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Comparisons of Isogenic Trisomic and Disomic Cells from People with Mosaicism for Down Syndrome Unmask Cellular Differences Related to Trisomy 21

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

by

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Acknowledgements

I have many people to thank for their help and support during the preparation of this dissertation.

First, I want to thank Dr. Colleen Jackson-Cook for being a tremendous research advisor. I am so grateful to you for the opportunity to be a member of your research laboratory, and I am especially thankful that you assigned this research project to me. You have helped me to develop my skills as a scientist and have provided me with constant encouragement and scientific expertise. I think that the advisor-advisee relationship is essential to a doctoral student's success, and I am so appreciative of your excellent mentorship.

I would like to thank the members of my doctoral committee, Dr. Kellie Archer, Dr. Lynne Elmore, Dr. Rita Shiang, and Dr. John Quillin for their interest in this research project. I am grateful for the advice, help, and expertise that you all have provided to me throughout this process.

I would also like to acknowledge the VCU Genetic Counseling faculty for supporting my development as both a research scientist and a genetic counselor.

I am thankful for the encouragement of my undergraduate research advisor, the late Dr. Craig Kinsley, who inspired me to pursue a PhD.

To my friends, I am grateful to you all for the many discussions that we have had about navigating the early years of our careers.

Lastly, I wish to thank my family for their love and support while I pursue my dreams. I have my brother and sister to thank for many laughs along the way. To my dear parents, I cannot thank you enough for your confidence in me. You have inspired me to pursue a fulfilling, meaningful career. I could not have accomplished this without you both.

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Abstract

COMPARISONS OF ISOGENIC TRISOMIC AND DISOMIC CELLS FROM PEOPLE WITH MOSAICISM FOR DOWN SYNDROME UNMASK CELLULAR DIFFERENCES RELATED TO TRISOMY 21

By Kelly Ann Rafferty, BS

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

Virginia Commonwealth University, 2017

Major Director: Colleen Jackson-Cook, PhD
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It is known that age-related changes impacting multiple organ systems occur earlier in people with Down syndrome (Ds), but the biological basis underlying this trisomy 21-associated propensity for premature aging is poorly understood. Given that the trisomic/normal cells from people with mosaic Ds (mDs) are identical with regards to environmental exposures and genes (except for chromosome 21 copy number), comparisons of these isogenic trisomic/disomic cells allow one to “unmask” the cellular consequences of trisomy 21 by removing extraneous factors. The primary aim of this study was to determine if trisomy 21 results in an increase in the acquisition of age-related somatic chromosomal changes. To meet this aim, chromosome-specific telomere lengths, senescence-associated distension of satellites (SADS), and chromosomal instability frequencies were compared between the isogenic

trisomic/disomic cells of people with mDs ranging from 1 to 44 years of age. Chromosome-specific telomere lengths were quantified using a Q-FISH (pantelomeric probe) method. The average trisomic cell telomere length (3.609 mean, +/- 0.082 SE) was significantly less than the average disomic cell telomere length (3.888 +/- 0.083) ($n=28$; $p<0.0001$). SADS frequencies were quantified by scoring chromosome 8 alpha satellite heterochromatin (using FISH) from 100 interphase nuclei. The frequency of SADS was significantly greater in trisomic cells ($\mu=0.15$, +/- 0.02) compared to disomic cells ($\mu=0.10$, +/- 0.01) ($p=0.001$). Somatic chromosomal instability frequencies were quantified by scoring 1000 cells using the cytokinesis-block micronucleus assay coupled with FISH (RUNX1 probe to distinguish trisomic/euploid nuclei). In the younger participants (ages 1-12; $n=25$), no significant difference was observed in micronucleus frequencies in the disomic (0.14 ± 0.02) compared to trisomic (0.17 ± 0.02) cells ($p=0.091$), but in the older participants (ages 15-44; $n=13$), the trisomic binucleates showed a significant increase in the relative proportion of cells with micronuclei (0.34 ± 0.05) when compared to their euploid counterpart (0.16 ± 0.03) ($p<0.0001$). Collectively, these results suggest that the cellular effects related to aging in Ds/mDs arise from a “network” involving multiple acquired chromosomal findings. They also support the use of this isogenic mDs model system for providing new insight about cellular changes that arise from a trisomy 21 imbalance.

Chapter 1. Introduction

Epidemiology of Down Syndrome

Down syndrome (trisomy 21) (OMIM #190685) is the most common autosomal aneuploidy condition in humans. Down syndrome is present in 1/700-1/800 live births (Parker et al., 2010). There are an estimated 206,000 people with Down syndrome living in the US (de Graaf et al., 2017). This condition was first described in 1866 by Dr. J. Langdon Down based on his observation that 10% of his patients with intellectual disabilities bore a striking resemblance to one another and had similar clinical features (Down, 1866). Almost 100 years later, Dr. Jerome Lejeune discovered that trisomy 21 is the cause of Down syndrome (Lejeune et al., 1959). In people with Down syndrome, the 493 genes located on chromosome 21 (which comprises 1.5% of the genome) are present in three copies.

Trisomy 21 is caused by the malsegregation of chromosome 21 in a gamete during meiosis. About 80% of trisomy 21 conceptions are spontaneously aborted (Freeman et al., 1991). An error in chromosomal segregation in the oocyte at meiosis I is the most common origin of trisomy 21, although the error can also occur during maternal meiosis II or either stage of meiosis in the spermatocyte (Hassold et al., 2001. Through the genotyping of polymorphic markers in a large population study, Freeman et al., (2007) showed that over 90% of the extra chromosomes in people with trisomy 21 were maternal in origin (Freeman et al., 2007; Sherman et al., 2007).

There have been many studies to determine risk factors for Down syndrome. The greatest association is with advanced maternal age (Sherman et al., 2007). The maternal age effect is limited to cases of Down syndrome caused by oocyte nondisjunction errors, which comprise the majority (Sherman et al., 2005). The extended timeline of oogenesis, which spans from fetal development through fertilization, is one factor that has been speculated to contribute to the maternal age effect.

Another factor that has been hypothesized to contribute to the maternal age effect is the biological aging of the ovary. It has been considered that women with aneuploid pregnancies may have reduced oocyte pools. Kline et al. (2004) directly tested this hypothesis by comparing levels of three ovarian-associated hormones and the number of antral follicles in the ovary between women with trisomic spontaneous abortions, women with other types of chromosomally abnormal spontaneous abortions, women with chromosomally normal losses, and mothers of normal livebirths. They did not find trisomy-related variability in these measures. Warburton (2005) reviewed the evidence for the biological ovarian aging hypothesis and surmised that the data are inconsistent and that there is a need for epidemiological reproduction studies in women with premature ovarian failure in order to test this hypothesis.

Another risk factor for nondisjunction of chromosome 21 is an altered recombination pattern, with perturbations in both the frequency and location of the recombinant events being observed for the chromosomes 21 from people with Down syndrome when compared to euploid controls (Warren et al., 1987; Sherman et al., 1991; Lamb et al., 2005). In addition to biological events, many environmental

influences have been investigated as possible risk factors for Down syndrome. While environmental factors have been clearly associated with aneuploidy in animal models (Hunt et al., 2003), to date, none have conclusively been identified in humans (Sherman et al., 2007).

Down syndrome can be diagnosed prenatally or postnatally. The use of prenatal screening to identify pregnancies at an increased risk for Down syndrome includes detection of ultrasound markers, analyte-based serum screens in the first or second trimester, and cell-free fetal DNA (cffDNA) screening. Since its introduction to clinical practice in 2011, the cffDNA-based Non-Invasive Prenatal Screen (NIPS) has become a popular option due to its superior sensitivity and lower false-positive rate than analyte-based serum screens. Although these screening methods can identify pregnancies that are likely to have trisomy 21, a prenatal diagnosis can only be made by fetal karyotyping achieved through invasive procedures including amniocentesis or chorionic villus sampling. In cases where Down syndrome is suspected postnatally, the diagnosis is usually confirmed with GTG-banding karyotyping in blood.

