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# NEW APROACHES IN BIOMONITORING HUMAN POPULATIONS EXPOSED TO GENOTOXIC AGENTS: EPITHELIAL CELL MICRONUCLEUS ASSAY

Irina Alexandrescu, Doina Havârneanu, Doina Popa

Institute of Public Health Iaşi, România

**Abstract. Aim.** The cytological diagnose of exfoliative epithelia has been increasingly used during the last years in biomonitoring human populations exposed to genotoxic agents. **Material and methods.** While peripheral blood lymphocytes are the tissues most frequently studied to assess cytogenetic damage in humans, in some cases, epithelial tissues may be better models than lymphocytes. **Results and discussion.** Epithelia are frequently the actual targets of carcinogens, as indicated by the sites of cancers related to exposure. Moreover, exfoliated epithelial cells from the mouth, nose, and bladder can be easily collected for large scale biomonitoring. In the last few years, increasing number of studies, also use other types of epithelia including bronchial, esophageal, intestinal, cervical, and breast duct. Micronuclei (MN) are formed as a result of chromosome damage and can be readily identified in exfoliated epithelial cells. Several methodological issues have to be considered to assure high quality results: timing of cell collection, sufficient number of cells scored, differentiation of normal and degenerated cells on the slides to eliminate false-positives. Factors of interindividual variability, including age and gender differences, are important aspects in the design of biomarker studies using MN assay. **Conclusions.** Broad spectrum of environmental factors is associated with increased level of MN in epithelial cells. The goal for the future is to validate predictive value of MN in the risk of cancer and other adverse health outcomes.
**Key words: micronuclei, biomarker, epithelial cells, genotoxic agents, ocupational cancer**

**Rezumat. Scop.** Diagnosticul citologic al epiteliului exfoliativ este utilizat tot mai mult în ultimii ani în biomonitorizarea populației umane expuse la agenți genotoxici. **Material şi metodă.** Deşi limfocitele din sângele periferic reprezintă elementul tisular cel mai frecvent studiat pentru evaluarea modificărilor citogenetice la oameni, în anumite cazuri țesuturile epiteliale pot fi modele de studiu mai reprezentative decât limfocitele. **Rezultate şi discuții.** Țesutul epitelial constituie în mod frecvent țintă de elecție pentru carcinogeni, fapt demonstrat de localizarea cancerelor în relație cu expunerile specifice. În plus, celulele epiteliale exfoliate din cavitatea bucală, nazală sau vezica urinară pot fi cu uşurință colectate pentru biomonitorizare la scară largă. În ultimii ani apar tot mai multe studii ce utilizează şi alte tipuri de epiteliu, incluzând pe cel bronşic, esofagian, intestinal, cervical şi al canalului galactofor. Micronucleii (MN) se formează ca rezultat al alterărilor cromozomiale şi pot fi cu uşurință identificați în celulele epiteliale exfoliate. Pentru a asigura rezultate concludente, trebuie să se țină seama de anumite elemente de metodologie: momentul prelevării celulelor, evaluarea unui număr suficient de celule, diferențierea celulelor normale de cele modificate pentru a înlătura rezultatele fals-pozitive. **Concluzii.** Factori cum sunt variabilitatea interindividuală, incluzând diferențele de sex sau vârstă, reprezintă aspecte importante în derularea studiilor de tip biomarker care utilizează testul micronucleilor. Creşterea frecvenței micronucleilor în

țesuturile epiteliale este asociată cu un spectru larg de factori de mediu. În perspectivă se urmăreşte să se stabilească cu certitudine valoarea predictivă a MN în evaluarea riscului de cancer sau a celui legat de alte efecte negative asupra sănătății.
**Cuvinte cheie: micronuclei, biomarker, celulă epitelială, agent genotoxic, cancer ocupațional**

INTRODUCTION

There is an increasing effort world-wide to determine the impact of environmental, genetic and life- style factors on genomic stability in human populations.

One technique that has been adopted by numerous laboratories is the measurement of micronuclei MN in peripheral blood lymphocytes and, to a lesser extent, epithelial cells, erythrocytes, and fibroblasts.

MN provide a measure of both chromosome breakage and chromosome loss and it has been shown to be at least as sensitive an indicator of chromosome damage as classical metaphase chromosome analysis. The key advantage of the MN assay is the relative ease of scoring and the statistical power obtained from scoring larger numbers of cells than are typically used for metaphase analysis (1).

Over the last five years, the number of laboratories working with MN in exfoliated cells has substantially increased. Many research groups are interested in exfoliated cells because these cells hold strong potential as a tool for biomonitoring human populations exposed to genotoxic agents or undergoing preventive treatments. In many cases, epithelial tissues are the actual targets of carcinogens, as indicated by the sites of cancers related to the exposures. More than 90% of cancers arise in epithelial tissues. Epithelial tissues are in immediate contact with genotoxic agents inhaled with the air and ingested with food and water, as well as with excreted metabolites in urine (2).

