

REVIEW

Micronuclei in neonates and children: effects of environmental, genetic, demographic and disease variables

Nina Holland*, **Alexandra Fucic¹**, **Domenico Franco Merlo²**, **Radim Sram³** and **Micheline Kirsch-Volders⁴**

School of Public Health, 733 University Hall, University of California, Berkeley, CA 94720-7360, USA, ¹Institute for Medical Research and Occupational Health, Zagreb, Croatia, ²Unit of Epidemiology, Biostatistics and Clinical Trials, Department of Cancer Epidemiology and Prevention, National Cancer Research Institute, Largo Roanna Benzi 10, 16132 Genoa, Italy, ³Institute of Experimental Medicine, Academy of Science of Czech Republic, Videnska 1083, 142 20 Prague 4, Czech Republic and ⁴Laboratory for Cell Genetics, Vrije Universiteit Brussel, Brussels, Belgium

*To whom correspondence should be addressed. School of Public Health, 733 University Hall, University of California, Berkeley, CA 94720-7360, USA. Tel: 510 665 2200; Fax: 510 665 2202; Email: ninah@berkeley.edu

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Children may be more susceptible to the effects of the environmental exposure and medical treatments than adults; however, limited information is available about the differences in genotoxic effects in children by age, sex and health status. Micronucleus (MN) assay is a well established method of monitoring genotoxicity, and this approach is thoroughly validated for adult lymphocytes by the Human Micronucleus Biomonitoring project (HUMN.org). Similar international undertaking is in progress for exfoliated buccal cells. Most of the MN studies in children are focused on analyses of lymphocytes but in the recent years, more investigators are interested in using exfoliated cells from the oral cavity and other cell types that can be collected non-invasively, which is particularly important in paediatric cohorts. The baseline MN frequency is relatively low in newborns and its assessment requires large cohorts and cell sample counts. Available results are mostly consistent in conclusion that environmental pollutants and radiation exposures lead to the increase in the MN frequency in children. Effects of medical treatments are less clear, and more studies are needed to optimise the doses and minimise genotoxicity without compromising therapy outcomes. Despite the recent progress in MN assay in children, more studies are warranted to establish the relationship between MN in lymphocytes and exfoliated cells, to clarify sex, age and genotype differences in baseline MN levels and the changes in response to genotoxicants. One of the most important types of MN studies in children are prospective cohorts that will help to clarify the predictive value of MN and other cytome end points for cancer and other chronic diseases of childhood and adulthood. Emerging ‘omic’ and other novel molecular technologies may shed light on the molecular mechanisms and biological pathways associated with the MN levels in children.

Introduction

The present article gives an overview of the micronucleus (MN) assay in lymphocytes and exfoliated epithelial cells of children from birth to adolescence. Special focus on children is important because they may have a higher sensitivity to genotoxic agents compared to adults and because genetic damage occurring at young ages may affect the lifetime risk of adverse health outcomes (1–3). The number of studies incorporating biomarkers of exposure and effect in children has significantly grown in recent years. Some aspects of genotoxicity assessment and specifically MN measurements in children have been addressed previously (4–6). The MN assay is increasingly used as the method of choice for evaluation of genotoxicity in children because of its affordability and efficiency. Various factors of importance to MN variability in children include tissue specificity, age, sex, health status and environmental and dietary factors.

The first section is a reflexion on the biological significance of MN in newborns and children. The following sections contain a brief description of the effect of age and sex in different cell types, the use of the MN assay for monitoring of environmental exposures and characterisation of MN levels in paediatric patients with various diseases and/or who are undergoing treatment. We discuss the relationship of MN as biomarkers of genotoxicity with other markers such as genetic polymorphisms and gene expression. In the last two sections, an attempt was made to (i) summarise the current state of knowledge and significant achievements in genotoxicity research in children and (ii) identify important knowledge gaps and provide recommendations for future research.

