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# Effects of age and gender on micronucleus and chromosome nondisjunction frequencies in centenarians and younger subjects

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**Studies have shown a significant increase in chromosome aneuploidy with age. The aim of this study was to elucidate whether the age-related changes in the level of hypoploidy correlate with the occurrence of micronuclei (MN) and chromosome nondisjunction (ND) in men and women. We analyzed cytokinesis-blocked (binucleated) lymphocytes treated with cytochalasin B, from 127 donors varying in gender and age including 53 centenarians. Fluorescent *in situ* hybridization with probes specific for several autosomes (1, 4, 6, 8, 20) and for the sex chromosomes was applied to analyze the chromosomal content of MN and to analyze the frequency of reciprocal loss and gain due to ND in binucleated interphase cells. The general level of MN in Giemsa-stained preparations was higher in women and in both genders increased with age until ~70 years and ranged, depending on age group, from 0.5 to 1.4% in men and from 0.9 to 1.8% in women. Gender-related differences were mostly observed in the younger age groups (≤50 years), with an almost two-fold difference between men and women ($P < 0.005$). Frequencies of autosome-positive MN in both genders and of sex chromosome-positive MN in men were comparable and remained unchanged in older groups. The frequency of X-positive MN in women was higher than the average frequency of autosome-positive MN and continued to increase until the oldest age. The frequency of NDs involving the analyzed chromosomes was on average two-fold higher in women than in men. In both genders, the frequency of NDs increased with age and was, on average, an order of magnitude higher than that of cells with MN, consistent with the previous reports that the efficiency of elimination of micronucleated cells is higher than of the cells presenting chromosome ND.**

## Introduction

Aging in humans appears to be associated with genetic instability (1). An age-related decline in the efficiency of repair processes and accumulation of mutations due to adverse endo- and exogenous conditions result in increased level of DNA damage, which—at the cytogenetic level—is reflected by an increased frequency of chromosomal aberrations (2–4). In fact, aberrations in chromosome number and/or structure were the first genetic lesions found to be specifically associated with age. Many studies have shown a significant increase in chromosome loss (hypoploidy), primarily of the sex chromosomes, in peripheral blood lymphocytes and skin fibroblasts, in both men and women of advanced age (4,5).

Aneuploidies observed in mitotic spreads from healthy, unexposed donors reflect spontaneous malsegregation events during mitoses. Chromosome lagging leading to nondisjunction (ND) at mitosis is a well-known mechanism of reciprocal gain and loss of chromosomes. The formation of micronuclei (MN) due to chromosome lagging represents another mechanism of chromosomal loss. MN appear in the cytoplasm of daughter cells as small additional nuclei and contain whole chromosomes or acentromeric chromosome fragments, left behind during nuclear division (6–9). Numerous reports have shown that the age-related increase in DNA loss is associated with the higher frequency of MN in peripheral blood lymphocytes from older individuals (10,11). The average number of MN per cell has been shown to differ depending on age and gender of cell donors and on the type of sequestered chromosomes (6,9).

In our recent cytogenetic study of human lymphocytes from donors varying in age and gender (4), we demonstrated that the pattern of age-related increase in the frequency of chromosomal aberrations in metaphase plates differed between men and women. Here, we extended that study by analyzing cytokinesis-blocked (binucleated) lymphocytes treated with cytochalasin B from the same donors. Our aim was to elucidate whether and how the age-related changes in the level of hypoploidy correlate with the occurrence of MN and ND observed in men and women.

## Material and methods

Heparinized whole-blood samples were obtained, with informed consent, from unrelated individuals representing a random sample of the ethnically homogeneous Polish population, men and women, stratified according to their age: 53 centenarians (46 women aged 100–108 years and 7 men aged 100–102 years), 20 individuals aged 69–78 years (11 women and 9 men), 14 aged 60–68 years (8 women and 6 men), 20 aged 40–50 years (9 women and 11 men) and 20 aged 21–30 years (9 women and 11 men). The stratification was designed to allow comparison with our previous study on frequencies of chromosomal aberrations in metaphase plates in the same group of individuals (4). The study group was recruited totally at random to randomize the environmental factors that could bias the comparison of age and gender groups. No diseases were reported in anamnesis and no specific environmental exposures were known to be present in excess in any of the gender or age groups.