Cytogenetically, Down syndrome can result from either: (1) primary trisomy (3 separate chromosomes 21); (2) secondary trisomy (where the trisomic imbalance results from either an isochromosome, of 21q, or a Robertsonian translocation between two chromosomes 21); (3) tertiary trisomy (where the trisomic imbalance is due to a reciprocal or Robertsonian translocation involving at least one chromosome that is not 21 [e.g. a rob(13;21)(q10;q10)]); or (4) mosaicism (2 or more types of cells, where one of the cell lines has a trisomic imbalance for chromosome 21). These types of Down syndrome can be distinguished with GTG-banding karyotyping. Approximately 95% of

individuals with Down syndrome have primary trisomy 21, and have a third copy of chromosome 21 in every cell. Secondary and tertiary trisomy 21 collectively account for 2-4% of people with Down syndrome. The most common type of rearrangement in translocation Down syndrome is a Robertsonian translocation between chromosomes 21 and 14. Down syndrome due to secondary or tertiary trisomy 21 can arise from the malsegregation of chromosomes in the gamete of a parent who carries a balanced translocation, or it can be caused by a de novo event. Mosaic Down syndrome also accounts for 2-4% of patients with Down syndrome. Mosaicism is defined as the presence of 2 or more cell lines that originate from a single gamete (Thompson and Thompson). Thus, an individual who has mosaicism for trisomy 21 has at least one cell line with a trisomic imbalance for chromosome 21. Mosaicism for Down syndrome is discussed in further detail later in this chapter.

Natural History of Down Syndrome

Dysmorphology

More than 80 dysmorphic traits have been described in people with Down syndrome (Epstein et al., 2001). The most common craniofacial abnormalities include brachycephaly, upslanting palpebral fissures, epicanthal folds, Brushfield spots, low nasal bridge, and overfolding of the upper helix of the ear (Jones, 2006). Additional dysmorphic traits include dental hypoplasia, short metacarpals and phalanges of the hands, single transverse palmar crease, and wide gap between first and second toes (Jones, 2006).

Neurological Phenotypes

Down syndrome is the most common genetic cause of intellectual disability (Daunhauer et al., 2014). There is variability in the cognitive skills of individuals with Down syndrome, and most have intelligence quotients that fall into the moderate to severe range of intellectual disability (Nelson et al., 2005; Lott and Dierssen, 2010). Specific deficits in cognitive processes have been described, including those in processing speed (Pennington et al., 2003; Vicari et al., 2004), language development (Abbeduto et al., 2008; Guralnick et al., 2002), working memory (Jarrold et al., 2002), problem solving (Lanfranchi et al., 2010) and attention (Costanzo et al., 2013). There is controversy over whether people with Down syndrome have deficits in visual and spatial skills, as this appears to be a comparative strength (Couzens et al., 2011; Yang et al., 2014; Silverman 2007).

Approximately 5-13% of children with Down syndrome have seizures (Lott, 2012). Seizure onset has a bimodal distribution and typically begins either in the first year of life or after age 30. Those with dementia are especially susceptible to seizures, which occur in 84% of this subgroup of people with Down syndrome (De Simone et al., 2010).

Psychiatric and Behavioral Characteristics

People with Down syndrome display atypical behaviors. Children with Down syndrome can be more socially motivated than typically developing children (Kasari et al., 2003). Parents and educators often report that stubbornness is an issue (Kasari and

Freeman, 2001). Disruptive, externalizing behaviors are more common at younger ages in children with Down syndrome (Capone et al., 2006). In contrast, following the transition into adulthood, people with Down syndrome are more likely to experience problems related to internalizing, such as depression and anxiety (Dykens, 2007; Foley et al., 2015). The reported prevalence of depression in people with Down syndrome ranges from 0-11.1% (Walker et al., 2011). Smith (2001) suggested that the observed behavioral problems in people with Down syndrome are sometimes the manifestation of an underlying medical issue.

Cardiological Abnormalities

About half of children with Down syndrome are born with a congenital heart defect, most commonly atrioventricular septal defect, ventricular septal defect, and atrial septal defect (Freeman et al., 1998; Bergström et al., 2016). An echocardiogram is usually completed shortly after birth for an infant with Down syndrome to ensure that any heart problems that might be present are detected and clinically managed.

Hearing and Vision Problems

Hearing and ophthalmologic exams are important evaluations in newborns with Down syndrome. The prevalence of hearing loss is estimated to be between 38% and 78% (Roizen et al., 1993; Balkany et al., 1979). This hearing loss can be conductive, sensorineural, or a combination (Roizen et al., 1993). Additionally, otitis media affects about 60% of children (Maris et al., 2014).

Ophthalmologic abnormalities are common in children with Down syndrome, including congenital cataracts, congenital glaucoma, refractive errors, strabismus, and nystagmus (Catalano 1990; Creavin and Brown, 2009). The frequency of ophthalmologic conditions increases with age (Roizen and Patterson, 2003).

Cancer

Hematological disorders and leukemia occur with greater frequency in children with Down syndrome, with 3-10% of newborns having transient myeloproliferative disease (Zwaan et al., 2010), nearly all of whom have a somatic mutation of the GATA1 gene (Vyas et al., 2007). Most cases are resolved by spontaneous remission, but 15-20% result in death (Klusmann et al., 2008; Malinge et al., 2009; Massey et al., 2006; Muramatsu et al., 2008). There is a 10-20 fold increase in acute myeloid leukemia (AML) and acute lymphocytic leukemia (ALL) in children with Down syndrome (Xavier et al., 2010). AML has an atypical presentation in those with Down syndrome but ALL has a classical presentation (Bruwier and Chantrain, 2012). The cure rate for ALL in people with Down syndrome is lower than that seen in people without Down syndrome due to treatment-related mortality (O'Rafferty et al., 2015).

Despite having factors that predispose to tumorigenesis, such as oxidative stress, chromosomal instability, and immunodeficiency, there is a low incidence of solid tumors in people with Down syndrome (Nižetić and Groet, 2012). It has been hypothesized that cells with trisomy 21 could have tumor suppressive activity that contributes to this low incidence (Nižetić and Groet, 2012). The reduced rate of solid tumors could also reflect epigenetic alterations that arise due a trisomic imbalance for

chromosome 21. A particularly interesting ‘Polycomb-opathy’ associated with trisomy 21 was identified by Adorno, et al. (2013). This epigenetic change results in a reduction of the cellular stress response threshold needed to signal the initiation of stem cell senescence and arises due to an increase in the expression of the CDKN2A gene, which has been associated with a decreased susceptibility to atherosclerosis and solid tumors, as well as an increased propensity for immunodeficiency (with each of these 3 traits being findings that are associated with Down syndrome) (Adorno, et al., 2013). However, the lower incidence of solid tumors could also reflect, at least in part, the diminished exposure that people with Down syndrome have to environment agents associated with some solid tumors (e.g. tobacco use; workplace toxins, etc).

Immunological Dysfunction

Immune system dysfunction is a defining feature of Down syndrome. About 15% of patients have autoimmune thyroid disease (American Academy of Pediatrics, Committee on Genetics, 2001). This can be hypothyroidism, or less frequently hyperthyroidism. Thyroid screening is a routine part of care for patients with Down syndrome. Another autoimmune condition with an increased prevalence in people with Down syndrome is celiac disease (Marild et al., 2013; Sharr et al., 2016). Also, respiratory tract infections occur more frequently in people with Down syndrome due to intrinsic abnormalities in the immune response, such as reduced thymus size and T lymphocytopenia (Kusters et al., 2009; Ram and Chinen, 2011).