Biological significance of MN in newborns and children

MN frequencies in human T lymphocytes represent accumulated genetic changes resulting from spontaneous or induced chromosome breakage/loss. Circulating T lymphocytes are thought to accumulate MN over several months or even years, after which cells with such abnormalities disappear from the cell pool, except if stable mutations are present in stem cells. Despite this limitation, MN frequencies in T lymphocytes from adults have been shown to be predictive for cancer (7) and are considered valuable biomarkers for early genetic effects. In newborns, MN in lymphocytes of umbilical blood reflect an average foetal genome instability plus *in utero* exposure to clastogens/aneugens. Indeed, four major facts need to be considered: (i) T lymphocytes to be analysed by the MN assay are circulating in peripheral blood for only ~6 months (8); (ii) the response of T lymphocytes to phytohaemagglutinin stimulation in umbilical cord blood is less efficient than in peripheral blood from adults (9); (iii) genome stability during foetal life might be different than later in ontogenesis and (iv) the baseline MN frequency is relatively low in newborns and its assessment requires large cohorts and cell sample counts.

At the present time, it is difficult to define the real biological significance of MN frequencies in newborns and children. In the next sections, these parameters that have been shown to influence the MN frequency in newborns and children will be discussed. Their predictive value for disease, and in particular childhood leukaemia, requires prospective studies combining data from different cohorts on exposure (food, lifestyle and environmental factors) during pregnancy, MN frequencies and other biomarkers of genetic/epigenetic effects in mother–child pairs and disease follow-up during childhood and adulthood. Some of the ongoing projects aiming to contribute to this issue are the EU-NewGeneris research project (www.newgeneris.org) in Europe, National Children's study (www.nationalchildrensstudy.gov) and ongoing birth cohorts such as CHAMACOS (chamacos.org) in the USA and other parts of the world.

Effects of age and sex on MN levels in lymphocytes and exfoliated cells

The effect of age and sex on MN levels in adult lymphocytes is well established, with women having ~30% higher levels of MN than men and with MN levels progressively increasing with age from early adulthood through the rest of life (10). The situation with the MN values in exfoliated epithelial cells is less clear in regard to age and sex (11). A meta-analysis of MN frequency in children (age range 0–18 years) and a pooled analysis of individual data available from both published studies as well as from the Human Micronucleus International Collaborative Study (HUMN) database (12) allowed the estimation of the effect of age and gender on MN level in peripheral lymphocytes (4). No influence of gender was found with very similar mean level of MN measured in lymphocytes of boys (5.9 MN/1000 cells, ‰; 95% CI = 4.4–8.0 ‰) and girls (5.5‰, 95% CI = 4.1–7.4) (4). MN frequency was low at birth (3.3‰ binucleated cells) and increased by 66% in children 1–4 years of age (5.4‰) by up to 116% in those aged 15–18 years. A 6.5% increase in MN frequency per year of age was estimated in Czech children aged 5–13 years (13). In buccal epithelial cells of 266 Ukrainian schoolchildren aged 14–18 years, the MN levels did not differ significantly between boys (2.5‰) and girls (2.6‰) (14). The mean MN levels in buccal cells reported by two other studies (15,16) in 0- to 6-year-old children differed by >2-fold (1.2 and 3.8‰, respectively), suggesting a major impact of environmental factors and technical variability on buccal cell MN frequency in human studies. In another study that involved lymphocytes and buccal cells of children (4–15 years old) and their mothers, ~30% higher MN frequencies were found in both cell types for adults in comparison to children (17). However, no statistically significant difference by age was observed due to a broad range of inter-individual variability.

In a study of newborns and their parents from Madrid, the mean number of MN in binucleated cells was lower in babies (3.9‰) than in their mothers (6.5‰) and fathers (6.1‰) (18). Further, a statistically significant positive correlation was found between newborns and their parents. In four mother–child groups from different parts of Mexico, researchers also found a correlation between mothers and newborn lymphocyte MN frequency; however, the absolute levels in this study were noticeably lower than in other studies (3.7‰ for mothers and 1.0‰ for newborns) (19). Interestingly, in a study with children of various ages, the strongest effect of air pollution on

MN frequency was observed in the youngest individuals (20). This observation is consistent with the potentially greater sensitivity of children to environmental exposures (3).

MN and environmental exposures

The MN assay has been widely used to study genome damage in children after transplacental (*in utero*) and post-natal exposures in a variety of rural or urban environmental settings as well as from accidental industrial/technological overexposures. Potentially genotoxic factors include unknown mixtures of environmental tobacco smoke, airborne nanoparticles, food contaminants (pesticides and chemicals generated by cooking), oil and coal combustion emissions and non-ionising and/or ionising radiation (indoor radon and nuclear plants).

