**REVIEW**

The effect of age, gender, diet and lifestyle on DNA damage measured using micronucleus frequency in human peripheral blood lymphocytes

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Micronucleus (MN) frequency in cytokinesis-blocked peripheral blood lymphocytes (PBL) has become one of the best-established biomarkers for studying DNA damage occurring in vivo in humans. The application of this method in population biomonitoring studies requires a deep understanding of how lifestyle and common host variables may influence MN frequency in PBL. In this mini-review, an update is provided on results from studies reporting on the impact of age, gender, diet and lifestyle factors (e.g., exercise, alcohol, smoking and recreational drugs) on this biomarker. Evidence from these studies shows that each of these factors, either in isolation or in combination, can significantly influence MN frequency. Proper control for these factors is required to enable better measurement of the impact of other conditions, such as environmental exposure to genotoxins or a susceptible genetic background, on MN frequency in PBL.

Background

Measurement of micronuclei (MN) in human peripheral blood lymphocytes (PBL) is one of the best-established cytogenetic methods for measuring chromosomal DNA damage in humans (1,2). MN are expressed in cells when either acentric chromosome fragments of whole chromosomes fail to be segregated to the daughter nuclei during mitosis. The most reliable method for measuring MN in PBL is the cytokinesis-block micronucleus cytoyte (CBMNcyt) assay. In the CBMNcyt assay, PBL are induced to divide ex vivo in culture and MN are scored specifically in cells that have completed one nuclear division; the latter are recognised as binucleated cells after inhibition of cytokinesis using cytochalasin-B. This review is focused solely on data obtained using the CBMNcyt assay in PBL. This is the best validated and most commonly used method for scoring MN in human cells both for in vitro and in vivo genetic toxicity studies as well as for investigating effects of ageing, gender, environmental exposure to genotoxins, nutritional imbalances and lifestyle factors (3,4). Furthermore, MN in PBL have been associated prospectively with increased risk of cancer and cardiovascular disease (5–7). Because of the widespread use of the CBMNcyt assay, it is particularly important to have a good understanding of those variables that may influence observed baseline MN frequencies in humans so that data in genotoxic exposure studies can be properly interpreted. Furthermore, some of this information may be useful to implement appropriate diet and lifestyle strategies for DNA damage prevention either on a personalised or population basis. The following segments of this paper describe our current knowledge on the effects of age, gender, diet and lifestyle factors on MN frequency in human PBL. Other papers in this special issue provide complementary information on the effects of genotoxin exposure, disease and genetic factors (8–11).

Age and gender

The effects of ageing and gender on MN in PBL was first recognised in studies done by Fenech et al. in the early 1990s (12–14). A typical example of such data is shown in Figure 1. Since then, several other laboratories around the world have consistently shown that MN in PBL increase steadily with age in both males and females starting from very young age groups onwards. Evidence was produced also showing that MN frequency tended to be greater in females relative to males. A meta-analysis of data from around the world was coordinated by the HUMN project (www.humn.org) and confirmed the statistical significance and consistency of these observations (15). The increase of MN with age is likely due to a combination of factors which include (i) the cumulative effect of acquired mutations in genes involved in DNA repair, chromosome segregation and cell cycle checkpoint and (ii) numerical and structural aberrations in chromosomes caused by exposure to endogenous genotoxins, inadequate nutrition, exposure to environmental or occupational genotoxins, as well as a wide range of unhealthy lifestyle factors (explained below). The increase in MN frequency in females can be accounted for by the greater tendency of the X chromosome to be lost as an MN relative to other chromosomes, and to the fact that females have two copies of the chromosome compared to only one in males (16,17).

Nutrition

The association of MN with nutrition was first noted in studies in anaemia caused by folate and/or vitamin B12 deficiency in which the formation of MN was observed in erythrocytes (18). The choice and the amount of foods and supplements intake have a strong influence on the cellular concentration of micronutrients required as cofactors in DNA synthesis and repair. Enzymes and proteins involved in these processes and in chromosome segregation require micronutrients either as a substrate (e.g. 5,10-methylene tetrahydrofolate as methyl
donor for synthesis of deoxythymidine-triphosphate by thymidylate synthase) or as a cofactor (e.g., Zn and Mg as cofactors for DNA polymerases) or as an integral part of the enzyme (e.g., hOGG1 required for repair of oxidised guanine which is a zinc finger protein). Nutrition is a complex aspect of environmental exposure that varies between individuals and communities depending on food choices, affordability or availability. Therefore, the impact of dietary patterns and multi-nutrient supplements will depend on the net effect of an individual’s or a community’s nutriome (i.e., the combination of nutrients) and its impact on the various DNA metabolism pathways (4). The occurrence of MN in the CBMNcyt assay is particularly sensitive to nutritional deficiency or excess, to the extent that even small differences in concentration of folic acid within the physiological range in vitro are observed to induce the same level of MN as an exposure to X-rays of 20 cGy [which is ~20 times the annual allowed safe exposure limit of ionising radiation (4)].

