Published in final edited form as:

Mutat Res. 2012 September 18; 747(2): 207–217. doi:10.1016/j.mrgentox.2012.05.004.

Mechanisms leading to the formation of micronuclei containing sex chromosomes differ with age

Kimberly H. Jones^{1,3}, Timothy P. York², and Colleen Jackson-Cook^{1,2}

Kimberly H. Jones: Kimberly.Jones@neodiagnostix.com; Timothy P. York: tpyork@vcu.edu; Colleen Jackson-Cook: ccook@mcvh-vcu.edu

¹Department of Pathology, Virginia Commonwealth University, Richmond, VA

²Department of Human & Molecular Genetics, Virginia Commonwealth University, Richmond, VA

Abstract

The frequency of spontaneously occurring micronuclei (MN) increases with age, with many of these MN containing sex chromatin. However, it is not known if this MN frequency increase is attributable to a higher number of the same cellular events that occur in younger people, or if a different sex chromosomal instability mechanism(s) arises with age. To gain insight regarding this question, the total number of signals present in MN and their corresponding binucleates, was scored in older (ages 40–80+ y.o.; n= 40) compared to younger (7–39 y.o.; n=19) individuals using probes specific for the X and Y chromosomes. In 19.9% of the cells scored at least one sex chromatin positive micronucleus was present. A significant decrease in cells having a “corrective” loss pattern (i.e. trisomy rescue, leading to euploid binucleates following sex chromatin exclusion into the MN) was observed with increasing age for the Y chromosome in males (p=0.022) and the X chromosome in females (p=0.004). In addition, a significant increase (p<0.001) in cells having multiple signals beyond those expected from a single cellular error was observed in the older compared to younger study participants, with these imbalances resulting from cells having either a single micronucleus with multiple signals, or cells having multiple MN. Collectively, these findings suggest that age-related increases in MN frequencies reflect both gains in the occurrence of similar cellular errors, as well as changes in the types of chromosomal findings that occur. Importantly, these results also illustrate that while MN frequencies reflect acquired abnormalities, they may also reflect cellular responses to “correct” an error, particularly when evaluated in young individuals. Therefore, when analyzing MN frequencies, one may also wish to evaluate the imbalances present in both the binucleates and MN to facilitate the recognition of varying cellular responses to environmental or genotoxic exposures.

© 2012 Elsevier B.V. All rights reserved.

Corresponding author: Colleen Jackson-Cook, ccook@mcvh-vcu.edu, Virginia Commonwealth University, Department of Pathology, PO Box 980662, Richmond, VA 23298-0662, Phone: 804-628-2992, Fax: 804-828-4242.

³Present address, Neodiagnostix, Inc, Rockville, MD

CONFLICT OF INTEREST STATEMENT

None of the authors has a conflict of interest (financial or otherwise) regarding this research study.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

Micronuclei; Spontaneous micronuclei; Acquired chromosomal abnormalities; Age-related chromosomal abnormalities; Mechanisms of micronuclei formation; Cytokinesis-block micronucleus assay

Introduction

Chromosomal instability and DNA damage have been associated with a variety of diseases (especially cancer), but have also been shown to occur spontaneously in healthy people, with the frequency of acquired somatic cell chromosomal aberrations increasing with age (¹⁻⁵). The cytokinesis-blocked micronucleus (CBMN) assay has become one of the most frequently used tools for assessing chromosomal abnormalities that have been acquired in the somatic cells of an individual, particularly in response to environmental exposures (^{6,7}). A micronucleus (MN) is a small structure that contains chromatin that was excluded from the daughter binucleates following cell division. The popularity of the CBMN assay stems, in part, from its ability to provide an accurate alternative to the time consuming process of identifying chromosomal damage through the analysis of metaphase chromosomes. By scoring micronuclei (MN), the CBMN interphase assay provides information regarding the frequency of chromosomal aberrations present in somatic cells that complete karyokinesis (but not cytokinesis) from only one round of cell division *in vitro*. Therefore, selective growth pressure that might occur against cells having chromosomal imbalances is also limited in this assay (⁸).

Studies from the Human Micronucleus (HUMN) consortium have shown advancing age to be consistently associated with an increased frequency of spontaneously occurring MN in adults (¹). These studies have also shown a gender difference in the frequency of MN, with differences in the female:male MN frequency ratios being most pronounced for people 40 years of age or older (¹). However, the underlying mechanisms leading to this age-related increase are not known. One approach that investigators have used to gain insight about the underlying chromosomal errors that result in the formation of spontaneously forming MN has been to characterize their chromatin content to determine if consistent patterns emerge. While one cannot directly visualize chromosomes in MN, their chromatin content can be inferred using fluorescence in situ hybridization methodologies (⁹). Through the use of pancentromeric and pantelomeric probes, investigators have inferred that the majority of spontaneously occurring MN present in healthy people contain a single, intact chromosome, but structural aberrations have also been observed (¹⁰⁻¹³). Furthermore, in adults the sex chromosomes (chromosomes X and Y) have been shown to be most frequently excluded into MN, with this frequency increasing with age (^{9,14-16}).

Few investigators have studied spontaneously occurring MN in children, in part due to their low frequency in younger individuals (reviewed by Holland et al. ¹⁷). Nonetheless, the studies that have been completed have shown some differences in the patterns of MN in children compared to adults. For example, the majority of investigators have not detected a clear gender difference in MN frequencies in children, but the results of these studies have been varied (¹⁷). Guttenbach et al. (¹⁸) specifically looked at the frequency of MN containing sex chromatin in 10 children (5 males and 5 females) and found 8% of MN in females (ages 1–10 years) to contain X chromatin, with Y chromatin being present in 14% of the MN in males (ages 0.5–4 years). In contrast, Hando et al. (¹⁹) and Nath et al. (²⁰) reported no MN containing sex chromosomes in the newborns they studied.

While there is a paucity of information regarding the frequency of MN containing sex chromatin in children, even less is known about the mechanisms leading to the formation of sex chromatin positive MN in children compared to adults. In adults mechanisms that have been purported to contribute to the formation of spontaneously occurring MN present in healthy people have included perturbations or defects in the assemblage or functioning of structures associated with mitosis, as well as alterations in the function of mitotic checkpoint genes (^{13,21}), with the majority of these anomalies leading to the loss of an intact chromosome. However, spontaneously occurring MN and/or nuclear buds have also been observed to arise through a “corrective” mechanism(s), whereby DNA imbalances are eliminated from the cells following the proper function of cell cycle and/or mitotic checkpoint genes (^{13,21–23}). The proportion of “corrective” type versus “error” related mechanisms that are associated with the formation of MN containing sex chromatin in healthy older adults compared to healthy children/young adults has not been previously established. Therefore, the primary aim of this study was to determine if these mechanism(s) varied with age. This goal was achieved by scoring the number of signals present in MN and their corresponding binucleates using probes specific for the pericentromeric regions of the X and Y chromosomes.

