential of the buccal MN assay for biomonitoring, the diversity of possible methodological variables, and their impact on assay performance, could hinder consistency among laboratories with regard to measuring the effects of dietary, lifestyle, and genetic factors.

In this context it is mandatory that a standardization of the buccal cell MN protocol and of the scoring criteria be undertaken, and that an inter-laboratory calibration exercise be organized. This would enable data from different laboratories and different countries to be more comparable and eliminate or minimize uncertainty due to methodological variables. In addition, the development of automated scoring systems is encouraged as a critical advancement for high-throughput and statistically powerful analysis. The HUMNXL project plans to address some of the important issues listed above in consultation with key scientists in this field.

The discussions about the best approach to carry forward the buccal cell MN project took place at the HUMN workshop during the 5th International Conference on Environmental Mutagenesis in Human Populations that was held in Antalya, Turkey, on 21 May 2007. The workshop was attended by 70 representatives from various laboratories, universities, private companies and government departments from around the world. A list of participants and their email addresses was collected during the meeting for future contacts.

The aims of the workshop were to: (1) discuss current state of knowledge on the buccal MN assay; (2) identify important gaps of knowledge regarding theory, biology and methods used for buccal cell analyses; (3) decide on a plan of action to resolve the key methodological and knowledge gap issues; (4) explore pooling databases to determine most important variables affecting the assay. Presentations were followed by an open discussion on priorities, prospects and plans for a collaborative study to improve and standardize the buccal MN assay. There was an energetic discussion on various aspects of the assay reflecting the urgency and importance of resolving methodological issues that would enable a harmonized application of this important method. A detailed report about this workshop is available at the HUMN website.

It was decided to initiate first phases that include creation of the protocol for collection of the datasets and publication of the
participation in this project and responses to the HUMN XL...

The next steps of the HUMNXL plan for the buccal cell MN assay include international validation efforts, starting with the currently planned scoring and staining validation exercise followed by initiating the predictive value of this assay for cancer and other diseases. Based on our previous experience with the MN assay in human lymphocytes, these efforts will require combined efforts of dozens of collaborators from many countries and will be carried forward over the course of several years. Expression of interest for participation in this project and responses to the HUMNXL questionnaire should be sent to the HUMN website contact list (http://www.humn.org).

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