In vivo studies in humans have shown that MN frequency in PBL is significantly associated with either dietary intake levels or plasma concentration of folate, vitamin B12, riboflavin, biotin, pantothenate, beta-carotene, vitamin E, retinol, and calcium (19–24). Results from a recent population study suggest that at least nine micronutrients affect genome stability in humans in vivo [Figure 2; (25)]. This latter study, performed in 190 healthy individuals (mean age 47.8 years, 46% males), was designed to investigate the association between dietary intake (estimated using a food frequency questionnaire) and genome damage in lymphocytes measured by the CBMNcyt assay. Multivariate analysis of baseline data showed that (i) the highest tertile of intake of vitamin E, retinol, folate, nicotinic acid (preformed) and calcium is associated with significant reductions in MN frequency, i.e. 28, 23, 33, 46 and 45%, respectively, (all \( P < 0.005 \)) relative to lowest tertile of intake and (ii) the highest tertile of intake of riboflavin, pantothenic acid and biotin was associated with significant increases in MN frequency, i.e. +36% (\( P = 0.054 \)), +51% (\( P = 0.021 \)) and +65% (\( P = 0.001 \)), respectively, relative to lowest tertile of intake. Mid-tertile \( \beta \)-carotene intake was associated with an 18% reduction in MN frequency (\( P = 0.038 \)), while the highest tertile of intake (>6400 µg/day) resulted in an 18% increment in MN frequency. In interpreting the results of this study, it is important to note that micronutrients usually exhibit metabolic dose–response curves in which both deficiency and excess can be deleterious (26–31). It is likely that in a specific mixed diet, depending on the intake level of an individual, some of the micronutrients may be outside the intake range that is optimal for prevention of genome instability. The results for \( \beta \)-carotene suggest an optimum for genome stability between 4000 and 6000 µg/day, with a tendency for marked increase in genome damage at higher or lower intake levels. This evidence from supplementation studies is in keeping with epidemiologic data suggesting increased cancer risk with deficiency or supplementation above recommended dietary intake levels for this vitamin (26, 28, 32). On the other hand, the apparent genome protection associated with vitamin E, retinol, folic acid, preformed nicotinic acid and calcium was still increasing at the highest tertile of intake, raising the question if the maximum beneficial effect is achieved at these levels of intake or if even higher levels of intake may be recommended. For example, the highest tertile of folate intake was >256 µg/day, which is consistent with a number of studies showing that the occurrence of developmental defects and cancer, as well as the level of risk biomarkers for cardiovascular disease, such as homocysteine, are minimised with folate intake levels of 400 µg/day or greater (33–37).

There are as yet no studies reporting on the association of MN frequency in PBL with the intake of macronutrients or food groups. There are, however, some reports on naturally occurring contaminants. For example, one study showed that MN frequency in PBL was significantly associated with blood mercury in fisherman who were consuming fish from a geological region of Italy that is naturally high in mercury (38); a similar association between blood mercury and MN in PBL was reported in a cohort of men in Madrid and it was hypothesised, that this may be associated with fish consumption (39). One study examined the impact of isoflavones consumption from soy foods, that are known to cause MN in vitro, possibly due to topoisomerase inhibition, but had no impact on baseline MN in PBL in vivo (40). There are no human in vivo studies that report on MN in PBL and consumption of pyrolysed foods (e.g. charcoal barbecued or panfried flesh foods, baked carbohydrate-rich products and cooking oils) despite the known presence of genotoxins such as acrylamide, polycyclic aromatic hydrocarbons, lipid peroxides and heterocyclic amines in these products that may induce MN (in vitro) (41–43).

Fig. 1. Baseline MN frequencies in PBL of healthy, non-smoking, males and females subdivided according to age-group in a South Australian cohort. MN frequency in PBL was measured using the CBMNcyt (1). Results represent the mean ± 1 SE; \( N = 14–33 \) within each subgroup.