Materials and Methods

Sample Ascertainment

A total of 59 individuals (37 males and 22 females) were ascertained through the Mid-Atlantic Twin Registry, which is based at Virginia Commonwealth University (VCU). Study participants were selected for inclusion in this project from a larger cohort of 145 individuals, the latter of whom were ascertained as part of a study to determine the influences of genetic and environmental factors on acquired aneuploidy conditions in healthy twins (Table 1)[²⁴]. To test the hypothesis that cytogenetic mechanisms giving rise to MN vary with age, binucleates having MN were scored using probes specific for the sex chromosomes, since sex chromosomes have been reported to be the chromosome most frequently present in age-related MN (^{9,14–16}). To enhance the probability of obtaining study subjects with a sufficient number of cells having sex chromatin positive MN (thereby avoiding the costly completion of experiments that were not informative) the selection criterion used was the presence of at least 5% of their MN containing chromatin from a sex chromosome [as determined from the successful evaluation of their specimen in our studies using spectral karyotyping (manuscript in preparation)]. After providing their informed consent (VCU IRB protocol 179), each participant submitted a blood sample. The peripheral blood specimen was obtained by a health care provider of the study participant's choosing (to optimize their convenience). After collection, the specimen was shipped to our laboratory via an overnight delivery carrier.

Cell Culture

The cultures to assess MN frequencies were established with lymphocytes [using Histopaque-1077 (Sigma)] according to the protocol of Fenech (²⁵). Briefly, cytochalasin B (3.0 µg/ml; Sigma) was added to the cells 44 hours after culture initiation. At 72 hours, binucleate interphase cells were harvested using standard techniques, which included a 10-minute incubation in hypotonic solution (0.075 M KCl), and serial fixation (three times using a 3:1 methanol: acetic acid solution). Slides were made following standard methods (¹⁰).

FISH Procedure

For males, the sex chromosomal contents of the MN were determined through the simultaneous hybridization of differentially colored pericentromeric probes for the X

chromosome (DXZ1; Spectrum Aqua) and the Y chromosome (DYZ3; Spectrum Orange) according to standard techniques (Abbot Molecular), with a probe for chromosome 17 (D17Z1; Spectrum Green) also being used as an autosomal control. For females, the same X (DXZ1) and autosomal (D17Z1) probes were used, but the Y chromosomal probe was not evaluated since previous GTG-banding studies confirmed that none of these females had a Y chromosome (²⁴). The target (interphase chromatin) and probe DNA were denatured separately. The interphase micronuclear and binuclear chromatin was denatured by immersing the slides in a 70% formamide solution (pH 7.0) at 73°C for 3 minutes. Following denaturation, the slides were briefly rinsed in cold water and then dehydrated using an ethanol series (70%, 85%, and 100% for 2 minutes each). The probes (which were prepared using a 1:100 dilution in a C-Denhyb solution, [Insitus Biotechnologies, Albuquerque, New Mexico]) were denatured at 94°C for 10 minutes, and then cooled on ice for 2 minutes. Following the addition of the denatured probe to the denatured cellular chromatin, the slides were placed in a humidified chamber and hybridized at 37°C overnight. Non-specific binding of probes was removed by washing in a 0.4xSSC/0.3%NP-40 solution at 73° for 2 minutes, followed by a one minute wash at room temperature (in a 2xSSC/0.1%NP-40 solution). To visualize the binucleates and MN, the chromatin in the nuclei and MN was counterstained using a DAPI II/antifade solution (Abbott).

FISH scoring

Slides were scored using an Axioskop equipped with triple- and single-band pass filters (Zeiss). Only cells that contained at least one MN, as visualized with DAPI staining, were scored. The number of fluorescent signals present in the daughter cells, as well as the MN, was scored for each probe. When scoring each cell, all focal planes were viewed to ensure the detection of all signals in the three-dimensional nuclei. Interphase nuclei or MN that did not have clear borders or that were overlapping were excluded from the analysis. The probes used in this study were required to have at least a 0.98 specificity and sensitivity value. Representative images of cells having MN containing sex chromosomes were captured using a Cytovision Imaging system (Applied Imaging)(Figure 1).

Statistical Methods

The total number of sex chromosome signals present in each cell (the number of FISH signals present in each binucleate plus the number present in the MN) were evaluated and categorized for: (1) their patterns of aneuploidy when present (Figure 1); and (2) the presence of cells (binucleate + MN) having signal values that were higher than those expected from a single cell cycle/mitotic divisional error involving a sex chromosome (Figures 2 [a–d] and Figure 3). The criteria used for partitioning individuals into the older (40+ y.o) and younger (7–39 y.o.) age groups was based on observations from the HUMN consortium, which have shown the age-related increase in MN frequency to be clearly observed in people 40 years of age or older (¹).

To better approximate a Gaussian distribution, a square root transformation of the data was performed as suggested by Ceppi et al. (²⁶) for MN studies, given that MN frequency is a count variable derived by summing the occurrence of a discrete variable. Following transformation, the relationship between the “corrective” categories of MN formation and age was estimated using a mixed effect modeling approach (²⁷) to account for the non-independence of family members, using the R statistical programming software (²⁸). Comparisons of the number of cells having multiple signals (category 2 above) that were observed in the younger compared to older study participants were completed using a contingency Chi-square test (²⁹). To limit type I errors, an alpha level of *p* less than 0.05 was used to assess significance for all statistical tests.

Results

The mean age of the participants in this study was not significantly different from that of the parental study cohort ($t = 0.846$, p -value = 0.399), with both groups having ages ranging from 7 to 85 years old (Table 1). However, as expected from the selection criterion used for this investigation (minimum of 5% of their MN that were sex chromatin positive), the MN frequency (mean [standard deviation]) for the individuals evaluated in this study (15.48 [7.90]) was significantly higher than that of the parental study group (12.36 [7.72]) ($t = 2.907$, p -value = 0.004) (Table 1). Thus, due to the selection criteria bias, one cannot be certain that the cellular patterns observed in this study are representative of those seen in people having low MN frequencies.