Obstructive Sleep Apnea

Sleep apnea is a common condition in children and adults with Down syndrome (Ng and Chan, 2004). Due to abnormalities in craniofacial development in those with Down syndrome, airflow through the upper respiratory tract is compromised (Chin et al., 2014). Another potential contributing factor to the increased risk for people with Down syndrome to develop sleep apnea is obesity. The obesity rate is higher in people with Down syndrome (Basil et al., 2016). The acquisition of sleep apnea has been associated with depression and poorer cognition and can be a confounding co-morbid factor for assessment of health problems in adults with Down syndrome (Chen et al., 2013; Breslin et al., 2014; Capone et al., 2013).

Gastrointestinal

Disorders of the gastrointestinal tract are common. Intestinal atresias affect about 6-7% of people with Down syndrome (Stoll et al., 1998; Frid et al., 1999). The incidence of Hirschsprung's disease is 2% (Holmes, 2014), compared to 0.02% of people in the general population (Goldberg, 1984). Other frequent gastrointestinal symptoms including diarrhea, constipation, gastroesophageal reflux, and abdominal pain are frequently reported (Holmes, 2014).

Musculoskeletal Abnormalities

At birth, newborns with Down syndrome have hypotonia (Lott, 2012). Cervical spine abnormalities are more frequent in people with Down syndrome than in the chromosomally typical population (Ali et al., 2006). About 15% of patients have atlantoaxial instability (Pueschel and Scola, 1987). Although this is usually asymptomatic, it can present as spinal cord depression (Ali et al., 2006). Additionally, spontaneous hip dislocation occurs in 5% of children in the first decade of life (Bennet et al., 1982).

Growth and Reproductive Issues

The birthweight of newborns with Down syndrome is lower than in euploid children (Zemel et al., 2015). Special growth charts are used to assess the growth of children with Down syndrome since they have a slower growth trajectory (Zemel et al., 2015).

Hypogonadism in males with Down syndrome has been thought to result in subfertility or sterility (Zuhlke et al., 1994). However, there have been several case reports of men with Down syndrome fathering euploid children with and without assisted reproductive technology (Aghajanova et al., 2015; Sheridan et al., 1989; Pradhan et al., 2006). In women with Down syndrome, menarche occurs in the typical age range but menopause happens earlier than in the euploid population. Women with Down syndrome are usually fertile. In a review of case reports about mothers with Down syndrome, Zhu et al., (2013) found that 10 out of the 28 children described in these case studies had Down syndrome.

Quality of Life and Social Issues

In a survey on parental attitudes toward children with Down syndrome, most respondents reported that having a child with Down syndrome improved their outlook on life (Skotko et al., 2011a). When surveyed about their own self-perceptions about living with Down syndrome, 97% of participants with Down syndrome reported that they are happy with their lives (Skotko et al., 2011b).

Over the past decade there has been a movement in favor of the inclusion of children with Down syndrome in general education as opposed to special education (Buckley et al., 2006). Despite these efforts to include children with Down syndrome, social connectedness remains a challenge. While adolescents with Down syndrome interact with their peers at school, these friendships are often limited to the classroom environment (D'Haem, 2008).

In addition to the educational integration of people with Down syndrome, there are increasing opportunities for them to become active community members as adults. In the US, high school students with Down syndrome develop a transition plan as part of their Individualized Education Program (IEP). This helps them to plan for post-secondary education, housing, and employment. The results of a recent study about the employment of people with Down syndrome in the US indicated that 56.6% were employed in a paying job and 25.8% were working in a volunteer capacity (Kumin and Schoenbrodt, 2016). People with Down syndrome sometimes reside in group homes, but can also live independently with limited support (Woodman et al., 2014). The outcome of a Danish study of social conditions for people with Down syndrome showed that 1.5% of participants were married and about 1% had a child (Zhu et al., 2013).

Premature Aging

The life expectancy of people with Down syndrome is currently 57.8 in females and 61.1 in males (Bittles and Glasson, 2004). Although people with Down syndrome today are living to older ages than in any previous generation, they experience a premature, pathogenic aging process. Symptoms of premature biological aging are evident in most body systems. The typical presentations of several age-related changes in people with Down syndrome are summarized in Table 1. Some of these changes include alopecia, immune system abnormalities, osteoporosis, menopause, obstructive sleep apnea, hearing loss, cataracts, and Alzheimer's disease (Zigman, 2013).

Between the ages of 45 and 60, Alzheimer's disease prevalence doubles every 5 years in people with Down syndrome (Coppus et al., 2006). Contrary to previous reports that all older adults with Down syndrome develop dementia, recent evidence suggests that Alzheimer's disease is common but not ubiquitous (Franceschi et al., 1990; Head et al., 2007; Krinsky-McHale et al., 2008). About one-third of adults with Down syndrome in the 55-59 age range have a diagnosis of Alzheimer's disease (Coppus et al., 2006). Personality changes, behavioral changes, and depression often precede the onset of dementia in adults with Down syndrome (Burt et al., 1992; Urv et al., 2010; Adams and Oliver, 2010).

The two cardinal brain lesions that define Alzheimer's disease neuropathology are plaques of amyloid beta protein in the cerebral cortex and neurofibrillary tangles in individual neurons (Perl, 2010). These neuropathological findings can be present in the brains of people with Down syndrome decades prior to the onset of recognized dementia (Hartley et al., 2015; Hyman, 1995; Lott, 2001; Head, 2012). The results of

postmortem studies have indicated that amyloid beta is deposited by age 40 and has been observed as young as age 12 (Lemere et al., 1996). This is likely influenced by the overexpression of the gene encoding amyloid precursor protein (APP) in trisomic cells since the APP gene is located on chromosome 21 (Prasher et al., 1998; Zigman, 2013). In agreement with the Lemere team's findings, Hamlett, et al. (2016) detected significantly increased levels of amyloid-β peptides and phosphorylated tau in neuronal exosomes of people with Down syndrome, with this effect being seen in even their youngest study participants, who were 8 years old. The similarities in neuropathology between Alzheimer's disease and Down syndrome have led to the hypothesis that the mechanisms underlying the neurological abnormalities in both conditions could be similar (Hartley et al., 2015).

Recognition of age-related health conditions based on clinical traits in people with Down syndrome can be challenging for several reasons. Firstly, impaired cognitive and communication skills can mask the onset of age-related decline. Secondly, medical problems that are common in Down syndrome (including depression, thyroid abnormalities, and sleep apnea), can mimic the symptoms of age-related decline (Wark et al., 2014; Breslin et al., 2014; Prasher, 1999). Third, in the case of dementia, obtaining the correct diagnosis is complicated by the fact that many people with Down syndrome never develop the skills that are used in assessment tools as determinants of decline (Krinsky-McHale and Silverman, 2013).

For these reasons, finding reliable biomarkers that could serve as indicators of pathological aging in people with Down syndrome has the potential to improve their quality of care. However, knowledge of the factors contributing to premature aging in

Down syndrome is incomplete. There is need for a better understanding of the acquired clinical findings that contribute to the premature aging. Also, it is important to improve our knowledge of the acquired changes that occur in trisomic cells compared to euploid cells as a result of the trisomic imbalance. Importantly, the recognition of the cellular changes that arise as a consequence of a trisomic imbalance could aid in our understanding of the etiologies of several of these age-related health conditions.

Table 1. Presentation of age-related phenotypes in people with Down syndrome versus adults with euploid chromosomal complements .