The simplicity of this approach led to its adoption for both in vitro genotoxicity testing and human population monitoring; but as can be expected at the early stages of the application of a new technique, there has been considerable diversion in methods and some inconsistencies with regard to variables that influence the measurements.

**Brief history**

Despite these protocol differences, the induction of MN is considered to be an effective biomarker of diseases and processes associated with induction of DNA damage. That is why, in order to validate the MN assay and to establish a standard protocol for all steps (biological material collecting, laboratory technique, diagnose, interpretation and data processing), an international team of specialists launched in 1997 a long-term and world-wide collaboration activity known as HUMN (Human MicroNucleus) – The International Collaborative Project on Micronucleus Frequency in Human Populations.

This program was designed to obtain baseline MN frequencies from different laboratories world-wide, and among different populations, in order to identify the confounding variables influencing the measurement of MN frequency in humans; compare the

different techniques used with a view to defining a standard protocol for population-based studies; and perform a prospective study, together with ongoing case-control studies, to examine the suitability of MN as a biomarker of risk for cancer and other diseases, including ageing.

Dr. Nina Holland, PhD, Professor at the University of Berkeley California, is responsible in the HUMN project for the specialty of MN assay in exfoliated cells. In this position, she made a preliminary presentation on micronucleus analysis in exfoliated cells at the 2nd HUMN Workshop in Bangkok 1998 sustaining – on serious scientific arguments – the necessity of developing the research in this direction inside an international multidisciplinary program, with the following aims (2):

1. Comparison and standardization of the protocol of cell collection, processing and scoring. The results will be used to establish an internationally acceptable procedure that will enable comparison of results between laboratories and across countries;
2. Comparison of spontaneous and induced MN levels in different epithelial tissues. This comparison will provide information on the extent to which there is agreement on the key variables (e.g. age, sex, smoking) that affect the base-line MN frequency;
3. Validation of MN as a biomarker of cancer risk in epithelial tissues, which will require a prospective study linking MN index with cancer risk, mortality and life-span;
4. A parallel study of the MN frequency in exfoliated cells and lymphocytes in the same subjects in order to obtain additional information on the predictive value of MN as a biomarker of cancer risk in humans.

Present situation for these goals and a plan of action are to be presented by Dr. Nina Holland in a Workshop of the International Conference on Environmental Mutagens in Human Populations programmed to take place in 20-23 May 2007 in Antalya Turkey.

**Current state of knowledge - HUMN**

**a. MN forming mechanism**

A micronucleus (MN) is formed during the metaphase/anaphase transition of mitosis – cell division (fig. 1).

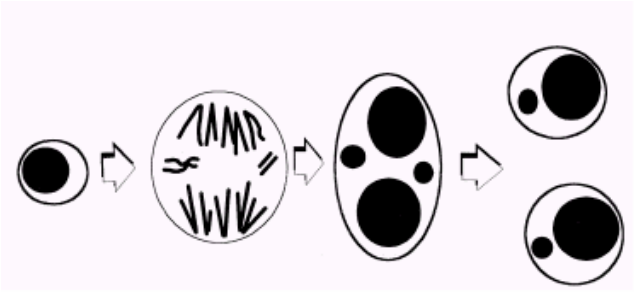

**Fig. 1 Micronucleus expression in a dividing cell (1)**

It may arise from a whole lagging chromosome (aneugenic event leading to chromosome loss) or an acentric chromosome fragment detaching from a chromosome after breakage (clastogenic event) which do not integrate in the daughter nuclei. Scoring of the micronuclei can be performed relatively easily and on different cell types relevant for human biomonitoring: lymphocytes, fibroblasts and exfoliated epithelial cells, without an extra in vitro cultivation step. MN observed in exfoliated cells are not induced when the cells are at the epithelial surface, but when they are in the basal layer.

**b. Laboratory standard procedure** (1)

1. exfoliated cells are collected - oral or nasal mucosa can be swabbed with a spatula or a cytobrush, urothelial cells isolated from urine sample by centrifugation;
2. slides are prepared by smearing or dropping washed cells onto a slide or using a centrifuge;
3. the cells are fixed and stained with Giemsa or DNA-specific dyes (Feulgen, Hoechst 33258, propidium iodide);
4. the minimum number of cells to be scored – between 1500 and 2000;
5. the frequency of MN is reported as cells with MN/ 1000 scored cells; other nuclear atypias as binucleated cells, broken-egg, karyolysis, karyorrhexis are to be mentioned in the reports, apart from MN frequency (scoring not being absolutely necessary).