From the 59 subjects in this study, a total of 4875 cells having MN were scored. In 19.9% of the total number of cells scored, at least one MN was detected that contained sex chromatin (Figure 4). The primary types of patterns observed in the sex chromatin positive micronucleated cells were: (1) one or both binucleates with a hypodiploid (loss) sex chromosome complement (57.2% [23.6% for X; 33.6% for Y]); (2) both binucleates with a euploid complement (loss corrective) (26.96%); (3) both binucleates with a gain in signal compared to euploid expectations (hyperdiploid) (1.49%); (4) one of the binucleates with a gain in signal while the other binucleate had a euploid complement (hyperdiploid corrective) (4.78%); (5) multiple sex chromatin signals in the MN (5 or more total signals for males and 7 or more total signals for females) that could not be accounted for on the basis of a single nondisjunctional or other gain-specific error (8.07%); and (6) other patterns that reflected imbalances that did not clearly fit into the above noted categories (1.49%) (Figure 4). The proportion of cells having sex chromatin positive MN that also showed a loss of signal for the autosomal control probe for chromosome 17 was 2.67%, which is significantly less than the value observed for either the X chromosomes (23.6%) ($p < 0.001$) or the Y chromosome (33.6%) ($p < 0.001$).

Cellular patterns for sex chromosomes (X and Y) in males

For the male study participants, 18.3% of the total number of MN scored (a portion of cells had more than one MN) contained Y chromatin, with 7.2% of the total number of MN scored containing X chromatin. Of the MN containing Y chromatin, the majority had a single signal (68.1%), followed by MN containing 2 (25.8%), 3 (4.2%), or at least 4 (1.9%) signals (Figure 5). Similarly, of the MN from the males that contained X chromatin, the majority contained one signal (51.6%), followed by MN having either 2 signals (42.6%), 3 signals (4%), or at least 4 (1.8%) signals (Figure 5).

To determine if the total cellular FISH patterns present in the binucleates and MN varied with age, the proportion of cells having the different types of FISH patterns were compared between the younger and older participants. A mixed effects regression analysis of the data showed that the percent of cells having loss of a Y chromosome into a MN that resulted in a “corrected” euploid binucleate pattern significantly decreased with age ($p = 0.022$) (Figure 6). However, no significant difference was observed with age for the proportion of cells having a “hyperdiploid corrective” pattern ($p = 0.911$) for Y chromatin (Figure 6).

For the MN seen in males that had X chromatin present, no significant divergence in the proportion of binucleates having “loss corrective” ($p = 0.100$) or “hyperdiploid corrective” ($p = 0.397$) patterns was observed with age (Figure 6).

Cellular patterns for the sex chromosome (X) in females

For the 22 female study participants, 15.9% of the MN scored (with a portion of cells having more than one MN) contained chromatin from the X chromosome. The majority of sex

chromatin positive MN contained a single centromeric region for the X chromosome (54.5%), with 2, 3, or at least 4 signals being seen in 37.0%, 4.1%, and 4.5% of the MN, respectively (Figure 5). A mixed effects regression analysis of the data showed that the proportion of cells having a “loss corrected” euploid binucleate micronuclear pattern with exclusion of X chromatin into a MN significantly decreased with age ($p=0.004$). In contrast, no significant difference in the proportion of “hyperdiploid corrective” cells was detected in the younger compared to older females ($p=0.454$) (Figure 6).

Cells having multiple sex chromosome signals

Interestingly, cells having MN containing multiple signals for either the X or Y pericentromeric region were seen in a significantly higher proportion of study participants (males and females) who were 40 years of age or older (Chi-square = 30.1, d.f.=1, $p<0.001$), with only 3 cells having multiple signals being observed in the participants who were 39 years of age or younger (Figure 7). When evaluated over the total number of cells having MN that were scored, a 12-fold increase in the incidence of cells having multiple signals was observed among the older adults (3% of all cells [sex chromatin positive and negative] having MN) compared to the children and younger adults (0.25%) (Figure 7). The greatest number of sex chromatin signals for a single cell (MN plus binucleates) was 14 for the X pericentromeric probe and 8 for the Y pericentromeric probe (Table 2 and Figure 3). Most of these multi-signal cells were seen as either a single or dual observation within a person, with 29 individuals (11 females [age range of 27–85] and 18 males [age range of 31–78]) having at least one multi-signal cell. However, 5 individuals (4 males and 1 female), all of whom were in their 60s, had at least 4 cells with multiple signals (range of 4 to 10 cells).

Discussion

This study was done to better understand the underlying basis for the observation of an age-related increase in spontaneously acquired MN containing sex-chromatin. We expanded upon previously reported data by analyzing the number of signals that were present for the pericentromeric region of the sex chromosomes in both the binucleates, as well as their corresponding MN, to determine the total cellular sex chromosomal complement. The most frequently observed pattern that was detected in the cells from our study participants was a hypodiploid sex chromosomal complement in at least one binucleate, due to the exclusion of that sex chromosome into a MN. Given that interphase nuclei were evaluated, one cannot rule out the possibility that a portion of these cells in females may have resulted from the convergence of the pericentromeric X chromosome satellite DNA sequences probed⁽³⁰⁾. However, one can use the frequency of “loss” that was observed for the chromosome 17 autosomal probe in these cells as an estimate of the proportion of cells that might arise from technical issues (such as probe convergence/co-localization), since the autosomal probe is also specific for alpha satellite sequences and may demonstrate similar levels of somatic pairing for this heterochromatic region⁽³⁰⁾. The frequency of cells having a single signal for this autosomal control probe (2.67%) was significantly less ($p<0.001$) than the proportion of cells having a loss of signal involving the X chromosome (23.6%). Furthermore, the observation of a loss of signal for a sex chromosome in males was not confounded by this co-localization artifact since these cells contained only one region for the sequences evaluated. Thus, it seems likely that the majority of the binucleates having a reduction in sex chromatin signals reflect true loss observations rather than sequence convergence/co-localization or technical artifacts. Hando, et al.⁽¹⁹⁾, who are among the few other investigators to include a description of the binucleate signal pattern in their study of MN containing sex chromatin, also reported patterns resulting in hypodiploid binucleates for the adult females they studied (ages 20 to 79 years [they excluded data from 8 newborns due to the low frequency of MN present]). However, they did not report the other cellular patterns

that were detected in this study, such as the patterns resulting in euploid complements in the binucleates, multiple signals beyond those expected from a single karyokinesis, or hyperdiploid complements.