Phenotype	Presentation in Down syndrome	Typical Presentation
Gray hair Daneshpazhooh et al., (2007); Schnohr et al., (1995)	14% in sample of 100 ages 3-20	Total gray hair <1% 30-39
Alopecia Daneshpazhooh et al., (2007)	10%	0.1%
Menopause Schupf et al., (1997); Ejskjaer et al., (2006); Blake (2006)	Likelihood of menopause 2x as high at age 40	Mean age 51.3 years
Cataracts Krinsky-McHale et al., (2012)	37.8% ages 40-49 years	2.5% ages 40-49
Hearing loss, moderate Van Buggenhout et al., (1999), Cruickshanks et al., 1998)	70% ages 50-59	33% ages 61-70
Osteoporosis Srikanth et al., (2011)	50% all adults	9% adults over age 50
Immune system dysfunction Ram (2011)	T and B cell lymphopenia, susceptibility to autoimmune disease	Less prevalent
Alzheimer's disease Coppus et al., (2006); Alzheimer's Association (2016); Hebert et al., (2013)	Prevalence doubles every 5 years from age 45-60: 8.9% age 45-49 17.7% age 50-54 32.1% age 55-59	11% age 65 and older 32% age 85 and older

The Cascade of Multiple Biological Changes Contributing to Premature Aging in Down Syndrome

The cascade of changes contributing to premature aging associated with trisomy 21 remains largely unknown, but is anticipated to show parallels to the biological mechanisms mediating aging in the general population due to overlaps in their aging phenotypes. Numerous biological changes that are associated with typical aging are also involved in the etiology of Down syndrome. In the overall population, epigenetic mechanisms, including histone modifications, changes to DNA methylation patterns, and chromatin remodeling, have been linked to human aging (Sen et al., 2016). In trisomic cells, the overexpression of several methylation pathway genes on chromosome 21 could have trans-acting effects on DNA methylation states in downstream target genes on other chromosomes (Do et al., 2017). However, little is known about the relationship between acquired methylation or other epigenetic changes and the propensity for premature aging in people with trisomy 21. Several mitochondrial abnormalities have been identified in cells with trisomy 21, including an increased frequency of somatic mitochondrial DNA mutations and mitochondrial dysfunction in neuronal cells, fibroblasts, blood, and urine (Jovanovic et al., 1998; Coskun et al., 2010; `Lott, 2012). Mitochondrial dysfunction contributes to the accumulation of reactive oxygen species, which, in turn, is thought to result in a chronic pro-oxidative state in the cells of people with Down syndrome (Lott, 2012). Helguera et al., (2013) suggest that mitochondrial downregulation is an adaptive response to oxidative stress, but contributes to conditions involving disruptions in energy metabolism. Oxidative stress is a driving force behind the mechanism underlying many of the acquired phenotypes seen in people with Down syndrome, including cognitive decline, the accumulation of

beta-amyloid plaques, and Alzheimer's disease. Telomere shortening is a classic hallmark of aging in the general population. The number of studies of telomeres in people with Down syndrome across their lifespan is very limited, but the investigators who have evaluated telomere lengths in adults with trisomy 21 have observed shorter average telomere lengths than in unaffected individuals (Vaziri et al., 1993). Similarly, increases in chromosomal instability are associated with aging in the general population, but there is a paucity of reports of the frequency of chromosomal instability in people with Down syndrome; especially as it relates to their propensity for premature aging (Thomas et al., 2008; Ferreira et al., 2009). Collectively, each of these cellular attributes are likely to contribute to chronic inflammation and immune system abnormalities, which are two key phenotypic findings that have consistently been observed with aging in people with Down syndrome (Wilcock and Griffin, 2013; Ram and Chinen, 2011).

Acquired Chromosomal Changes and Premature Aging

Considering this cascade of biological changes that contributes to premature aging in people with Down syndrome, we hypothesize that acquired chromosomal changes are likely to contribute to the acquired health conditions and premature aging seen in people with Down syndrome.

The association between telomere shortening and cellular aging has been well established in euploid cells (reviewed in Blasko, 2005), but there is a paucity of studies evaluating telomere lengths in people with Down syndrome. When compared to healthy, age-matched controls, Vaziri, et al. (1993) observed shorter telomeres in their probands

who had Down syndrome. Interestingly, Jenkins et al. (2010) observed that people with Down syndrome who have mild cognitive impairment have shorter telomeres compared to people with Down syndrome who do not have early-stage cognitive impairment.

Another cytogenetic attribute that has been associated with aging in euploid cells is an alteration in heterochromatin structure. Senescence-associated distension of satellites (SADS) is a recently described biological phenomenon in senescent cells characterized by the distension of the pericentromeric (α-sat) and satellite II (sat II) sequences of chromosomes (Swanson et al., 2013). This change in the chromatin compaction of the centromeric regions has been observed in cells during early-stage senescence. To date, there has not been a reported investigation of SADS in the cells of people with Down syndrome. However, like telomere shortening, this cellular attribute is an indicator of the senescence progression.

Acquired chromosomal instability represents another subset of biomarkers associated with aging in the general population. In the few previous studies of this biomarker in people with Down syndrome, investigators have observed increased somatic cell micronucleus frequencies in the buccal mucosa cells (Thomas et al., 2008; Ferreira et al., 2009) and mitomycin-treated lymphocytes (Scarfi et al., 1990) of people with Down syndrome compared to the levels seen in healthy, age-matched controls. In contrast, Maluf and Erdtmann (2001) found no difference in micronucleus frequency between cells from people with Down syndrome and cells from healthy control participants. However, their participants with Down syndrome were significantly younger than their control participants ($p=0.005$). Overall, genetic and environmental background differences between the participants in the trisomic and control groups have limited the

investigators' ability to attribute the observed changes to influences directly reflective of a trisomic imbalance (Jones et al., 2011).

Mosaic Down syndrome: An isogenic trisomic-disomic model system

Interpersonal background genetic and environmental variation observed for the above noted cytogenetic attributes (as well as most biomarkers and phenotypic traits) presents a significant challenge for determining trisomy 21-specific relationships of cellular alterations. One approach to “unmask” the effects of a trisomic imbalance is to study people with mosaicism since they have both trisomic and normal cells that differ only for the presence of an additional chromosome 21 (Davidsson, 2014). Importantly, this “mosaic” study design approach not only removes the confounding effects of inter-individual differences due to total genetic make-up, but also controls for the effects of environmental influences, since the trisomic and normal cells in people with mosaicism share identical exposure histories (Figure 1).

Mosaic Down syndrome can result from two primary mechanisms. In the majority of cases, there is a meiotic malsegregational event that leads to a trisomy 21 zygote (Pangalos et al., 1994; Papavasiliou et al., 2009). This is followed by a mitotic error involving chromosome 21 during one of the early somatic divisions in the developing zygote. The mitotic error can be a nondisjunctional event or an anaphase lagging occurrence, both of which can result in the loss of one chromosome 21 in that cell (Papavassiliou et al., 2014). This mitotic error gives rise to a disomic cell, which, through proliferation, results in a disomic cell line. Less frequently, mosaic Down syndrome can arise from a single nondisjunctional error of chromosome 21 in an initially euploid zygote during an early mitotic division (Papavasiliou et al., 2009).

Mosaic Down syndrome encompasses the same spectrum of physical stigmata as non-mosaic Down syndrome, but patients may have fewer phenotypic findings.

Papavasiliou et al., (2009) reported an association between a low percentage of trisomic cells and few overall phenotypic findings. They also determined that the percentage of trisomic cells in buccal mucosa is inversely correlated with IQ score. Similarly, a high proportion of trisomic lymphocytes was found to be associated with the incidence of congenital heart defects (Papavasiliou et al., 2009). In addition to having a less severe phenotype than non-mosaic trisomy 21, those with mosaicism may have fewer social differences from the typically developing population. People with mosaic Down syndrome are more likely than those with non-mosaic Down syndrome to complete post-secondary education, work full-time, marry, and parent a child (Zhu et al., 2014).