The majority of such exposure studies revealed higher MN frequencies in children exposed to environmental pollutants compared to referents (6). For example, air pollution in different parts of the world was associated with a 30–130% increase in the mean MN level in exposed children in comparison to referent groups (16,21,22) and a 30% increase in those exposed to indoor environmental tobacco smoke (23). An increased level of MN in lymphocytes and buccal cells of children was associated with regional ozone levels in California (17). Children aged 3–7 years who were exposed to airborne chemical industry mixtures of formaldehyde, dust, solvents, nitrates and ozone had significantly higher MN frequency in buccal cells than control children (range 3–13‰ versus 0–3‰ MN, respectively) (24,25). A significantly higher MN frequency in binucleated lymphocytes was detected in two groups of Czech children aged 5–9 and 10–13 years living in a highly polluted region as compared with those living in a rural area of low environmental pollution (7.0 versus 4.9‰ and 9.2 versus 6.6‰, respectively) (13). Of particular note, here are the higher MN levels in older children that may be reflective of age-related changes or of longer periods of exposure.

A significant increase in MN (240%) was also observed in children living near a chemical disposal site (26); a 730% increase in MN in children exposed to heavy metals and industrial pollutants (15) and a 630% increase in those exposed to arsenic in drinking water (27). Significantly elevated MN frequencies were detected in lymphocytes from 9-year-old children living in Bukowno, a town in southern Poland, who had higher blood lead levels in comparison to a control group (2.96 and 1.16‰, respectively) (28). Additionally, with the use of the fluorescence *in situ* hybridization (FISH) assay, this study has shown that lead caused genome damage primarily by an aneugen mechanism.

Natural sources of ionising radiation were also associated with significantly elevated MN frequencies in schoolchildren exposed to high indoor radon levels (29). The largest body of evidence on the effects of ionising radiation in children is available from studies conducted during and after the Chernobyl, Techa river and Guiana nuclear accidents (30,31). Children chronically exposed to elevated levels of radiation after the Chernobyl nuclear power plant accident in 1986 and children of clean-up workers ('liquidators') showed significantly increased MN levels in comparison to reference values (32–34). It was shown that children from Gomel (Belarus), one of the areas most severely affected by radioactive contamination following the accident, had higher MN frequencies than children from Pisa, Italy. Further, there

was a significant correlation with the level of ^{137}Cs , and this factor appeared to have a more pronounced effect on the MN frequency than the presence of thyroid tumours in Gomel children (34). A more comprehensive application of the MN assay for biomonitoring of genetic damage compared children's MN in contaminated regions of Belarus and the non-contaminated city region of Minsk within the same country (35). Overall, the evidence of the effects of early life environmental exposures on genetic damage in children is strong, and all efforts have to be made to eliminate such exposures to pregnant mothers and children to protect their health.

Effects of disease and treatment on MN in children

A number of studies have monitored the level of genetic damage of children with cancer and other diseases who have undergone different forms of treatment, including radio- and chemotherapy. No evidence of chromosome damage by MN assay was reported in lymphocytes of children and adolescents with differential thyroid carcinoma at 48 h after receiving ^{131}I radio metabolic therapy (36). This study also evaluated changes in gene expression and found that the majority of patients (9 of 11) had altered expression levels of DNA repair and apoptosis genes. Two examples of studies that attempted to monitor genotoxicity of local radiation therapeutic or diagnostic exposures showed no significant long-term changes in MN in blood or exfoliated cells as a result of dental X-rays (37) or radiosynovectomy (38).

In contrast, children in the vicinity of Chernobyl, who received ^{131}I application for thyroid cancer, had marked increases in MN frequency 5 days after the treatment followed by a subsequent decrease across a 4- to 7-month interval. However, pretreatment baseline levels were not restored (39). In an earlier study of children affected by Chernobyl accident, the researchers observed a broad range of MN levels in different parts of the former Soviet Union, in some cases sharply exceeding typical baseline levels of MN in children (40). The same authors followed genotoxic effects associated with different forms of radiotherapy and chemotherapy for thyroid cancer. Most of them were treated with ^{131}I and had comparatively lower MN levels post-treatment (10‰) in lymphocytes in comparison to ^{60}Co (26‰) radiotherapy or combined radio- and chemotherapy (26–34‰).