**c. Criteria for MN analysis in exfoliated cells (1, 12)**

MN (fig. 2) must:

- be less than 1/3 diameter of the main nucleus;
- be on the same plane of focus with the main nucleus;
- have the same color, texture and refraction as the main nucleus;
- have smooth oval or round shape;
- be clearly separated from the main nucleus;
- Questionable MN will be disregarded.

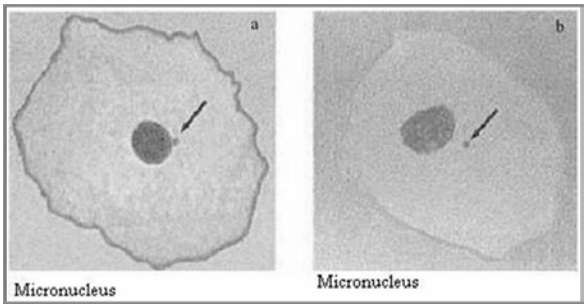


**Fig. 2. Micronuclei in epithelial cells, Feulgen + Fast green staining, 1000x (13)**

**d. Normal values for MN frequency in epithelial cells**

The average reported health population MN frequency is 1 – 3 per 1000 cells, with no significant variation between different types of exfoliated cells. Repeated scoring of MN in epithelia from the same individuals showed variation between 30 and 103%. This may be considered a measure of intraindividual variability, which reflects random variation in the observation of relatively rare events in a limited number of trials (1). Unlike what has been reported in lymphocytes, there are no consistent sex or age effects on the frequency of MN in exfoliated cells. Although the spontaneous frequencies of MN are similar in all types of exfoliated cells, these levels can increase significantly at different sites in response to specific exposures: in buccal cells at betel and tobacco chewers, buccal and nasal cells of people exposed to formaldehyde. Increases in the MN frequencies in exfoliated cells were also observed as a result of exposure to radiotheraphy, arsenic in drinking water, smoking, pesticides or anti-neoplastic drugs (14, 15, 16, 17, 18).

**Why MN in exfoliated cells?**

• Epithelial tissues are in immediate contact with inhaled and ingested genotoxic agents, and kidney and bladder cells are also in contact with metabolites of the chemicals;

• Genotoxic changes in bronchial, esophageal, cervical, breast duct, and other types of epithelia have been reported;

• More than 90% of cancers arise in epithelial tissues; in many cases, these tissues are the actual targets of carcinogens, as indicated by the sites of cancers related to the exposures;

• Epithelial cells can be easily collected from the mouth, nose, and bladder by noninvasive procedures;

• The standard laboratory procedure is feasible, cheap and accurate; final results can be obtained in 2 hours.

**Why MN as biomarker in genotoxic exposures?**

A series of reliable findings come to support the association of the frequency of MN in target or surrogate tissues and cancer development:

a) a significant increase of the MN frequency in target tissues as well as in peripheral lymphocytes in cancer patients (3, 4);

b) subjects affected by certain congenital diseases (Bloom syndrome, ataxia telegiectasia) have both abnormally high MN frequencies and an increased risk of cancer (5, 6);

c) certain clinical chemoprevention trials on oral premalignancies have used MN in oral mucosa as surrogate endpoint of cancer (7, 8);

d) there is a strong correlation between carcinogenicity and genotoxicity for some agents able to increase MN frequencies in humans and animals – ionizing radiation, ethylene oxide, benzene, tobaco smoke (9);

e) MN frequency is associated with the blood concentration of vitamines and folates, which are positively correlated with increased risks for some cancers (10, 11).

**Our experience**

Cytological examination of exfoliated cells has been for a long time a

currently used investigation method for the Laboratory of Cytology from the Occupational Health Department – Institute of Public Health Iasi, through the cytological sputum analysis. Our own experience, as well as the current international knowledge on this issue, proved, without any doubt, that sputum cytology is a very efficient tool in early diagnose of respiratory disorders for chronically exposed subjects to occupational hazards as asbestos, artificial mineral fibres, oil derivates, organic dusts, cumulative occupational exposures to ionizing radiations, air-born microflora, solvents, heavy metals, acids, formaldehyde. The cytological alterations found in the exposed groups consisted not only in evidences of ferruginous bodies and inflammatory processes, but also in nuclear atypias, mainly micronuclei in respiratory cells, plus other abnormalities as karyolysis, karyorrhexis, broken-egg type or binucleated cells. This was the reason for us to start, in 2004, a documentation activity that successfully transformed a hypothesis into certitude: micronuclei in epithelial exfoliated cells represent a trustful diagnose element, and have a great potential in being considered and used as biomarker in chronic exposures to genotoxic hazards (19). As part of a broader project, namely "Sputum cytology – indicator for the assessment of occupationally induced alterations", the implementation of the MN assay in exfoliated respiratory cells became one of the main professional targets for the entire team of the Cytology Laboratory.