Lymphocyte cultures were established from 0.3 ml samples of whole blood, added to 4 ml Eagle's Medium 1959 (Biomed, Poland) supplemented with 15% heat-inactivated fetal calf serum (Biomed, Poland), 1% penicillin (200 U/4 ml) solution (Polfa, Poland) and phytohemagglutinin (LF-7 Biomed). After incubation for 44 h at 37°C, cytochalasin B (6 μg/ml; Sigma, St Louis) was

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added to the medium and cells were incubated for another 28 h at 37°C. Then, colcemid (Gibco, Scotland, UK) was added to the medium and 1 h later binucleated lymphocytes were harvested by centrifugation (10 min at 150× *g*). Cells were resuspended in 75 mM KCl, incubated for 20 min at 37°C and gently fixed three times with methanol : acetic acid (3 : 1) for 20 min at +4°C. Finally, fixed lymphocytes were mounted on clean glass slides and air dried (8).

The incidence of MN was determined in Giemsa-stained preparations. Typically, for each individual, ∼250 (100–400) binucleated cells were examined for the presence of MN in each of the analyzed age/gender groups. Cells were classified as micronucleated either if the MN were directly attached to the nucleus or if the distance between nucleus and MN was not obviously larger than the diameter of the MN.

Fluorescent *in situ* hybridization (FISH) with molecular direct-labeled probes (Cytocell, UK) was preformed on binucleated lymphocytes by using commercial procedures and probes specific for selected human chromosomes; 1, 4, 6, 8, 20, X and Y were chosen on the basis of previous data showing their potential involvement in the process of aging (6,9,12). Four hybridization mixes were prepared: for chromosomes 1 (biotinylated probe) and 6 (digoxigenated probe), for chromosomes 4 (biotinylated probe) and 8 (digoxigenated probe), for chromosomes 20 (biotinylated probe) and X (digoxigenated probe) in women and for chromosomes X (biotinylated probe) and Y (digoxigenated probe) in men. Nuclei and MN were counterstained with DAPI in antifade solution. Signals from FISH were visualized by using an Olympus microscope with a UPlanFlx100/1.3 oil objective. Analyses were conducted by using MetaSystems imaging systems. Typically, for each individual, ∼150 binucleated cells were scored after hybridization with centromere-specific probes (for the alphoid sequences of each of the analyzed chromosomes); another ∼150 cells were scored after hybridization with painting probes specific for the whole chromosome. Binucleated cells were considered to contain ND of a given pair of chromosomes when three chromosome-specific FISH signals were present in one of the binuclei and only one in the other nucleus.

The average number of MN per cell was calculated by dividing the total number of MN observed by the number of binucleated lymphocytes analyzed. To compensate for the low number of cells scored per individual (∼250 cells per individual in Giemsa-stained preparations and ∼150 in FISH experiments), the frequency of MN was assessed in a substantial number of individuals grouped according to gender and age (such that the total number of cells scored per group ranged from 1500 to 4000). Statistical tests [Kruskal–Wallis analysis of variance (ANOVA) by Ranks and Friedman ANOVA, implemented in Statistica package] were used to determine the significance of the effect of independent variables (age or gender) on the frequency of aberrations in different groups. Regression analysis (Statistica package) was performed on the square-root-transformed data to assess the slope and significance of the correlation of the frequency of aberrations with age. Because of the unequal sample size and unequal variance in the analyzed gender/age groups, the raw data (average number of MN per cell) were subjected to square-root transformation $[x' = \mathrm{sqrt}(x) + \mathrm{sqrt}(x + 1)]$ prior to the analysis.

## Results

The frequency of MN in Giemsa-stained preparations was assessed by dividing the total number of MN by the number of binucleated lymphocytes analyzed in each individual or group of individuals. Wherever found, one MN per cell was observed, such that the average number of MN per cell corresponded to the average proportion of binucleated cells with MN.