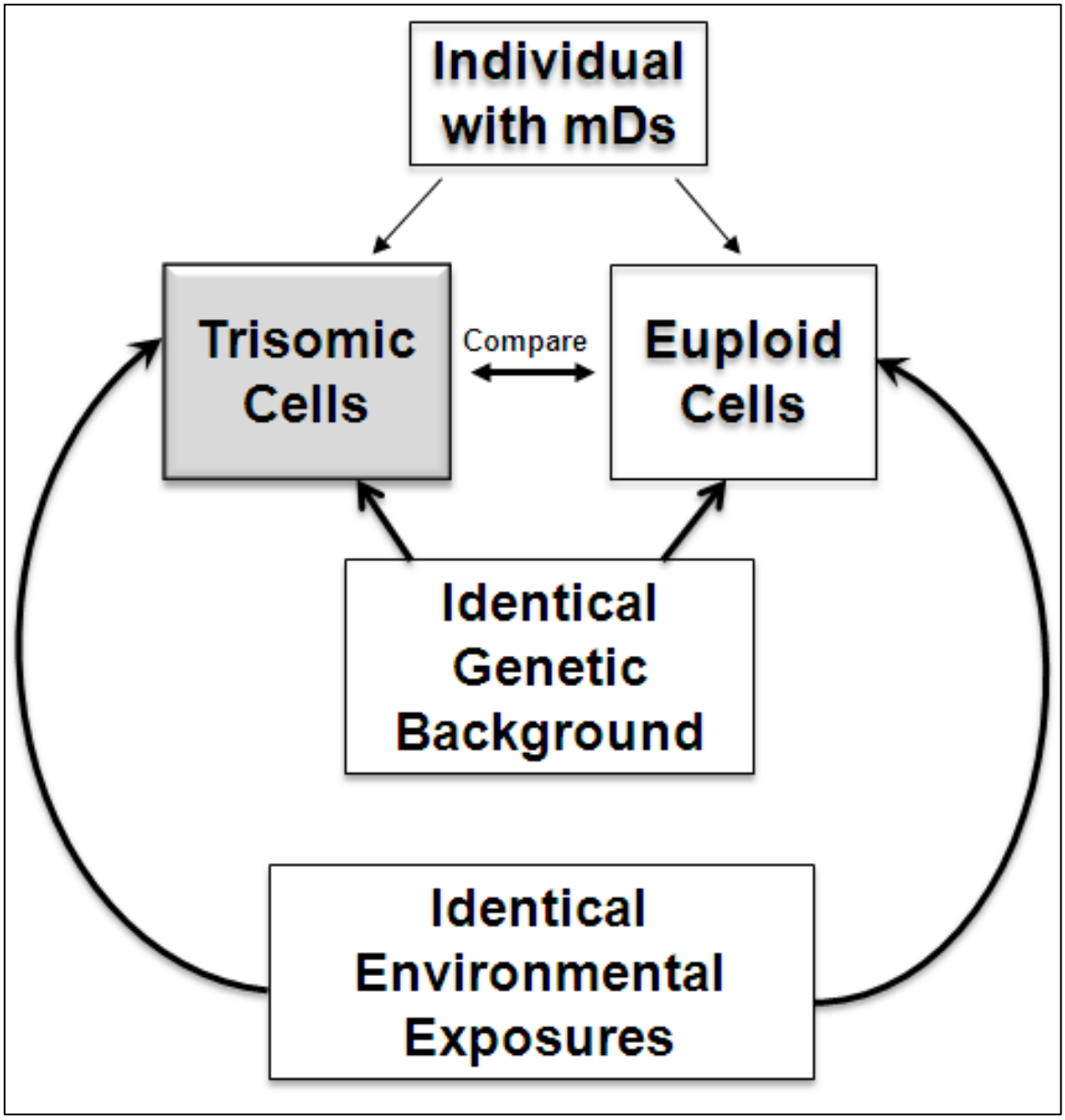

Figure 1. Isogenic trisomic-disomic mosaic Down syndrome study design. An individual with mosaicism for trisomy 21 has both trisomic and disomic cells as a constitutional finding. These trisomic and disomic cells have identical genetic backgrounds (except for the trisomy 21 imbalance) and identical environmental exposures. By measuring trisomic compared to disomic cellular attributes, individual variation is eliminated to allow for direct assessments of the trisomy 21-specific influence(s).

Purpose of Study

Based on the scientific premises reviewed above, we hypothesize that: acquired chromosomal changes, including telomere shortening, senescence-associated distension of satellites, and chromosomal instability, contribute to the acquired health conditions and premature aging seen in people with Down syndrome.

The three primary aims of this study were:

1. Determine if there are differences in chromosome-specific telomere lengths between isogenic trisomic and disomic cells, and assess whether telomere length is influenced by age in the trisomic and/or disomic cells.
2. Determine if there are differences in the frequencies of senescence-associated distension of satellites between isogenic trisomic and disomic cells, and assess whether SADS frequencies are influenced by age in the trisomic and/or disomic cells.
3. Determine if there are differences in the somatic chromosomal instability frequencies between isogenic trisomic and disomic cells, and determine if chromosomal instability frequencies are influenced by age in the trisomic and/or disomic cells.

To our knowledge, this is the first study of these three acquired chromosomal changes in individuals with mosaicism for trisomy 21, and also the first to directly compare these cytogenetic attributes in isogenic trisomic and disomic cells within an individual using a mosaic model system/study design.

Chapter 2. Telomere length assessment in isogenic trisomic and disomic cells from people with mosaic Down syndrome

Introduction

Since the discovery of telomeres 76 to 79 years ago (Muller, 1938; McClintock, 1941), our understanding of this chromosomal structure, and its association with numerous health conditions, has greatly expanded (Starkweather et al., 2014). The term “telomere” was derived from the Greek words “telos” (which means “end”) and “meros” (which means “part”). As the name implies, telomeres are located at the tips of chromosomes and are required to maintain chromosomal structural integrity (de Lange, 2002). Telomere shortening has also been recognized as one of the primary hallmarks of aging cells (López-Otín et al., 2013). A portion of individuals who have pronounced telomere shortening and/or defects in genes related to telomere length maintenance/replication have been shown to have conditions that are collectively called “telomeropathies” (reviewed by Opresko and Shay, 2017). A common trait observed in most people with a telomeropathy is premature aging. Telomere shortening has also been associated with inflammation (reviewed by Zhang et al., 2016). Given that people with Down syndrome have been noted to have alterations related to inflammation, as well as premature aging, it follows that a trisomy 21 imbalance may be related to early or accelerated telomere atrophy. To test this hypothesis, we compared chromosome-specific telomere lengths in the isogenic trisomic and disomic cultured lymphocytes from people with mosaicism for Down syndrome.

Review of Telomere Attrition in People with Normal Chromosomal Complements

As noted above, telomeres are essential to the maintenance of genomic stability in human cells. These structures, which are located at the ends of chromosomes, consist of highly-conserved, double-stranded TTAGGG repeat sequences. In human cells, the range of normal telomere length is from 5-15 kb (Samassekou et al., 2010). In the absence of telomeres, the natural ends of chromosomes would activate DNA repair mechanisms and result in deleterious chromosome fusions (O'Sullivan and Karlseder, 2010).

Telomere lengths vary widely between individuals. The results of several twin pair studies have suggested that telomere length is influenced by genetic and shared environmental factors (Slagboom et al., 1994; Andrew et al., 2006), while other twin study outcomes have indicated that environmental influences are less important than heritable contributions (Graakjaer et al., 2004). Several loci that influence telomere length have been mapped through human linkage studies (Vasa-Nicoterra et al., 2005; Andrew et al., 2006). In their review of telomere length inheritance, Kappei and Londono-Vallejo (2008) discuss the possibility that inherited polymorphisms in genes which control telomerase expression, such as the genes/promoters of the telomerase or shelterin complexes, could contribute to telomere length differences between individuals. Tedone et al. (2014) compared the telomere lengths in offspring of centenarians and offspring of parents who died at the typical lifespan. They observed that the offspring of centenarians had significantly longer telomeres than control offspring, which supports the concept of telomere length heritability.