Fig. 2. Percentage variation in MN frequency in peripheral blood lymphocytes for mid- and highest tertile of intake of vitamin E, calcium, folate, retinol, nicotinic acid, beta-carotene, riboflavin, pantothenic acid and biotin relative to the lowest tertile of intake. MN frequency was measured using the CBMNcyt. For more information, refer to Fenech et al. (25).
Several studies have reported on the beneficial or null effects of single or multi-vitamin supplementation on MN frequency in PBL; the outcomes of these studies are detailed in a separate paper in this special issue (44).

Three studies have compared vegetarians and non-vegetarians with respect to MN in PBL as well as plasma micronutrients such as folate and the vitamins B12, C and E. (45–47). None of these studies have found significant differences in MN frequency in PBL between these two distinct dietary habits despite significant differences in the blood levels of specific micronutrients (e.g. folate and vitamin C being higher in vegetarians and B12 lower in those on vegan diets who do not supplement with this micronutrient). B12 deficiency in vegan males was shown to be associated with a higher MN frequency value (19,47). The association between vitamin B12 deficiency and increased MN in PBL was also observed in other nutritional studies in humans (48–50).

Despite an apparent positive association between MN in PBL and insulin resistance in obese women (51), neither body mass index nor consumption of low-calorie weight loss diets in obese individuals was associated significantly with MN frequency in PBL (25,52). In the latter study (52), the group on high protein-high red meat diet did not differ with respect to MN frequency from the group on high-carbohydrate weight loss diet after 12 months on these diets that caused a mean weight loss of 9.3 kg.

Physical exercise

Six studies have reported on the effects of physical exercise on MN in PBL (53–58). They essentially range from a reduction in MN in association with moderate exercise or following a triathlon race in fit subjects (53,54), to null effects (55,56) or even an increase in MN in healthy volunteers after acute exhaustive exercise (57,58). Effects of extreme exercise were usually observable within the first 24 h post-exercise but could also take up to 19 days for recovery to pre-exercise values in the case of triathlons.

Smoking of tobacco cigarettes

The effect of tobacco smoking on the frequency of MN in human lymphocytes has been the object of many population studies, which often have been negative or equivocal often because of small sample size and possibly mis-classification. To overcome these limitations, a pooled re-analysis of 24 databases from the HUMN international collaborative project (www.humn.org) was performed with the aim of understanding the impact of smoking habits on MN frequency in PBL (59). The complete database included 5710 subjects, with 3501 non-smokers, 1409 current-smokers and 800 former-smokers, among subjects in occupational and environmental surveys. The overall result of the re-analysis confirmed a small non-significant decrease of MN frequencies in current-smokers [frequency ratio (FR) = 0.97, 95% confidence interval (CI) = 0.93–1.01] and in former-smokers (FR = 0.96, 95% CI = 0.91–1.01), when compared to non-smokers. MN frequency was not influenced by the number of cigarettes smoked per day among subjects occupationally exposed to genotoxic agents, whereas a typical J-shaped curve is observed for non-exposed smokers, showing a significant increase of MN frequency in individuals smoking ≥30 cigarettes/day (FR = 1.59, 95% CI = 1.35–1.88). This analysis confirmed that smokers generally do not experience an overall increase in MN frequency, although when the interaction with occupational exposure is taken into account, heavy smokers were the only group showing a significant increase in genotoxic damage as measured by the MN assay in lymphocytes. From these results, some general recommendations for the design of biomonitoring studies involving smokers were formulated as follows: (i) quantitative data about smoking habit should always be collected because, in the absence of such data, the simple comparison of smokers versus non-smokers could be misleading, (ii) the subgroup of heavy smokers (≥30 cigarettes/day) should be specifically evaluated whenever it is large enough to satisfy statistical requirements and (iii) the presence of an interaction between smoking habit and occupational exposure to genotoxic agents should be always tested.