The frequency of MN in all age groups pooled together was slightly (∼1.3-fold) higher in women than in men (0.011 ± 0.007 versus 0.008 ± 0.007, respectively; Figure 1a); the difference was marginally significant by a nonparametric statistical test (Kruskal–Wallis ANOVA by Ranks, $H_{\mathrm{df}\,=\,1,n\,=\,123} = 4.17$; $P = 0.04$). The difference between women and men was more pronounced (∼1.9-fold) and also more significant ($H_{\mathrm{df}\,=\,1,n\,=\,40} = 8.04$; $P = 0.0046$) when the youngest groups (20–50 years) were compared (0.012 ± 0.006 and 0.008 ± 0.005 in women and men, respectively); no significant difference between genders was observed in people aged ≥60 years (Figure 1a).

The correlation of MN frequency with age in men and women was further analyzed by linear regression. Square-root-transformed average numbers of MN per cell were plotted against each donor's age. In both genders, an age-related increase in MN frequency was seen in donors aged 20–68 years. An opposite trend, i.e. age-related decrease, was observed in individuals aged 60–110 years (Figure 1b). The positive correlation was statistically significant only in men ($P = 0.004$, $r^2 = 0.26$). In women, only the negative correlation was statistically significant ($P = 0.012$, $r^2 = 0.1$).

In order to analyze the presence of specific chromosomes in MN, FISH with centromere-specific and chromosome painting probes for five autosomes (1, 4, 6, 8, 20) and for sex chromosomes (X and Y) was applied to binucleated interphase cells (Figure 2a and b). In ∼90% of the MN, a positive signal was obtained with both types of probes (centromere-specific and painting) indicating that in most cases MN contained whole chromosomes rather than their fragments. This is consistent with what is usually found in nonexposed donors (13). No MN with signals from more than one chromosome per MN were observed.

Positive FISH signals were found in MN from men aged ≥60 years and women aged ≥40 years (Figure 3a). Within these groups, no age-related increase in the frequency of signals from autosome-specific probes was seen, and the frequencies of MN with the FISH-probed autosomes in men and in women were comparable. MN with the sex chromosomes (both X and Y) in men aged ≥60 years were found at frequencies similar to those of MN with the FISH-probed autosomes and no significant age-related change in their frequency was noted. In women, the frequency of MN with the autosomes resembled that in men and remained unchanged in the age range from 40 to >100 years. In contrast, the frequency of MN with chromosome X in women aged 41–50 years was three-fold higher than the average frequency of MN with the FISH-probed autosomes and increased with age, to become twice as high in centenarian women ($r^2 = 0.96$). The frequency of Y-positive MN in centenarians was two-fold higher than in the younger men; in both genders, the frequency of MN with chromosome 1 was two-fold higher, compared to other autosomes, but it did not change with age.

FISH-based methodology was applied to analyze the frequency of reciprocal loss and gain due to chromosome NDs in binucleated interphase cells (Figure 2c and d). FISH allows visualization of the reciprocal segregation patterns in daughter nuclei and is a convenient way of analyzing aneuploidies, since most of the problems related to the artifactual loss and gain of