An association between cellular aging and telomere shortening was first demonstrated in human fibroblasts and lymphocytes (Harley et al., 1990; Allsopp et al., 1992; Vaziri et al., 1993). With each cellular division, the telomeres lose 40-200 base pairs in length (Harley et al., 1990; Counter et al., 1992). Eventually, they become critically short, resulting in replicative senescence (Levy et al., 1992).

The relationship between telomere shortening and human chronological age has been studied extensively. Cooke and Smith (1986) found that the telomeres in sperm cells were longer than those in adult somatic cells. In a twin study with three age groups, Slagboom et al., (1994) observed that donor age was inversely correlated with telomere length. Shortened telomeres have been observed in cells from people with various age-related health problems, including Alzheimer's disease (Panossian et al., 2003), ulcerative colitis (O'Sullivan et al., 2002), atherosclerosis (Samani et al., 2001; Bentos et al., 2004), and myocardial infarction (Brouilette et al., 2003). The role of telomeres in all-cause mortality is controversial, with several researchers reporting an association between short telomeres and all-cause mortality (Cawthon 2003; Ehrlenbach 2009), while other investigators have not observed such an association (Martin-Ruiz 2005; Epel 2009; Njajou 2009). Recently, in a population study of 64,637 participants, Rode et al., (2015) found an association between short peripheral blood leukocyte telomeres and mortality.

Due to the association between age-related health problems and shortened telomeres, telomere length has been considered as a candidate biomarker for human aging. In a meta-analysis of 19 publications about the validity of telomeres as a biomarker, Mather et al., (2010) concluded that telomere length meets some, but not all,

of the three primary requirements for an aging biomarker, as defined by the American Federation of Aging Research (American Federation of Aging Research, 2016).

Review of Telomere Length in People with Down syndrome

There is a paucity of knowledge about telomere lengths in people with Down syndrome and how this might be related/contribute to premature aging. Using the terminal restriction fragment (TRF) technique, Vaziri et al., (1993) were the first to show that trisomy 21 cells had a faster rate of telomere loss compared to disomic (normal) cells. Jenkins et al. (2006, 2008, 2010, 2016) have used a semi-quantitative fluorescent in situ hybridization (FISH) methodology to measure telomere lengths in older adults with Down syndrome. They first reported that lymphocyte telomeres of female probands with Down syndrome and dementia were shorter than age-matched controls who had Down syndrome, but did not have dementia (Jenkins et al., 2006). They next determined that the frequency of very short telomeres, such that they lacked fluorescent signals, was greater in adults with Down syndrome and dementia or mild cognitive impairment compared to those without decline (Jenkins et al., 2008). In their two most recent publications, Jenkins et al. (2010; 2016) provided evidence that shortened telomere length may indicate dementia status in adults with Down syndrome. Using a longitudinal study design, they measured telomere lengths in 5 individuals with Down syndrome at multiple time points as they progressed from preclinical dementia to mild cognitive impairment (MCI) or frank dementia. Prior to decline, telomere lengths were similar to those of other adults with Down syndrome who do not have dementia. After dementia progression, the telomere lengths were significantly shortened. Based on their

findings, this research team suggested that telomere length could be a biomarker for assessing dementia status in people with Down syndrome.

Knowledge of telomere lengths in people with Down syndrome at young ages is especially limited. While some researchers have reported shorter telomere lengths in young people with Down syndrome, others have reported longer telomere lengths. Recent studies of telomere lengths in newborns and children with Down syndrome are summarized in Table 2.

Table 2. Summary of telomere length studies in young people with Down syndrome and mothers of children with Down syndrome.

Reference	Participants	Method	Outcome
Sukenik-Helevy et al., (2011)	7 trisomy 21 conceptuses 6 diploid conceptuses	Semi-quantitative FISH in amniocytes Telomere length reported as fluorescence intensity	Shorter telomeres in trisomy 21 amniocytes
Nakamura et al., (2014)	11 trisomy 21 newborns 10 trisomy 18 newborns 10 diploid newborns	Semi-quantitative FISH in lymphocytes Telomere lengths were calibrated by comparing with results of Southern blotting/TRF in fibroblast cell lines	No telomere length difference between diploid and trisomy 21 newborns
Wenger et al., (2014)	3 trisomy 21 newborns 7 diploid newborns	Semi-quantitative FISH in blood In metaphase cells, length reported as ratio of telomeres to whole chromosome length In interphase cells, length reported as fluorescence intensity	Shorter telomeres on chromosome 1 in trisomy 21 newborns compared to diploid newborns
Gruszecka et al., (2014)	68 trisomy 21 youths (age 2-21) 56 diploid controls	qPCR and TRF confirmation in DNA from blood	Longer telomeres in trisomy 21 children/young adults
Albizua et al., (2015)	404 mothers of trisomy 21 newborns from maternal NDJ 24 control mothers of trisomy 21 children from paternal/post-zygotic NDJ	qPCR in DNA from blood	Young MII mothers had shorter telomere lengths compared to young MI and control women Evidence for “premature biological aging” hypothesis in mothers of trisomy 21

	18 control mothers of diploid children		children
Bhaumik et al., (2017)	170 newborns with trisomy 21 and their mothers 186 diploid newborns and their mothers	TRF	Longer telomeres in trisomy 21 newborns No effect of maternal NDJ on trisomy 21 newborns' telomere lengths

Terminal restriction fragmentation (TRF); Nondisjunction (NDJ); Meiosis II Nondisjunction (MII); Meiosis I Nondisjunction (MI)

One potential explanation for the huge discrepancies in the results of previous telomere length studies in people with Down syndrome could be the reliance on case-control study designs. Due to this approach, the interpretation of previous results is limited by interpersonal differences in telomere lengths. In addition to heritable genetic influences, another challenge to the determination of the true consequences of trisomy 21 on telomere length is the number of demographic and lifestyle factors that affect this cellular attribute. For example, telomere length has been shown to be influenced by inflammation, tobacco and alcohol use, exercise, sex, race, socioeconomic status, weight, and diet (Mather et al., 2010). Additionally, as previously discussed, heritable genetic factors influence telomere lengths. To limit the effects of person-specific variation, we compared the telomere lengths in the trisomic and disomic cells from individuals with mosaic Down syndrome. This isogenic trisomic-disomic system enables the assessment of telomere length differences caused specifically by the trisomy 21 imbalance.

A second potential factor toward the conflicting results observed by different groups investigating telomere attrition between study groups is the methodology used to assess telomere length. Telomere length can be measured in human cells using a number of different techniques (reviewed by Montpetit, et al., 2014). These methods include DNA-based techniques like quantitative polymerase chain reaction (qPCR) (O'Callaghan and Fenech 2011; Cawthon 2009) and terminal restriction fragmentation (TRF) (Kimura et al., 2010), as well as cell-based methods like flow fluorescence in situ hybridization (FISH) (Baerlocher et al., 2006). A disadvantage to these approaches is

that they do not allow for chromosome-specific analysis of telomere length (Aubert et al., 2012; Montpetit et al., 2014).

There is evidence suggesting that chromosome-specific variations in telomere length exist (Lansdorp et al., 1996; Henderson et al., 1996; Martens et al., 1998). Lansdorp et al. (1996) observed that in a single metaphase cell, telomere signal size varied up to three-fold among the different chromosomes. In research focused on chromosomal instability and/or senescence, the shortest telomere, rather than mean telomere length, has been shown to be the critical sentinel for signaling these events (Hemann, et al., 2001; Zou Y, et al., 2004). Therefore, for our analysis of telomere length in isogenic cells from people with mosaic Down syndrome, we elected to use a semi-quantitative FISH method to capture information regarding chromosome-specific differences for the telomere lengths of each chromosome arm in a cell. Another advantage to this chromosome-specific FISH approach to telomere length assessment is that individual cells can be analyzed and karyotyped to determine whether the cell is trisomic or disomic. The primary aims of this study were: (1) to measure telomere length differences in isogenic trisomic and disomic cells from people with mosaic Down syndrome and (2) to determine if there is an association between age and telomere length differences in isogenic trisomic and disomic cells.