Of particular interest was the category of cells having binucleates in which the exclusion of a sex chromosome(s) into a MN resulted in the establishment of a balanced cellular complement (possible trisomy rescue). The proportion of these “corrective” cells was significantly increased in the younger compared to older females for the X chromosome and in the younger compared to older males for the Y chromosome. However, no significant difference in the proportion of “corrective” cells was seen with age for the X chromosome in males.

One can theorize that the “corrective” cells observed in this study might have arisen from a mitotic error (possibilities include [but are not limited to] a segregational error in the parental cell; a replication error in the current cell; or possibly DNA damage in the current cell) such that the total sex chromatin contents in the parental cell included an extra copy of the pericentromeric region of a sex chromosome (3 rather than 2)(Figure 2). The formation of the MN in this cell may then have resulted from the optimal functioning of the cell cycle checkpoints (or alternative mechanisms) to correct this error (trisomy rescue). The cellular processes involved in detecting these types of aberrations may decrease in efficiency with age or function in a differential manner with age. Alternatively, these “corrective” mechanisms may represent the lowest level of spontaneously acquired abnormalities that occur in humans, since the absolute frequency (but not relative frequency) of “corrective” cells was as high among the older individuals as the younger people. As humans age, they may maintain this “baseline” occurrence of cells with acquired aberrations, but also acquire additional types of chromosomal instability, especially those involving loss specific mechanisms, that lead to an overall age-related increase in the frequency of MN.

Support for the conjecture that there are not only differences in the frequency of cells having MN, but also differences in the types of errors that occur with age comes from our observation of an increased frequency of cells having multiple signals (exceeding the number arising from a single cell cycle/mitotic division) in the older compared to younger subjects studied. We are among the few investigators who have previously reported the observation of these rare, multi-signal cells (^{21,31,32}). In the few previous reports only adults were evaluated, with no previous data being available regarding the potential presence of these cells in children. Given that these cells are rare in both younger and older people, one might hypothesize that the paucity of observations of cells having multiple signals in younger individuals simply reflects stochastic processes attributable to an overall decreased frequency of MN in younger people. However, given that we evaluated more micronucleated cells (total of 929) from individuals in the 7 to 19 year old cohort compared to the 40–49 year old cohort (total of 642) from this current study, our data suggest that the observed increase in multi-signal cells (for example, none seen in the 7 to 19 year-olds, but 10 seen in the 40–49 year-olds) is indeed reflective of an age-related factor(s) rather than chance alone.

To our knowledge, there have not been any previous reports of the multi-signal cells in which the total number of probe signals present in both the binucleate and MN was denoted. However, a study completed by Catalan, et al. (¹⁴) reported the proportion of all sex chromatin positive MN that had 1, 2, or at least 3 signals in the 10 men and women they studied. When comparing similarly aged individuals from their study and ours (Table 3), the proportion of cells having MN with 1, 2, and 3 or more signals was not significantly different for either the X chromosome in females ($p=0.384$) or the Y chromosome in males ($p=0.215$). However, differences in the percentage of MN having 1, 2 or at least 3 signals for

the X chromosome were observed between the males in these studies ($p=0.010$). This difference could reflect the fact that the mean age of the men in our study was 40.5 years compared to a mean age of 36.8 years for the males in their study. Alternatively, it could reflect genetic/environmental differences that were present in the small number of men sampled in each of these investigations. While the study design varied between this investigation and one completed by Bakou, et al (³³), they also observed a higher frequency of MN having multiple (2+) signals in the 50 year-old women they evaluated (6.13 ± 2.84) when compared to women in their twenties (0.48 ± 0.19), with the 12.8-fold increase in the percentage of cells having multiple signals they observed being comparable to the 12-fold increase in the percentage of multi-signal cells we observed in our older compared to younger study participants.

In contrast to our expanding knowledge regarding the mechanism(s) leading to the formation of a single MN in a cell (²¹), the mechanism(s) leading to cells having multiple copies of a chromosome/chromosomal region are not well understood. We and others [reviewed by Kirsch-Volders](²¹) have suggested that a single MN having multiple signals could result from: (1) the retention of a MN from a previous division(s) within the cytoplasm of a cell, with the MN continuing to complete replication (but not karyokinesis due to the lack of the necessary cytoplasmic components) resulting in increasing numbers of signals that are acquired through multiple cell cycles; or (2) multiple errors in a single cell cycle/karyokinesis (for example, a chromosome or chromosomal region may experience replicative as well as segregative and/or structural alterations [such as bridge-fusion-bridge initiated rearrangements, the latter of which could result in the presence of multiple centromeric signals])(^{13,21,31,32}). Cells having multiple signals due to the presence of 2 or more MN could have arisen from multiple errors in a single cell cycle. Alternatively, they could reflect the retention (possibly with replication of chromatin) of a MN from a previous cycle, along with the acquisition of an additional MN from this current cycle.

The observations reported herein were focused on lymphocytes having MN containing sex chromosomes. Thus, we do not know if a similar age-related mechanism(s) exists for binucleated cells having MN containing autosomes, or if there is a difference in the mechanisms leading to MN acquired in the somatic cells of healthy individuals versus people having constitutional chromosomal aneuploidy conditions. However, it is interesting to note that several investigators studying people having Down syndrome have reported age-related changes in the proportion of cells having a “corrective loss” of chromosome 21 (2 versus 3 copies of chromosome 21 (due to loss mechanisms or possibly reflecting selective growth pressure). The total frequency of micronuclei has also been observed to change with age in people having Down syndrome, with results varying between investigators but generally showing an increased frequency with age (³⁴⁻⁴¹).

Based on the collective information gained from the findings of other investigators [reviewed by Kirsch-Volders](²¹), as well as the results of this study, one could speculate that the increase in acquired sex chromosomal aneuploidy observed in the lymphocytes of older individuals is attributable to a variety of factors (Figure 8), and may include additional mechanisms for malsegregation/structural aberration formation beyond those present in children/young adults. These age-related differences may arise in direct response to environmental exposures, or as an indirect environmental effect, with the environmental exposure leading to epigenetic alterations (such as heterochromatin reorganization and histone modifications), which, in turn, could lead to age-related acquired chromosomal abnormalities. Moreover, epigenetic changes that arise in response to normal aging may also contribute to the observed increase in the frequency and type of acquired chromosomal aberrations (^{13,42}). Additionally, given that we and others have shown a heritable component to the frequency of spontaneous MN frequencies (^{24,43}) and that MN frequencies have been

shown to be influenced by genetic polymorphisms (⁴⁴), it seems likely that genetic effects (such as genes involved in chromatid cohesion; checkpoint and repair functions; or centrosomal duplication function) and/or gene by environment interactions (especially involving dietary intake) could be associated with the development of age-related acquired chromosomal instability.