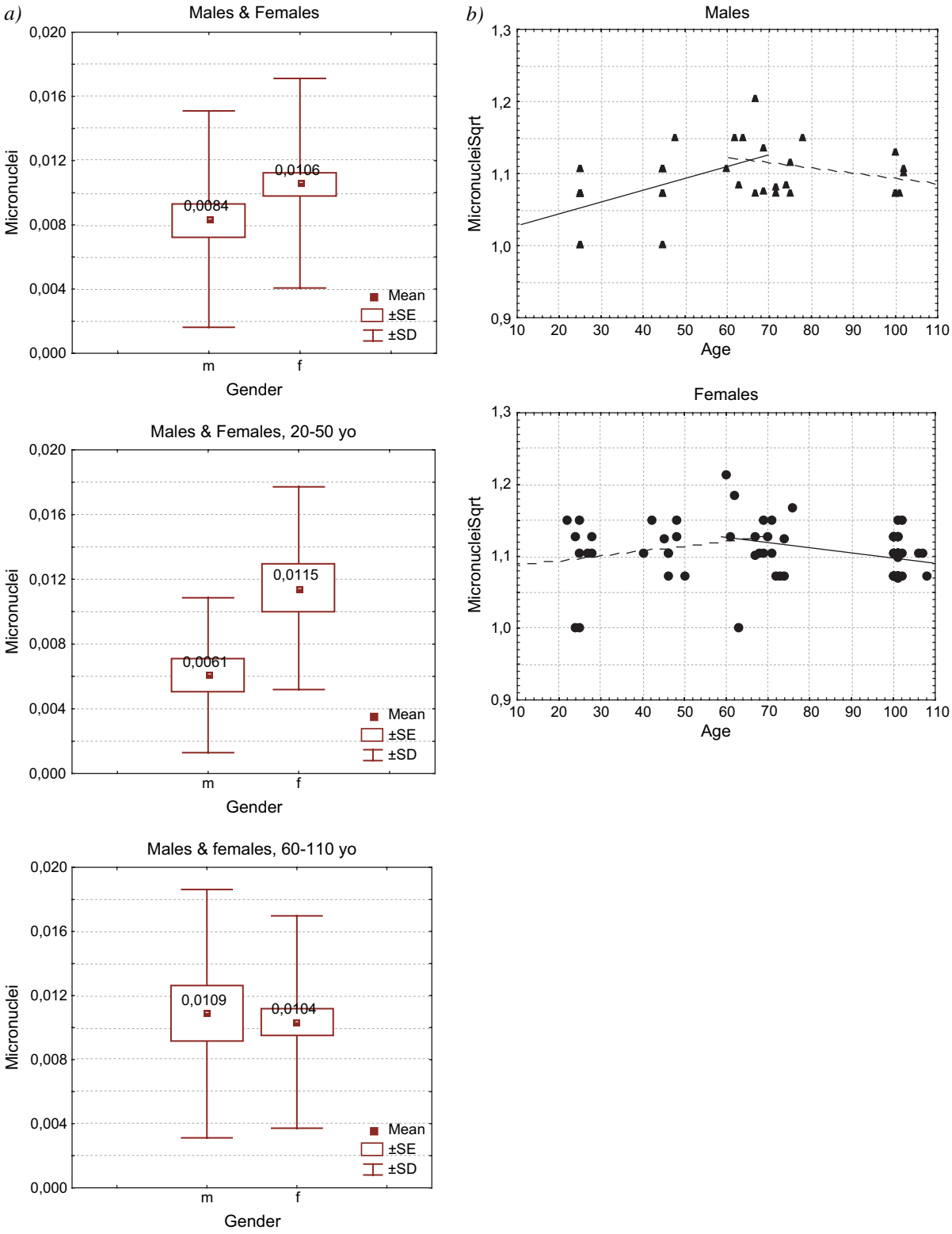


**Fig. 1.** Age-related changes in the frequency of MN in men and women. (**a**) The average number of MN per Giemsa-stained cell in men (m) and women (f)—different age groups. To compensate for the low number of cells scored per individual, the frequency of MN was assessed in a substantial number of individuals grouped according to gender and age. (**b**) Correlation between the frequency of MN (square-root-transformed proportion of cells with MN) and age. Solid lines indicate statistically significant correlations: men aged 20–69 years ($r^2 = 0.26$; $P = 0.004$; $y = 0.0015x + 1.02$) and women aged 60–110 years ($r^2 = 0.10$; $P = 0.012$; $y = -0.0007x + 1.17$).

chromosomes, which flaw other methods, are avoided. Cells with NDs involving any of the seven analyzed pairs of chromosomes were present already in the youngest age groups in both genders, and their frequency was, on average, an order of magnitude higher than that of cells with MN analyzed by the same method.