Materials and Methods

Ethics Statement

Human subjects' research was approved by the Virginia Commonwealth University IRB (protocol number #HM179 CR3). Written documentation of informed consent/assent was obtained from all research participants, with parental informed consent being obtained for children or adults who demonstrated intellectual disability levels that might compromise their ability to provide fully informed consent.

Study Participant Ascertainment

A total of 28 participants with mosaicism for Down syndrome were ascertained through their membership/participation with the International Mosaic Down Syndrome Association. The only selection criterion was that the study member had a chromosomally confirmed diagnosis of mosaicism for Down syndromeEach of these study participants submitted a blood sample to allow for the assessment of chromosome-specific telomere lengths. The people with a diagnosis of mosaic Down syndrome who were evaluated in this study included two participant cohorts. The first cohort (also called "new cases") included 10 individuals (4 males and 6 females) who were ascertained from 2013 to 2017 . The chromosome-specific telomere values for cohort 1 were evaluated by Kelly Rafferty. The second cohort (also called "previous cases") included 18 participants who were ascertained prior to 2013 who were evaluated as part of a pilot study that was completed by a former member of our laboratory team (C. Charalsawadi). The subset of participants with mosaicism who were evaluated in either of these cohorts were selected based on their "minor" cell population

being present in at least 10% of cells, to allow for the recognition of both isogenic cell types in the analysis (e.g. cut-off of 90% disomic; 10% trisomic or 90% trisomic; 10% disomic).

Lymphocyte Culturing

Mononucleated cells were collected from a peripheral blood specimen using Histopaque (1077; Sigma). Following standard methodology, the cells from the buffy coat were washed and then placed into culture media (RPMI 1640 media, supplemented with 15% Fetal bovine serum [FBS]) containing phytohemaglutinin [PHA], the latter of which stimulates lymphocytes to proliferate). The cells were incubated (at 37ºC in 5% CO_2) for 72 hours, with Colcemid being added at 71.67 hours (20 minutes prior to harvest). The metaphase chromosomes from lymphocytes were harvested using standard techniques (Rooney and Czepulkowski, 1992), which included incubating the cells in a hypotonic solution (0.075 M KCl) for 20 minutes, followed by fixation (three times using a 3:1 methanol:acetic acid solution). Slides were made in a Thermatron unit with the chamber temperature being set at 23°C and the humidity level being set at 47 +/- 2 %.

Semi-quantitative, chromosome-specific FISH for telomere assessments

FISH was completed with a fluorescein-conjugated peptide nucleic acid (PNA) probe according to the manufacturer's protocol (Dako). The FITC-labelled probe has the pan-telomeric sequence $(CCCTAA)^3$. Slides were prepared for FISH with the following washes at room temperature: TBS buffer 2m, 3.7% formaldehyde 2m, TBS buffer 5m,

TBS buffer 5m, pretreatment solution (Dako) 10m, TBS buffer 5m, TBS buffer 5m. Following these washes, slides were dehydrated in an ethanol series at increasing concentrations of 70%, 85%, and 100% (2m each). The target chromatin, which was affixed to a microscope slide, and the probe PNA were co-denatured at 80°C for 3m. Following denaturation, the slides were placed in a dark chamber and hybridized at room temperature for 2 hours. Non-specific binding of probe was removed by washing in a rinse solution at room temperature (1m) and a wash solution at 65°C (5m) (Dako). The slides were again dehydrated in the ethanol series. To visualize chromosomes and determine relative fluorescence intensity of the PNA probe, the chromatin was counterstained with a 5:1 solution of DAPI II: antifade/propidium iodide (Abbott Molecular).

Determination of Telomere Length

An Axioskop equipped with single- and triple-band pass filters was used (Zeiss) to evaluate and capture images of the metaphase spreads. For each participant, a maximum of 10 disomic and 10 trisomic metaphase cells were included. Each cell was karyotyped using the reverse DAPI banding patterns of the chromosomes. Images were captured with JAI progressive scan camera (JAI) and analyzed with the Comparative Genomic Hybridization software program developed for the CytoVision image analysis system (Leica Biosystems). For each cell, 3 fluorescent images were captured: (1) DAPI (to visualize chromosomes and banding patterns); (2) spectrum green (to visualize the FITC-labelled PNA probe); and spectrum orange (to define the location of the structure [or “backbone”] of the propidium iodide-stained chromosome) (Figure 2).

Only telomeres that could be readily viewed were scored. If either end of a chromosome was in contact with other chromosomes or appeared clustered or was underlying another chromosome in a manner that obscured assessment of at least one of the individual telomeres, this chromosome was excluded from the analysis. Also, any chromosome that could not be unequivocally identified, based on its reverse DAPI pattern, was excluded from the analysis. Because many homologous chromosomes cannot be distinguished from one another in metaphase preparations, values obtained for both homologs of a chromosome were averaged to calculate the chromosome-specific values. The resulting telomere length values are given in semi-quantitative units based on the comparative intensity values of the stains, with more intense staining being reflective of longer telomeres and higher values.

Statistical Analyses

For each participant, a paired t-test was used to determine whether there was a significant difference in the telomere intensities of the trisomic and disomic cells. To control for multiple comparisons, a Bonferroni correction was applied by dividing the original α of 0.05 by the number of comparisons per test (48 chromosome arms in males, 46 chromosomes in females). This calculation resulted in a corrected α of 0.001 (0.05 / 48 ≈ 0.001; 0.05 / 46 ≈ 0.001). To determine chromosome-specific telomere intensity differences, a paired t-test was completed for each chromosome arm to compare the values in the trisomic and disomic cells of all participants. To compare the categories of telomere length outcomes (i.e. shorter telomeres in trisomic cells, shorter telomeres in disomic cells, or no difference between trisomic and disomic cells), a Chi-Square test was used. For the overall assessment of telomere length differences in trisomic and disomic cells, the average value for each chromosome arm was determined (average of all participants) and a paired t-test was completed between trisomic and disomic cells. To determine the association between age and average telomere length, a correction was applied to account for batch effects between the two cohorts. The mean telomere intensity value of the cohort 1participants was divided by the mean telomere intensity value of the cohort 2 participants to obtain a correction value.