Alcohol consumption

Excessive consumption of alcohol is an established risk factor for cancer (60). Studies that have specifically and carefully investigated the association of alcoholism with MN formation have found that alcohol consumption is associated with higher MN frequency in PBL (60–66). One study examined the type of MN induced by alcoholism using centromere probes and concluded that the increase in MN frequency in alcoholics relative to controls is mainly due to MN containing whole chromosomes (64). Alcohol is metabolised to acetaldehyde via alcohol dehydrogenase-2 (ADH1B) or cytochrome p4502E1 (CYP2E1), and acetaldehyde is detoxified to acetate by acetaldehyde dehydrogenase-2 (ALDH2) (60–62). Common genetic polymorphisms of ADH1B, CYP2E1 and ALDH2 influence the metabolism of alcohol. The ADH1B*1/*1 genotype encodes the low-activity form of ADH1B and ALDH2*2/*2 and ALDH2*1/*2 genotype encode inactive ALDH2. A large study of >200 healthy Japanese men showed that there was a significant trend for the MN frequency in PBL to increase in habitual or moderate drinkers without a smoking habit as the numbers of the *1 allele in ADH1B increased and the *2 allele in ALDH2 increased. Habitual drinkers who were homozygous CYP2E1*1/*1 and heterozygous ALDH2*1/*2 or homozygous ALDH2*2/*2 genotype showed the highest mean MN frequency in PBL in the same cohort (61) (Figure 3).

Fig. 3. The association between alcohol consumption and ALDH2 polymorphism with MN frequency in PBL in a cohort of Japanese men. For more detail, refer to Ishikawa et al. (61); binucleated cells.
The hypothesis that the non-alcoholic fraction of wine protects against genome damage induced by oxidative stress was studied in a crossover intervention study involving six young adult males aged 21–26 years (67). The participants adhered to a low plant phenolic compound diet for 48 h prior to consuming 300 ml of complete red wine, de-alcoholised red wine or ethanol on separate occasions 1 week apart. Blood samples were collected at 30, 60 and 120 min after beverage consumption. Baseline and radiation-induced genome damage was measured via the MN frequency in PBL using the CBMN cyt assay, and total plasma catechin concentration was measured. Consumption of de-alcoholised red wine significantly decreased the gamma radiation-induced MN frequency in PBL at 60 and 120 min post-consumption by 20%. In contrast, alcohol tended to increase radiation-induced MN frequency and complete wine drinks to 120 min post-consumption by 20%. These preliminary data suggested that only the non-alcoholic fraction of red wine protects DNA from oxidative damage. An earlier study from the same group using a similar design had shown protective \textit{ex vivo} effects of moderate wine consumption against hydrogen peroxide-induced MN in PBL (68). Neither of these studies showed a significant effect of wine consumption on baseline MN frequency in PBL measured on the same day after the 300 ml wine was consumed.

**Use of recreational and illicit drugs**

Another lifestyle variable that potentially could produce genotoxic effects is the use of recreational and illicit drugs such as methamphetamines (METHs), cocaine, marijuana and heroin (69). However, there is as yet only one study reported showing that MN in PBL are increased in METH users (70). This study on 76 long-term METH abusers and 98 unexposed controls demonstrated that total METH exposure was positively correlated with MN and sister chromatid exchanges frequencies in \textit{ex vivo} cultured lymphocytes. The results of this study, which was also supported by positive results from \textit{in vitro} tests in the same study, indicated that METH is a genotoxic agent and that reactive oxygen species may play a role in METH-induced genotoxicity. Surprisingly, this is the only reported study on the effect of recreational or illicit drugs use on MN frequency in humans. Nitrous oxide abuse by inhalation in adolescents and young adults is not uncommon and could lead to inactivation of vitamin B12 with consequent inhibition of methionine synthesis, which is essential for folate bioavailability (71,72). Genetic defects in methionine synthase and folate deficiency are both associated with increased MN in PBL (4,73). Moderate exposure to nitrous oxide in the medical occupational setting in operating theatres was shown to increase MN in PBL up to 4-fold (74,75). It is therefore quite possible that abuse of nitrous oxide for recreational purposes could cause an increase in MN in PBL and deserves investigation.

**Effect of healthy lifestyle habits measured using the health practice index**

It is plausible that lifestyle habits causing cancer and cardiovascular disease may be indirectly associated with MN frequency in PBL because this biomarker has been prospectively associated with these diseases (3–5). Huang \textit{et al.} (53) evaluated the effect of such lifestyle habits in a study conducted among 208 healthy adult (19–59 years) male Japanese hard-metal workers. The subjects were divided into groups according to their self-reported lifestyle, i.e. good, moderate and poor, according to their responses to a questionnaire regarding eight health practices (cigarette smoking, alcohol consumption, sleeping hours, working hours, physical exercise, eating breakfast, balanced nutrition and mental stress) and the presence or absence of each parameter was summed to obtain a health practice index (HPI: range 0–8, with a higher score indicating healthier lifestyle habits). Total lifestyle quality as measured by the HPI was strongly negatively associated with MN frequency in cultured human lymphocytes \((P < 0.01)\). Nutritional imbalance, lack of regular exercise \((< 2\) times per week), insufficient sleep \((< 6\) h per day) and overtime working \((> 9\) h per day) each contributed significantly to higher MN frequency \((all P < 0.05)\). In the smoker group, a significantly higher MN frequency was only found in heavy smokers \((P < 0.05)\). Mental stress, eating breakfast and alcohol drinking had no effect on MN frequency in this study group. Taken together, these findings indicated that poor lifestyle habits significantly increase MN frequency in human lymphocytes.