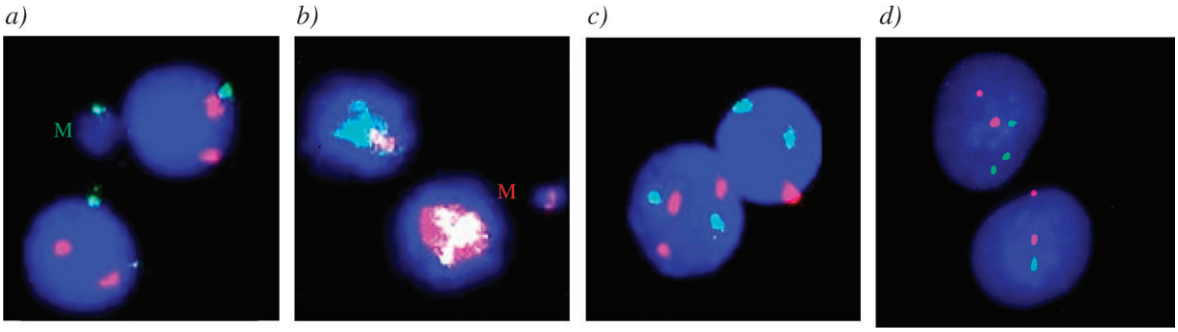


**Fig. 2.** Binucleated lymphocytes after FISH with chromosome-specific probes. (**a**) FISH with centromere-specific probes for chromosomes 1 (green) and 6 (red); green probe reveals signal for chromosome 1 in the MN; (**b**) FISH with painting probes for chromosomes 4 (green) and 8 (red); red probe reveals signal for chromosome 8 in the MN; (**c**) FISH with centromere-specific probes for chromosomes 4 (green) and 8 (red); red probe reveals ND of chromosome 8; (**d**) FISH with centromere-specific probes for chromosomes 1 (green) and 6 (red); green probe reveals ND of chromosome 1.