Chemotherapy can result in a significant increase in MN frequencies in lymphocytes and exfoliated buccal cells of paediatric patients with acute lymphocytic leukaemia in comparison to the levels before treatment and those in healthy controls (41,42). Two examples of long-term therapies with potential genotoxicity include treatment of sickle-cell anaemia by hydroxyurea and treatment of attention deficit/hyperactivity disorder with Ritalin (methylphenidine). Follow-up of children treated by hydroxyurea using flow cytometry of reticulocytes (MN-CD71+) and mature erythrocytes demonstrated a significant increase in MN by 3 months of therapy compared to baseline (43). Further, this elevated MN frequency remained consistent over next 12 years of continuous hydroxyurea exposure. An initial report of the statistically significant increase in cytogenetic damage in children treated with Ritalin as measured by chromosome aberrations (3-fold), sister chromatid exchanges (4.3-fold) and MN assay in lymphocytes (2.4-fold) (44) was not confirmed by later studies in lymphocytes and buccal cells (45), including prospective follow-up for 12 months (46).

Beyond treatment, another important angle of MN studies in children is to characterise the differences in cytogenetic damage associated with disease status itself. For example, it was demonstrated that the buccal cytome and MN frequency are substantially altered in Down's syndrome (47), which corroborates other reports about genomic instability in blood and oral mucosa of children with this condition (48–50). Other diseases associated with elevated MN include ataxia-telangiectasia, anaemia Fanconi (51) and some forms of inflammatory bowel disease (52).

In summary, the evidence of genotoxicity of some diseases as well as radio- and chemotherapy in children points out to the necessity to minimise the doses that would assure clinical effect without excessive risk to children's DNA and chromosomes. Furthermore, it is essential to monitor the genotoxicity of new forms of treatment as well as the effect of combined therapies that may have a synergistic effect.

MN and other biomarkers in children: genotypes, gene expression and DNA adducts

Data on the relationship between the frequency of MN and genetic polymorphisms in children are very limited. Decordier *et al.* (53) studied the impact of oxidative stress induced by H_2O_2 , on the MN frequencies in the group of newborn girls. Additionally, they also evaluated possible impact of DNA repair (*hOGG1*, *XRCC1*, *XRCC3* and *XPD*) and folate metabolism (*MTHFR*) polymorphisms on the level of chromosomal damage. Higher background frequencies of MN were observed in children carrying *XRCC1*¹⁹⁴ variant genotype (5.0 versus 1.3‰, $P < 0.05$). Newborns carrying the variant *XRCC3*²⁴¹ genotype might be at higher risk for the induction of MN by oxidative stress. However, an interpretation of these findings is limited by a small number of study subjects ($n = 17$), especially in regard to the effects of genotypes.

Recently, Rossnerova *et al.* from Czech Republic (A. Rossnerova, M. Spatova, I. Solansky, R. Sram, unpublished data) studied the MN frequency in the peripheral lymphocytes of children diagnosed with asthma in Ostrava City, Silesia. This region has a high level of air pollution assessed by concentrations of small particulates, PM_{2.5}, carcinogenic polycyclic aromatic hydrocarbons and benzo[*a*]pyrene (B[*a*]P), likely to be some of the highest found in Europe. As an example, the level of B[*a*]P measured in November 2008 by the stationary monitoring of the Health Institute in Ostrava-Bartovice was $11.4 \pm 9.8 \text{ ng/m}^3$. MN levels and the modifying impact of genetic polymorphisms of *GSTM1*, *GSTT1* and *EPHX1* were analysed in asthmatic children 6–15 years of age ($n = 81$), matched with controls ($n = 94$). The MN frequency in binucleated lymphocytes in asthmatic children did not differ from controls (3.3 versus 3.2‰). However, *GSTM1*-positive subjects had on average higher MN (3.5‰ versus *GSTM1* null 2.9‰, $P < 0.05$). The difference was also significant for *GSTT1* (3.4‰ for positive versus null 2.8‰, $P \leq 0.05$). In more detailed statistical analyses, the plasma levels of vitamins A, C and E, cotinine level in urine and parental smoking were also taken into account. Multivariate linear regression indicated that effect of *GSTM1* gene (-0.45 ± 0.21 , $P = 0.03$) was significant for the overall cohort. Further, it was also significant in non-smoking families (-0.69 ± 0.31 , $P = 0.03$), while in smoking families, effects of two other genes significantly affected MN frequency (*GSTT1*, -0.63 ± 0.30 , $P = 0.04$) and