In summary, we have shown that in males and females who are 40 years of age or older, the proportion of cells having MN that arise from a “trisomy rescue” or “corrective” mechanism is decreased compared to that seen in younger people, but the proportion of cells having multiple signals is increased. While it is clear that MN frequencies serve as an indication of DNA damage and/or imbalance, in some cases, particularly when seen in children/young adults, their presence may also represent a favorable “corrective” or protective response of that individual’s cells when challenged with an exposure/stress situation. Therefore, when analyzing MN frequencies, it may be informative to examine the contents of the binucleates, in addition to the MN, to facilitate the recognition of varying cellular responses to environmental or genotoxic exposures.

Acknowledgments

This study was completed through the cooperation of many generous individuals, most of whom are members of the Mid-Atlantic Twin Registry. This study was funded by a grant (CJC) from the National Institute of Environmental Health (R01 ES12074). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIEHS, NIH.

References

1. Fenech M, Holland N, Zeiger E, Chang WP, Burgaz S, Thomas P, Bolognesi C, Knasmueller S, Kirsch-Volders M, Bonassi S. The HUMN and HUMNxL international collaboration projects on human micronucleus assays in lymphocytes and buccal cells--past, present and future. *Mutagenesis*. 2011; 26:239–245. [PubMed: 21164208]
2. Bonassi S, El-Zein R, Bolognesi C, Fenech M. Micronuclei frequency in peripheral blood lymphocytes and cancer risk: evidence from human studies. *Mutagenesis*. 2011; 26:93–100. [PubMed: 21164188]
3. Migliore L, Coppede F, Fenech M, Thomas P. Association of micronucleus frequency with neurodegenerative diseases. *Mutagenesis*. 2011; 26:85–92. [PubMed: 21164187]
4. Andreassi MG, Barale R, Iozzo P, Picano E. The association of micronucleus frequency with obesity, diabetes and cardiovascular disease. *Mutagenesis*. 2011; 26:77–83. [PubMed: 21164186]
5. Murgia E, Maggini V, Barale R, Rossi AM. Micronuclei, genetic polymorphisms and cardiovascular disease mortality in a nested case-control study in Italy. *Mutat Res*. 2007; 621:113–118. [PubMed: 17448506]
6. Fenech M. Cytokinesis-block micronucleus assay evolves into a "cytome" assay of chromosomal instability, mitotic dysfunction and cell death. *Mutat Res*. 2006; 600:58–66. [PubMed: 16822529]
7. Battershill JM, Burnett K, Bull S. Factors affecting the incidence of genotoxicity biomarkers in peripheral blood lymphocytes: impact on design of biomonitoring studies. *Mutagenesis*. 2008; 23:423–437. [PubMed: 18678752]
8. Fenech M, Morley AA. The effect of donor age on spontaneous and induced micronuclei. *Mutat Res*. 1985; 148:99–105. [PubMed: 3918259]
9. Norppa H, Falck GC. What do human micronuclei contain? *Mutagenesis*. 2003; 18:221–233. [PubMed: 12714687]
10. Leach NT, Jackson-Cook C. The application of spectral karyotyping (SKY) and fluorescent in situ hybridization (FISH) technology to determine the chromosomal content(s) of micronuclei. *Mutat Res*. 2001; 495:11–19. [PubMed: 11448638]
11. Wojda A, Witt M. Manifestations of ageing at the cytogenetic level. *J Appl Genet*. 2003; 44:383–399. [PubMed: 12923314]

12. Lindberg HK, Falck GC, Jarventaus H, Norppa H. Characterization of chromosomes and chromosomal fragments in human lymphocyte micronuclei by telomeric and centromeric FISH. *Mutagenesis*. 2008; 23:371–376. [PubMed: 18502768]
13. Fenech M, Kirsch-Volders M, Natarajan AT, Surrallés J, Crott JW, Parry J, Norppa H, Eastmond DA, Tucker JD, Thomas P. Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. *Mutagenesis*. 2011; 26:125–132. [PubMed: 21164193]
14. Catalan J, Autio K, Kuosma E, Norppa H. Age-dependent inclusion of sex chromosomes in lymphocyte micronuclei of man. *Am J Hum Genet*. 1998; 63:1464–1472. [PubMed: 9792875]
15. Wojda A, Zietkiewicz E, Witt M. Effects of age and gender on micronucleus and chromosome nondisjunction frequencies in centenarians and younger subjects. *Mutagenesis*. 2007; 22:195–200. [PubMed: 17284771]
16. Song Y, Chen Q, Zhang Z, Hou H, Zhang D, Shi Q. Effects of age on segregation of the X and Y chromosomes in cultured lymphocytes from Chinese men. *J Genet Genomics*. 2009; 36:467–474. [PubMed: 19683669]
17. Holland N, Fucic A, Merlo DF, Sram R, Kirsch-Volders M. Micronuclei in neonates, children: effects of environmental, genetic, demographic and disease variables. *Mutagenesis*. 2011; 26:51–56. [PubMed: 21164182]
18. Guttenbach M, Schakowski R, Schmid M. Aneuploidy and ageing: sex chromosome exclusion into micronuclei. *Hum Genet*. 1994; 94:295–298. [PubMed: 8076947]
19. Hando JC, Nath J, Tucker TD. Sex chromosomes, micronuclei and aging in women. *Chromosoma*. 1994; 103:186–192. [PubMed: 7924621]
20. Nath J, Tucker JD, Hando JC. Y chromosome aneuploidy, micronuclei, kinetochores and aging in men. *Chromosoma*. 1995; 103:725–731. [PubMed: 7664620]
21. Kirsch-Volders M, Plas G, Elhajouji A, Lukamowicz M, Gonzalez L, Vande Loock K, Decordier I. The in vitro MN assay in 2011: origin, fate, biological significance, protocols, high throughput methodologies and toxicological relevance. *Arch Toxicol*. 2011; 85:873–899. [PubMed: 21537955]
22. Mateuca R, Lombaert N, Aka PV, Decordier I, Kirsch-Volders M. Chromosomal changes: induction, detection methods and applicability in human biomonitoring. *Biochimie*. 2006; 88:1515–1531. [PubMed: 16919864]
23. Utani K, Kawamoto JK, Shimizu N. Micronuclei bearing acentric extrachromosomal chromatin are transcriptionally competent and may perturb the cancer cell phenotype. *Mol Cancer Res*. 2007; 5:695–704. [PubMed: 17606478]
24. Jones KH, York TP, Juusola J, Ferreira-Gonzalez A, Maes HH, Jackson-Cook C. Genetic and environmental influences on spontaneous micronuclei frequencies in children and adults: a twin study. *Mutagenesis*. 2011; 26:745–752. [PubMed: 21765037]
25. Fenech M. The cytokinesis-block micronucleus technique: a detailed description of the method and its application to genotoxicity studies in human populations. *Mutat Res*. 1993; 285:35–44. [PubMed: 7678131]
26. Ceppi M, Gallo F, Bonassi S. Study design and statistical analysis of data in human population studies with the micronucleus assay. *Mutagenesis*. 2011; 26:247–252. [PubMed: 21164209]
27. Faraway J. Extending the linear model with R. Hall, RC., editor. R, CRC; New York, NY: 2006.
28. R Development Core Team, R. R Foundation for Statistical Computing; Vienna, Austria: 2010. A language and environment for statistical computing.
29. Mendenhall, W. Analysis of Enumerative Data. In: Mendenhall, W., editor. Introduction to Probability and Statistics. Duxbury Press; North Scituate, MA: 1975. p. 288–298.
30. Schmid M, Grunert D, Haaf T, Engel W. A direct demonstration of somatically paired heterochromatin of human chromosomes. *Cytogenet Cell Genet*. 1983; 36:554–561. [PubMed: 6196158]
31. Leach NT, Jackson-Cook C. Micronuclei with multiple copies of the X chromosome: do chromosomes replicate in micronuclei? *Mutat Res*. 2004; 554:89–94. [PubMed: 15450407]
32. Terradas M, Martin M, Tusell L, Genesca A. Genetic activities in micronuclei: is the DNA entrapped in micronuclei lost for the cell? *Mutat Res*. 2010; 705:60–67. [PubMed: 20307686]