At the present moment (beginning of 2007), as (from our knowledge) the only Romanian laboratory that currently uses this technique, we are entitled to present the following achievements on this issue:

♦ Accumulation of a broad and up-to-date database concerning the experience on this topic, on international level;

♦ Implementation of a standard procedure in collecting, processing, evaluating and diagnosing the samples, according to the international standards:

- the exfoliated cells are collected from the inner cheek by gentle scrapping;
- the smears are air-dried, fixed and stained by the May-Grumwald & Giemsa method;
- the number of micronucleated cells is reported (‰) for 2000 counted epithelial cells;
- any other nuclear atypias are observed and registered;
- statistical processing of the data uses modern reliable methods.

♦ Current application of the MN assay on epithelial exfoliated cells on certain groups occupationally exposed to respiratory hazards, which lead to a database of case-studies, images, group-studies, some of them already presented in scientific papers, on national and international level: combined air pollutants in the workplace environment, ionizing radiation, asbestos (20, 21, 22).

**Figures 3 – 8** present some images from our studies, in subjects exposed to respiratory hazards; the images present both MN and other types of nuclear atypias in oral exfoliated epithelial cells:

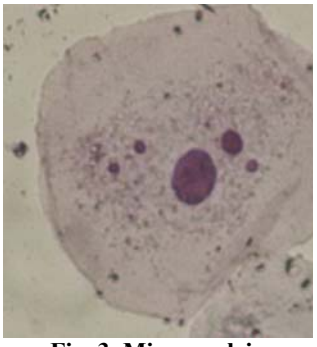

**Fig. 3. Micronuclei (MGG, x400)**

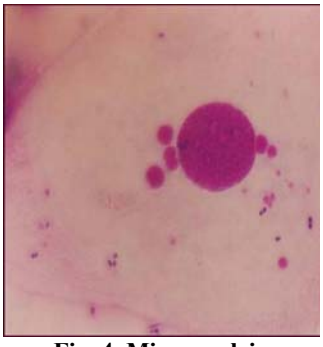

**Fig. 4. Micronuclei (MGG, x1000)**

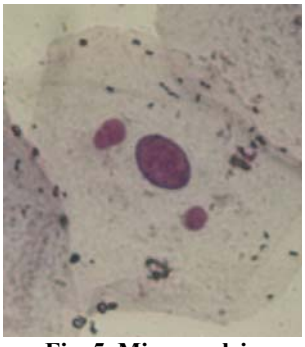

**Fig. 5. Micronuclei (MGG, x400)**

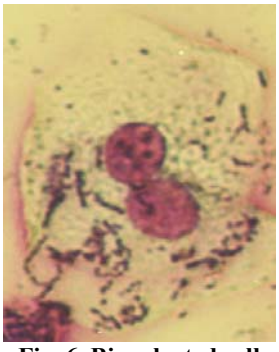

**Fig. 6. Binucleated cell (MGG, x400)**

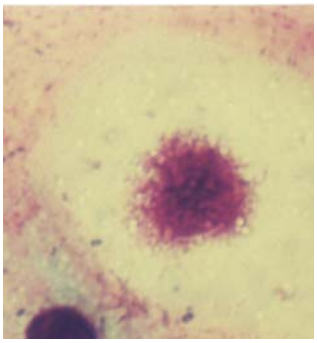

**Fig. 7. Karyolysis (MGGx1000)**

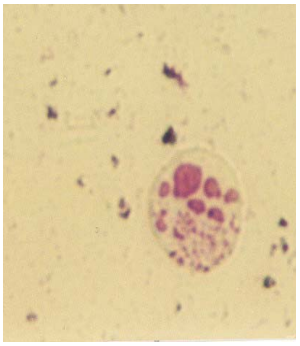

**Fig. 8. Karyorrhexis (MGG x200)**

**Figures 3-8. Different types of MN atypias in oral exfoliated epithelial cells**

CONCLUSIONS

At the present stage of this project, we can appreciate that is that the MN assay in exfoliated cells holds promise as a site-specific biomarker of exposure to genetic toxins, and for cancer risk. With an understanding of the variables that influence baseline MN frequencies it will be possible to better evaluate the contributions to other factors that are usually the focus of environmental and genetic studies, such as exposure to specific chemicals and radiation, the relationship of MN to disease, and the effects of chromosomal instability syndromes.

In this context we intend to continue the study, following as much as possible the objectives and the methodology established by the international project HUMN:

1. Collect and summarize data on exfoliated cells from different specific exposed groups;
2. Standardize protocols;
3. Analyze possible sources of variability in the results;
4. Establish correlations between MN frequency in exfoliated cells and cancer risk for both site-specific cancers and general cancer incidence;
5. Conduct a parallel study of the MN frequency in exfoliated cells and lymphocytes in the same subjects in order to obtain additional information on the predictive value of MN as a biomarker.

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