(*EPHX1*, 1.01 ± 0.46 , $P = 0.03$). Results from these studies support a role of *GSTM1* and possibly *GSTT1* and *EPHX1* genes in affecting genetic damage in children by different detoxification processes that may be especially important in the context of polluted environments. However, much larger studies similar to the combined cohort analysed by the HUMN in adults to evaluate effects of genetic polymorphisms on the MN levels (54) will have to be conducted in children to address this issue more reliably.

In the last 2–3 years, several studies attempted to address genetic damage in children by new methodologies such as analyses of etheno adducts in mother–newborn pairs (55) and microarrays (36). Of a particular interest was a comparison of MN frequencies and differentially expressed genes in the lymphocytes of children and adults from two areas with distinctly different levels of air pollution (56). This study demonstrated that very little overlap at the transcriptome level exists between children and adults, and the two most important functions modulated in children, but not in adults, are nucleosome and immune response related. Expression of multiple genes was significantly correlated with the MN frequencies in children (678 positively and 572 negatively); however, these data are difficult to interpret in terms of molecular mechanisms of chromosome instability so far, and this issue will require additional studies.

Summary of main achievements in MN studies in children

MN assay in children is increasingly used because of its public health significance for monitoring environmental exposures such as pollution, radiation, industrial toxicants and various medical treatments. It has been also used to assess genotoxicity associated with diseases such as leukaemia, Down's syndrome, genetic conditions with DNA repair deficiencies, as well as chronic conditions such as asthma and inflammatory bowel disease. Some of the MN studies in children have taken into account effects of diet and vitamin supplementation. MN frequency in children in available studies was measured in lymphocytes, buccal epithelium and rarely in reticulocytes. Most of the studies were focused on analyses of lymphocytes but in the recent years, more investigators are interested in using exfoliated cells from the oral cavity and other cell types that can be collected non-invasively, which is particularly important in conducting paediatric studies (57).

It has been established that baseline MN levels in newborn children are relatively low in comparison to adults; however, the effects of age and sex on the MN levels in lymphocytes and exfoliated cells during childhood and adolescence are not clear and warrant further investigations. Developmental changes in response to environmental exposures, such as ionising radiation, chemical genotoxicants and various medical treatments that are hazardous to the genome, are currently not well understood.

In addition to the measurement of MN, some of the studies applied cytome approach that allows more detailed assessment of genotoxicity and cell toxicity (47, 57). Some of the studies used FISH analyses of MN (13), flow cytometry (43) and automatic scoring of MN in different cell types (58,59) (see other papers in this issue for more details). New 'omic' and other molecular methodologies have been recently used in conjunction with MN studies (13) and may help to shed light on the molecular mechanisms and biological pathways associated with the MN levels in children.

Knowledge gaps and future research

Available data on the MN results are particularly hard to interpret in paediatric populations due to complex interactions between environment and genotype within the matrix of growth dynamic, development and adaptation. All these processes may have significant impacts on the level of genome damage measurable by the MN assay.

The impact of demographic parameters such as age, gender, lifestyle and diet-related factors (alcohol consumption, obesity, meat consumption and smoking) is often not evaluated when the primary focus of the authors is on a certain environmental factor or disease. Information about the health status and infectious diseases are also important for interpretation of MN assay results.

The dynamics of cell division in children may be different than in adults. In the case of epithelial cells from the oral cavity, it is known that in adults it takes 2–3 weeks for damaged cells in the basal layer to migrate to the surface. However, cell proliferation may vary in children by age and in different cell types. This information is important to the timing of the cell collection to assess exposure of genotoxicity events.

At the present time, it is difficult to define the real biological significance of MN frequencies in newborns and children. Further standardisation of the protocols and improvement of study design can facilitate creation of a worldwide database of MN data for children that should help establish the predictive value of MN not only for cancer but possibly for other health conditions of childhood and adulthood. It would require large prospective studies combining exposure data (beginning from pregnancy and during childhood) with comprehensive assessment of lifestyle factors and health status. Use of new 'omics' and other novel molecular technologies presents a very promising line of future mechanistic research of MN in children.

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