33. Bakou K, Stephanou G, Andrianopoulos C, Demopoulos NA. Spontaneous and spindle poison-induced micronuclei and chromosome non-disjunction in cytokinesis-blocked lymphocytes from two age groups of women. *Mutagenesis*. 2002; 17:233–239. [PubMed: 11971995]
34. Jenkins EC, Schupf N, Genovese M, Ye LL, Kapell D, Canto B, Harris M, Devenny D, Lee JH, Brown WT. Increased low-level chromosome 21 mosaicism in older individuals with Down syndrome. *Am J Med Genet*. 1997; 68:147–151. [PubMed: 9028448]
35. Percy ME, Markovic VD, Dalton AJ, McLachlan DR, Berg JM, Rusk AC, Somerville MJ, Chodakowski B, Andrews DF. Age-associated chromosome 21 loss in Down syndrome: possible relevance to mosaicism and Alzheimer disease. *Am J Med Genet*. 1993; 45:584–588. [PubMed: 8456829]
36. Borsatto B, Smith A, Garcez EM, Peres CA. Age-associated mosaicism and polyploidy in Down's syndrome. *Mech Ageing Dev*. 1998; 100:77–83. [PubMed: 9509397]
37. Shi Q, Adler ID, Zhang J, Zhang X, Shan X, Martin R. Incidence of mosaic cell lines in vivo and malsegregation of chromosome 21 in lymphocytes in vitro of trisomy 21 patients: detection by fluorescence in situ hybridization on binucleated lymphocytes. *Hum Genet*. 2000; 106:29–35. [PubMed: 10982178]
38. Thomas P, Fenech M. Chromosome 17 and 21 aneuploidy in buccal cells is increased with ageing and in Alzheimer's disease. *Mutagenesis*. 2008; 23:57–65. [PubMed: 18048581]
39. Scarfi MR, Cossarizza A, Monti D, Bersani F, Zannotti M, Lioi MB, Franceschi C. Age-related increase of mitomycin C-induced micronuclei in lymphocytes from Down's syndrome subjects. *Mutat Res*. 1990; 237:247–252. [PubMed: 2150431]
40. Maluf SW, Erdtmann B. Genomic instability in Down syndrome and Fanconi anemia assessed by micronucleus analysis and single-cell gel electrophoresis. *Cancer Genet Cytogenet*. 2001; 124:71–75. [PubMed: 11165325]
41. Ferreira FL, Pra D, Martino-Roth MG, Garcias GL. Buccal micronucleus frequency is associated with age in Down syndrome. *Genet Mol Res*. 2009; 8:1231–1237. [PubMed: 19876863]
42. Herrera LA, Prada D, Andonegui MA, Duenas-Gonzalez A. The epigenetic origin of aneuploidy. *Curr Genomics*. 2008; 9:43–50. [PubMed: 19424483]
43. Surowy H, Rinckleb A, Luedeke M, Stuber M, Wecker A, Varga D, Maier C, Hoegel J, Vogel W. Heritability of baseline and induced micronucleus frequencies. *Mutagenesis*. 2011; 26:111–117. [PubMed: 21164191]
44. Dhillon VS, Thomas P, Iarmarcovai G, Kirsch-Volders M, Bonassi S, Fenech M. Genetic polymorphisms of genes involved in DNA repair and metabolism influence micronucleus frequencies in human peripheral blood lymphocytes. *Mutagenesis*. 2011; 26:33–42. [PubMed: 21164180]

Highlights

- Corrective mechanisms account for a higher portion of MN in cells from young people
- A higher portion of multi-signal MN/multiple MN are seen in cells from older people
- MN can reflect the optimal functioning of cells to correct chromosomal errors
- Scoring FISH patterns in MN and binucleates provides insight as to mechanisms

Figure 1. FISH patterns in cells having sex chromatin positive MN

The general types of FISH patterns seen in binucleates and their respective MN from females (top row) and males (rows 2–3) are shown schematically and include: (1) cells in which one binucleate has a hypodiploid complement as a result of the exclusion of a sex chromosome into the MN (column 1); (2) balanced (euploid) binucleates in which an apparent imbalance was “corrected” by exclusion of sex chromatin into the MN (column 2); (3) cells in which both binucleates have a hyperdiploid complement, with sex chromatin also being present in the MN (column 3); and (4) one hyperdiploid binucleate, along with a balanced (euploid) binucleate whose complement was “corrected” by the exclusion of sex chromatin into the MN (column 4). In the bottom row (row 4), representative images of binucleates (Y probe [red]; X probe [cyan]; 17 probe [green]) and their respective MN having the above noted patterns are shown for the Y chromosome, with the micronucleation in these cells resulting in either: loss of Y chromatin (column 1); correction of an imbalance in a binucleate through loss of Y chromatin (2 signals) into a MN (column 2); gain of Y chromatin in the cell, with both binucleates having 2 signals for the Y chromatin, along with the MN being Y chromatin positive (2 signals)(column 3); and correction of a hyperdiploid complement in one binucleate through the exclusion of Y chromatin (2 signals) into a MN (column 4).