**Knowledge gaps**

The dominating role of age and gender among variables affecting MN frequency in PBL is virtually beyond dispute. However, our knowledge on the effects of nutrition and lifestyle factors on MN in PBL is not yet complete. The following are some important knowledge gaps that need to be addressed in future research.

- It is likely that genotype differences may influence the degree to which age and lifestyle variables affect MN frequency; this needs to be investigated thoroughly with large and adequately powered studies that can accurately evaluate the effect modification of most common polymorphisms.
- Nutrition studies in lymphocytes clearly show the sensitivity of the MN to differences in micronutrient intake, although the effect of macronutrients and food groups remains unexplored. This information would help identify dietary patterns that are likely to be optimal for DNA damage prevention and to design appropriate controlled interventions to test such hypotheses.
- The potential effects of obesity needs further investigation because the induction of MN in PBL may be evident only if the insulin resistance phenotype is present, as suggested in a recent study on polycystic ovary syndrome (51).
- The apparent lack of a strong effect of smoking on MN frequency in PBL even in heavy smokers is somewhat puzzling; the possibility of an adaptive response to the chronic genotoxin exposure, however, should be considered (76). On the other hand, the effect of smoking on MN frequency may be evident only in those smokers who are more susceptible to the genotoxic effects of cigarette smoke and who are at a higher risk of developing lung cancer as was shown recently (77). Measuring MN frequency in different lymphocyte subsets and performing studies stratified by susceptibility to genotoxins from cigarette smoke may better define the impact of smoking on this biomarker. Another explanation for the weak effect of smoking could be \textit{ex vivo} cell death of damaged cells or their failure to divide in culture because they already contain MN induced by chronic exposure to cigarette
smoke genotoxins. Use of the cytoe biomarkers within the CBMNcyt assay (1) could shed light on these possibilities because this allows measurement of cell death (necrosis and apoptosis), cytoptosis (via the nuclear division index) as well as MN in non-divided mononuclear cells.

- The abuse of recreational and illicit drugs is unfortunately not rare. Its effect on MN frequency is only reported in one study despite the association with DNA damage induction being highly plausible. More studies are required to verify the extent to which recreational illicit drugs use may influence MN frequency in PBL and whether these effects are confounded by association with other unhealthy lifestyle habits.

- This review was not intended to focus on the effects on MN frequency of environmental radiation or chemicals. However, we acknowledge that contamination of foods with radionuclides or genotoxic chemicals, exposure to routine radiological medical checkups, including Computerised Tomography scans, as well as lifestyles that increase exposure to ultraviolet radiation may also contribute to the observed MN frequency. For this reason, every effort should be made to quantify such exposures as accurately as possible when studying MN frequency in human populations.

- More studies are needed using standardised and validated tools that capture reliable measures of multiple healthy/unhealthy behaviours and evaluate their integrated effects on MN frequency in PBL. The results of the study using the HPI are promising for identifying strategies for prevention of DNA damage; however, replication in other populations to verify the robustness of this association is required.

- Psychological stress is increasingly recognised as being associated with deleterious genomic effects including telomere shortening in blood cells (78–80). Whether MN frequency in PBL is affected by psychological stress deserves intensive exploration.

Conclusions

It is evident that the MN frequency in PBL measured using the CBMNcyt assay is affected by age, gender and multiple dietary and lifestyle factors. A thorough understanding and measurement of these factors is therefore essential when performing biodosimetry studies of suspected exposure to known or unknown genotoxic agents. The fact that diet and lifestyle have a profound effect on the occurrence of MN in PBL, which is one of the best validated biomarkers of DNA damage in humans, emphasises the plausibility and importance of a disease prevention strategy based on MN frequency reduction by appropriate improvements in nutrition and lifestyle habits both at the individual and population level.

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References


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