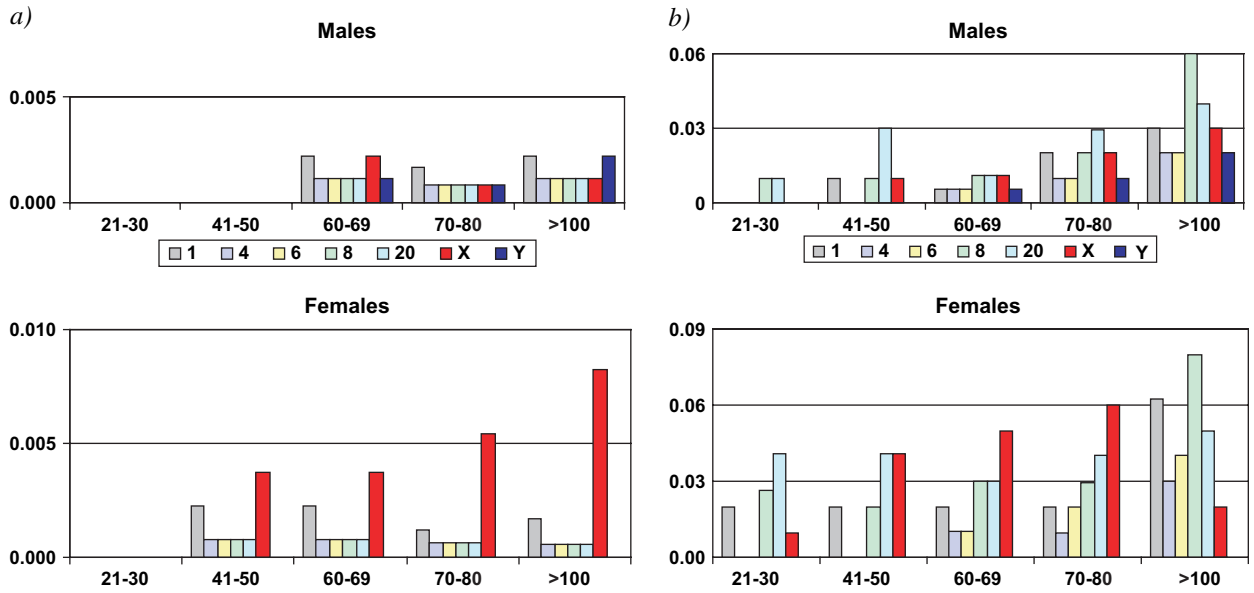


**Fig. 3.** Frequency of positive FISH signals for chromosomes 1, 4, 6, 8, 20, X or Y (centromere-specific probes), in different age and gender groups. In each group, the number of MN or cells positively stained with the chromosome-specific probe was divided by the total number of binucleated cells scored. (**a**) The frequency of MN displaying chromosome-specific FISH signals; (**b**) The frequency of NDs involving FISH-probed chromosomes.

The overall frequency of NDs was on average two-fold higher in women than in men and increased with age in both genders; the gender difference was more pronounced in the younger groups. In men, the frequency of NDs involving autosomes and sex chromosomes increased with age (Figure 3b), to reach the highest level in centenarians (autosomes and chromosome X: $r^2 = 0.91$; chromosome Y: $r^2 = 0.89$). The frequency of NDs involving autosomes increased with age also in women ($r^2 = 0.64$). Surprisingly, the increase in NDs involving chromosome X was not significant in women ($r^2 = 0.09$), due to a rapid decrease in the contribution of X in NDs observed in centenarians (for the noncentenarian women $r^2 = 0.91$).

## Discussion

In our study, the mean frequency of MN (estimated in Giemsa-stained preparation) ranged, depending on age group, from 0.5 to 1.4% in men and from 0.9 to 1.8% in women. This is consistent with previous reports, where MN frequency in women was higher than in men (11,14). Many authors reported an age-related increase in MN frequency in both sexes (9,10), especially in individuals aged >70 years. For example, Fenech and Morley (15) noted a several-fold increase in MN frequency in lymphocyte cultures from 80-year-old donors compared to young children. Guttenbach *et al.* (16) observed an increase in MN frequency from 0.15% in people aged <10 years. However, some authors reported that in the oldest age classes, the MN frequencies leveled off; for example, frequency ratios, which express the increase in cytogenetic damage with respect to the first age class, reaches the value of 2 at the age class 50–59 and remains substantially unchanged thereafter (12). Our study is consistent with this observation.

Overall, the frequency of MN (or micronucleated lymphocytes, since in most cases one MN per binucleated cell was present) was higher in women than in men, but this discrepancy was significant only in younger age groups (≤60 years). The highest MN frequency, observed in the group aged 60–68

years, was comparable in men and women. The significant increase in MN frequency with age was observed only in men aged ≤70 years, reflecting the higher incidence of MN in women than in men from youngest age groups. MN frequency decreased in the oldest age groups under study (>70 years); the effect was significant only in women, presumably due to the low number of centenarian men available for the study. The deficiency of MN in the oldest groups might be explained by the fact that micronucleated cells are preferentially eliminated by apoptosis (6,17,18). In fact, some studies have shown age-related changes in the level of proteins and factors that regulate apoptosis (19) and demonstrated that inhibition of apoptosis could increase the proportion of micronucleated cells (8).

It has been shown that a higher MN frequency directly corresponds to a decreased efficiency of DNA repair and an increased genome instability (3,4). The age-related increase in the frequency of MN in binucleated cells from individuals aged ≤70 years reported in our study corresponds well with the age-related increase in the occurrence of hypoploidy demonstrated in our earlier study of metaphases in lymphocytes from the same individuals. Moreover, the decrease in MN frequency observed in older women is consistent with the plateau in hypoploidy level observed in women but not in men aged >70 years (4,20).