Figure 2. FISH Patterns in cells having multiple signals for X or Y chromatin

In a normal male somatic cell (a), the X (aqua) and Y (red) chromosomes replicate, with the sister chromatids segregating at anaphase to give rise to two daughter cells that, at the completion of karyokinesis (bottom of diagram), each have a single sex chromosome. (b) If an extra Y chromosome (red) is present in the cell, after replication and segregation, one could anticipate seeing a total of four Y chromosome signals in the binucleates/MN as a result of this single error. In this example, one replicated Y chromosome does not align on the metaphase plate and does not establish spindle fiber attachment, leading to its exclusion into a MN. The extra chromosome in this cell may be present as a result of a malsegregational event during a previous parental cell division cycle, or it could possibly have occurred as a result of a replication (or other) error in this cell cycle. (c) An observation of 5 or more total cellular signals (binucleates plus MN) for the Y chromosome is indicative of the presence of 2 or more acquired changes that occurred for that cell. Thus, cells having 5 or more total signals (for the X or Y chromosome in males) were categorized as having “multiple” signals. In this example, the binucleates are balanced, but 3 Y chromatin signals are present in a single MN due to the misalignment and subsequent lack of spindle fiber attachment of either an extra chromosome and chromatid, or possibly the presence of a structurally altered dicentric chromosome, along with a chromatid (or other structurally altered Y chromosome) (dotted insert). (d) For X chromatin in a female, the observation of 7 or more total cellular signals (binucleates plus MN) would indicate that 2 or more acquired changes occurred for that cell. Therefore, binuclear/micronuclear X chromosome signal patterns of 7 or more were categorized as having “multiple” signals in females. In this example, the binucleates are balanced, but 3 X chromatin signals are present in a single MN due to the exclusion (following misalignment and lack of spindle fiber attachment) of an extra replicated chromosome, along with a chromatid (but this pattern could also reflect the presence of a structural alteration such as, but not limited to, a monocentric and dicentric chromosome). The most frequently observed patterns of multi-signal cells involving Y chromatin were those having 5 total signals as a result of either; (e) balanced binucleates, with a single MN having three signals; or (f) one binucleate having loss of a Y, and the presence of 2 MN having a total of 4 signals (3 and 1 as shown here; or 2 and 2). For females, the most frequently observed X chromatin patterns leading to multi-

signal cells were those having 7 signals due to either: (g) a loss of signal in one binucleate, with 4 signals being present in a single MN; or (h) a loss of signal in one binucleate with 4 signals being present in 2 MN (either 3 and 1 as shown here; or 2 and 2). The origin of these multi-signal cells is not known (indicated by ? shown by the parental cell). Those having multiple signals within one MN could reflect the presence of multiple errors within a single cell cycle, or the retention (and possibly replication) of a MN from a previous cycle. The cells having multiple signals with 2 or more MN could have arisen from multiple errors in a single cell cycle. Alternatively, they could reflect the retention (and possibly replication of chromatin) of a MN from a previous cycle, along with the acquisition of an additional MN from this cycle. In figures e-h, the potential complement of the parent cell is shown, along with the appearance of the cells at karyokinesis (K), following their exposure to Cytochalasin B. The time at which the additional changes occurred [either the Synthesis phase of the cell cycle (S) or during/following the mitotic division (M)] could not be unequivocally determined for these cells.

Figure 3. Multiple cellular signals for X or Y chromatin in binucleates and their MN

Although rare, cells having a greater number of signals than could result from the occurrence of a single segregational/replication error were seen in both males and females. The largest number of signals that was observed for the Y chromosome probe (red) was seen in a male who had a cell with a total of 8 signals (a) including one binucleate having 2 Y signals (left); one binucleate having 1 Y signal (right), and 3 MN having 2, 2, and 1 signal each for the Y probe. This cell had a balanced complement for the chromosome X (aqua) and 17 (green) probes. For the X chromosome (aqua), the greatest total number of cellular signals observed was 14, with this pattern being detected in a female. These binucleate/micronuclei signals (shown in b) were distributed as follows: 2 signals (euploid number) were present in each of the binucleates, with a total of 10 signals being present in 3 MN [6 signals in the top MN and 2 signals in the other 2 MN]. This cell had a balanced complement for the chromosome 17 (green) probe signals.

Figure 4. Proportion of cells having sex chromatin positive MN

Of the cells that were evaluated on the basis of having at least one MN, 19.9% were sex chromatin positive (X or Y; shown by black dotted segment of pie chart). The most frequent pattern observed in sex chromatin positive MN was for loss, followed by cells having corrective loss, multiple signals, corrective gains, gains, or other types of patterns.

Figure 5. Number of X or Y chromatin signals present in MN

The majority of MN having Y chromatin contained a single signal, but MN were observed that contained 2, 3, 4 or more signals (left histogram group). Similarly, MN containing X chromatin were observed that contained 1, 2, 3, or at least 4 signals in both males (center histograms) and females (right histograms).

Figure 6. The proportion of cells with a “corrective” cellular imbalance varies with age
 The percentage of cells (Y axis) having: (a) loss corrective; or (b) hyperdiploid corrective complements are shown for the X (males and females) and Y chromosomes (males) with respect to the age of the study participants (X axis). Correction of a cellular imbalance through loss of Y chromatin into a MN (i.e. trisomy rescue resulting in euploid binucleates) (upper left scatter plot) was inversely correlated with age ($p=0.022$). In contrast, no significant differences in the proportion of cells having hyperdiploid corrected complements for Y chromatin (upper right scatter plot) were observed with age. Also, no significant relationship between loss corrective (left center scatter plot) or hyperdiploid corrective (right center scatter plot) cellular patterns were observed for the X chromosome in males. For females, the proportion of cells having the correction of a cellular imbalance through loss of X chromatin into a MN (i.e. trisomy rescue resulting in euploid binucleates) significantly decreased with age (left, bottom scatter plot; $p=0.004$), but no significant relationship between the proportion of hyperdiploid corrected complements and age (right, bottom scatter plot) was detected ($p=0.454$).

Figure 7. The number of cells having multiple binucleate/micronuclear signals increased with age

The incidence of cells having multiple signals was low. However, a significantly higher number of multi-signal cells was observed in the older (40–80+) compared to younger (7–39) individuals ($p < 0.05$). This increase reflected gains for both the number of cells (Y axis) having multiple signals in a single MN (black), as well as cells having multiple signals as a result of multiple MN (gray). The total number of MN scored is also shown for each age group (note the scale shift on Y axis for the total number of cells scored compared to the number of cells having multiple signals).

Figure 8. Mechanisms leading to sex chromatin positive MN with age

In humans, sex chromatin positive MN are thought to be influenced by heritable genetic (confirmed effects indicated by solid arrows)^[24,43], environmental ^[21], and epigenetic influences (hypothesized influence, indicated by dotted arrow). In younger individuals (lower left of figure), a significant proportion of cells having MN may arise through the optimal functioning of checkpoints to “correct” for chromosomal errors, but a portion of cells having MN also result from chromosomal loss, as well as other mechanisms. As people age (lower right), the relative proportion of cells having sex chromatin positive MN due to “corrective” processes decreases, while those resulting from multi-signal processes increases. Also, with age, loss appears to be the most frequent mechanism leading to the presence of sex chromatin positive MN.

Table 1

Ages and Total MN Frequencies of Study Participants

Attributes of Study Participants	This study (n=59)	Parent Study (n=145) ^I
Age		
<u>Males</u>		
7–9	2	4
10–19	5	6
20–29	1	1
30–39	3	8
40–49	6	9
50–59	5	7
60–69	8	11
70–79	5	15
80+	<u>2</u>	<u>2</u>
Total	37	63
<u>Females</u>		
7–9	0	2
10–19	3	20
20–29	4	6
30–39	2	8
40–49	5	20
50–59	1	7
60–69	4	11
70–79	2	6
80+	<u>1</u>	<u>2</u>
Total	22	82
Mean Age (s.d.)	47.17 (22.46)	45.71 (23.07)
Age Range	7–85	7–85
Mean Total MN Frequency (s.d.)	15.48 (7.90)	12.36 (7.72)
Total MN Frequency Range	5–45	1–45

^I See Jones, et al., 2011 (24) for further information about the parent study.

Table 2

Cells having multiple signals for the pericentromeric probes localized to the X (males and females) or Y (males) chromosome(s).

Number and distribution of total cellular signals ¹	Number of Cells Having FISH Signal Pattern		
	X in Males ²	X in Females ²	Y in males ²
5 total cellular signals¹			
3 of 5 signals in single MN	1	-	5
3 of 5 signals in 2 or more MN ³	3	-	8
4 of 5 signals in single MN	3	-	4
4 of 5 signals in 2 or more MN	2	-	3
5 of 5 signals in 2 or more MN	-	-	3
6 total cellular signals			
3 of 6 signals in single MN	4	-	-
3 of 6 signals in 2 or more MN	-	-	1
4 of 6 signals in 2 or more MN	1	-	2
5 of 6 signals in single MN	-	-	1
5 of 6 signals in 2 or more MN	-	-	3
7 total cellular signals			
3 of 7 signals in single MN	-	4	-
3 of 7 signals in 2 or more MN	-	1	-
4 of 7 signals in single MN	-	3	-
4 of 7 signals in 2 or more MN	1	3	1
5 of 7 signals in 2 or more MN	2	-	1
6 of 7 signals in single MN	-	-	1
6 of 7 signals in 2 or more MN	2	-	-
7 of 7 signals in single MN	1	-	-
8 total cellular signals			
3 of 8 signals in 2 or more MN	-	2	-
4 of 8 signals in single MN	-	3	-
4 of 8 signals in 2 or more MN	-	1	-
5 of 8 signals in 2 or more MN	-	-	1
7 of 8 signals in 2 or more MN	-	-	1
8 of 8 signals in single MN	-	-	1
10 total cellular signals			
3 of 10 signals in single MN	-	1	-
8 of 10 signals in 2 or more MN	-	1	-
12 total cellular signals			-
3 of 12 signals in single MN	-	1	-
14 total cellular signals			-
10 of 12 signals in 2 or more MN	-	2	-

¹Total number of cellular signals was calculated by adding # signals in binucleate 1 + # signals in binucleate 2 + # signals in MN 1 (and if more than one MN was present the signals in each MN were added to the total).

²By definition, cells having fewer than 5 signals for males or 7 signals for females were not included in the multi-signal category

³Cells having 2, 3, 4, or more MN are pooled into this category of cells having 2 or more MN. For example: (1) a cell might have 5 total signals with 1 signal in each binucleate, and 1 signal in each of the 3 MN present; or (2) a cell could have 5 signals with 1 signal in each binucleate, 1 signal in one MN and 2 signals in a second MN.

Table 3

Comparison of signals present in micronuclei between studies.

Study	Age range (mean ± s.d.)	Total # MN scored	% of micronuclei with # of signals			P value ³
			1	2	3	
X chromosome						
<u>Males</u>						
Catalan et al. (n=10) ¹	21–29; 51–54 (36.78 ± 14.57)	107	62.6	37.4	0	
Current study (n= 14) ²	26–56 (40.46 ± 8.96)	66	51.5	40.9	7.6	0.010
<u>Females</u>						
Catalan et al. (n=4) ¹	26–29; 58–59 (37.99 ± 17.94)	167	61.1	34.7	4.2	
Current study (n=11) ²	25–49 (34.98 ± 10.81)	167	54.5	38.9	6.6	0.384
Y chromosome						
Catalan et al. (n=10) ¹	21–29; 51–54 (36.78 ± 14.57)	60	78.0	21.7	0	
Current study (n= 14) ²	26–56 (40.46 ± 8.96)	222	77.9	17.6	4.5	0.215

¹Data from Catalan, et al (1998)

²These subjects represent a subset of the total number of people evaluated in this study, being limited to those individuals who had ages in the same range as those evaluated by Catalan, et al.

³P values are from Contingency Chi-square tests (df=2) comparing data from the Catalan et al. and current studies for the number of sex chromosome signals present in males (X and Y) or females (X).