Many studies have demonstrated that the age-related increase in the hypoploidy level is associated with the loss of sex chromosomes (4,6,9,11,21) and indicated that the age-related increase in sex chromosome loss correlates with the increased level of MN formation (11). Here, we applied FISH with probes specific for several autosomes and for the sex chromosomes to analyze the chromosomal MN content of binucleated lymphocytes and to compare the frequency of chromosome-positive FISH signals in MN and in ND. In contrast to the earlier observation that the presence of centromere-positive MN was significantly higher among the older donors than among the younger donors (22), the levels of positive staining obtained with both painting and centromere-specific probes were in essence identical in all the age groups.

Positive FISH signals in MN were obtained with all the FISH probes used (specific for chromosomes 1, 4, 6, 8, 20, X and Y), consistent with an earlier demonstration that all of the 23 chromosomes could be present in MN (6). MN with the FISH-probed chromosomes were detected in women aged >40 years and in men >60 years. Although the frequencies of autosome-positive MN in both genders and of the sex chromosome-positive MN in men were comparable and did not change with age in older groups, the absence of FISH-positive nuclei in the youngest groups is consistent with the observation that the age-dependent increase of MN concerns both MN with X chromosome, Y chromosome and autosomes (6,9). MN contained whole chromosomes rather than their fragments, which is consistent with the observation that with age kinetochores become dysfunctional (6,20). The frequency of the X-positive MN in women was higher than the average frequency of autosome-positive MN and continued to increase until the oldest age (two-fold increase in centenarians compared to the group aged 40–50 years). This is consistent with the earlier reports; for example, Guttenbach *et al.* (23) estimated that exclusion of sex chromosomes into MN doubles during human life, from 11% in young to 20% in old donors; Catalan *et al.* (22) demonstrated that the X chromosome was highly overrepresented in MN, the older women showing a higher proportion of X-positive MN (24%) than the younger women (14%); Hando *et al.* (24) described a ten-fold difference in the frequency of X-positive MN in women, as compared to men. Differential survival of various aneuploid cell types could cause a higher frequency of cells aneuploid for X than for any of the autosomes or chromosome Y. There is apparently a continuous loss of autosomes by MN formation. Monosomic cells created in this way seem to have a selective disadvantage, most of them being unable to survive. This situation is likely to be similar for the single-copy X chromosome in men. A different proliferation activity may also contribute to the discrepancy between genders in the age-related changes in MN frequency (25).

FISH analysis was applied to analyze the frequency of reciprocal loss and gain due to chromosome ND in binucleated interphase cells. All analyzed chromosomes (1, 4, 6, 8, 20, X or Y) were involved in NDs; the overall frequency of NDs was higher in women. In contrast to MN, where no specific FISH signals were present in the youngest groups, NDs involving the analyzed chromosomes were found already in the group aged 20–30 years, and their frequency increased with age, in both genders. In both genders, the frequency of NDs was, on average, an order of magnitude higher than that of cells with MN. These results, consistent with the previous reports that the efficiency of elimination of micronucleated cells is higher than of the cells presenting chromosome ND (17,26), suggest that ND is the main mechanism leading to the aneuploidy of autosomes as well as the sex chromosomes. Interestingly, the decreased frequency of NDs involving chromosome X in centenarian women is accompanied by the steady age-related increase in X chromosome-positive MN in women. This might be related to the observation that MN in women preferentially contain the inactive X chromosome (24,27).

## Conclusions

The changes in MN frequency in lymphocytes from men and women of different age groups observed in this study appear to correlate well with the previously described changes in the level of hypoploidy in metaphase plates from the same groups of donors (4). The overall frequency of MN increases with age until ~70 years and within this age range is higher in women, but in older donors, it levels off to become comparable in centenarians of both genders. This suggests a kind of threshold of genome instability that cannot be crossed if one is to survive until the centenarian age; in general, women appear to reach this threshold faster than men. The individuals surviving until the oldest age most likely represent a fraction accumulating these lesions at a slower pace.

On the other hand, the higher frequency of NDs compared to MN involving analyzed chromosomes, in donors of all age and both genders, suggests that the efficiency of elimination of micronucleated cells is higher than of the cells presenting chromosome ND and that ND could be the most essential contributor to the observed age-related increase in the frequency of spontaneous aneuploidies.

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