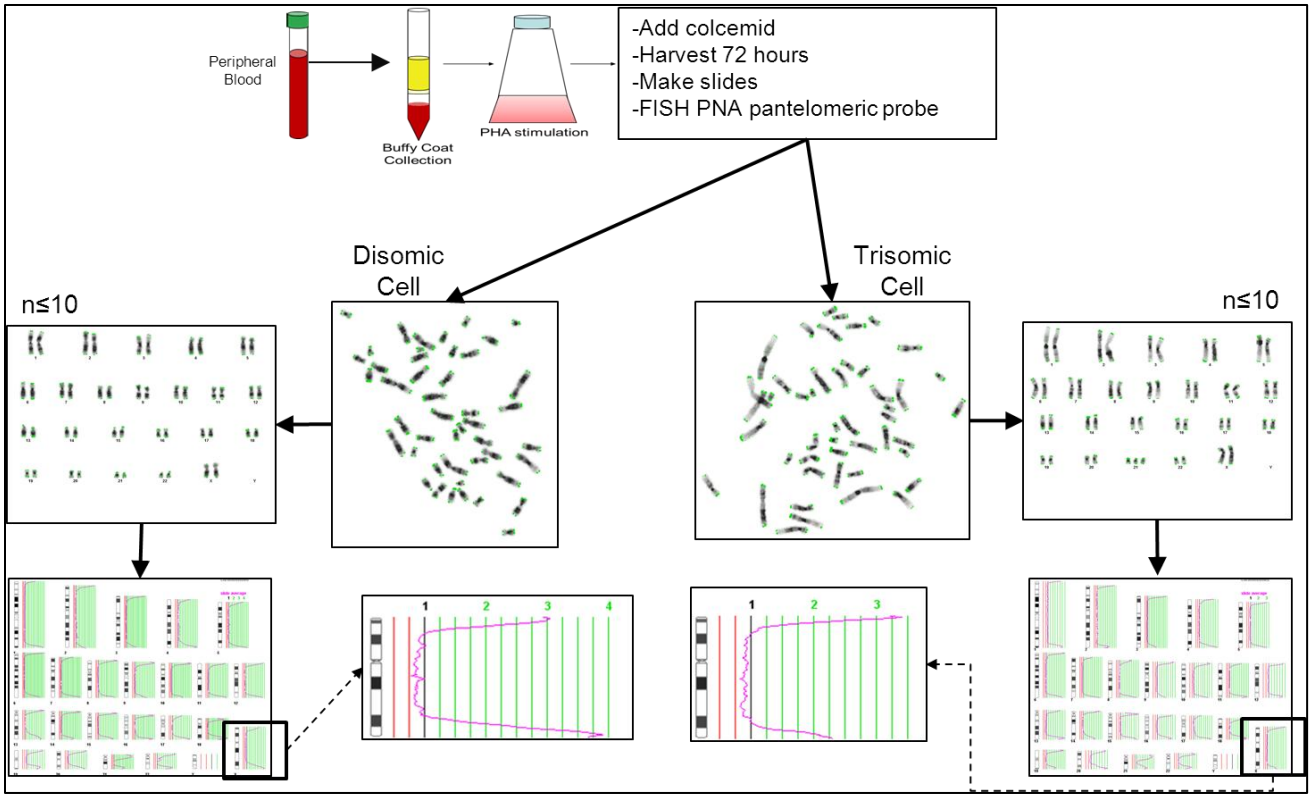

Figure 2. Summary of methods for telomere length assessment using a chromosome-specific semi-quantitative FISH method. After collecting cells from the buffy coat of a peripheral blood specimen, metaphase chromosomes were harvested and their telomeres hybridized using a PNA pantelomeric FISH probe. Metaphase spreads were karyotyped to distinguish trisomic from disomic cells and to allow for an assessment of chromosome-arm specific telomere lengths. A total of 10 disomic cells and 10 trisomic cells were analyzed per individual. The telomere lengths were calculated by quantifying the average fluorescence intensity across the 10 trisomic and disomic cells for the short arm and long arms of each chromosome. For example, the inset shows that in this study participant, the short arm telomere values for the X chromosome were similar in the disomic cells (value of 3) and trisomic (value of 3.25) cells, but the long arm had a longer telomere value in the disomic cells (3.9) compared to the trisomic cells (2.25).

Results

Cohort Ages and Frequencies of Trisomic Cells

The ages of the participants in cohort 1 (4 males and 6 females) ranged from 1.2-45 years (μ=16.29) (Figure 3). The ages of the participants in cohort 2 (8 males and 10 females) ranged from 0.3-28 years (μ=7.16) (Figure 3). There was no significant difference in the mean ages of the two cohorts (p=0.084). Also, the percentages of trisomic cells in blood specimens from cohort 1 (range 11.3 to 89.5, μ=33.27) did not differ significantly from the percentages of trisomic cells in blood specimens from cohort 2 (range 10.3 to 90.2, μ=32.43) (p=0.942).

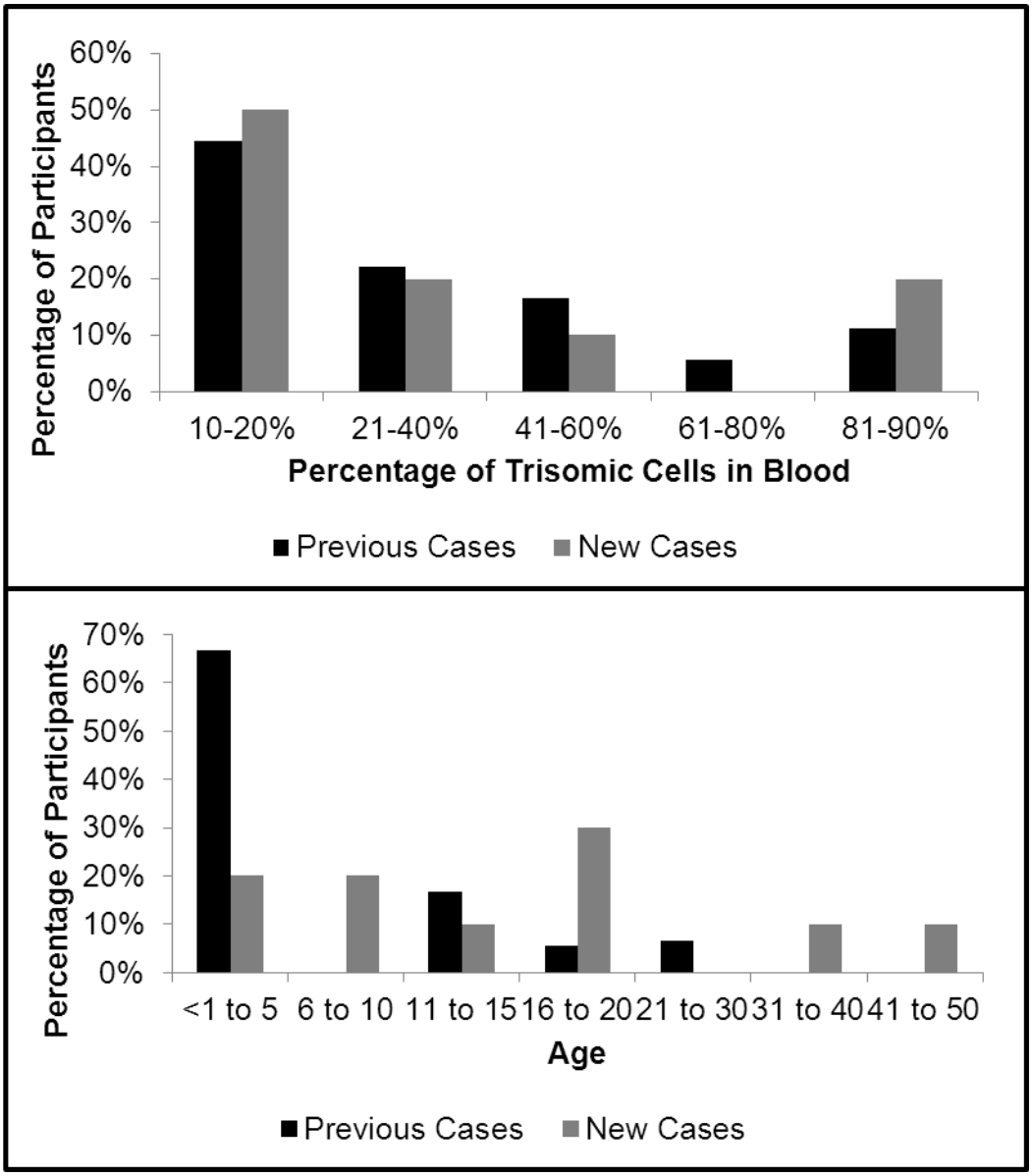

Figure 3. Distribution of the ages and percentages of trisomic cells in blood of previous and new cohort participants selected for inclusion in this study.

Age and Telomere Intensity Values

While a trend toward shorter telomeres with increasing age was seen, this correlation was not significant for the total pool of participants evaluated from both cohorts ($r= -0.196$, $p=0.327$) (Figure 4). Also, age was not correlated with the differences in telomere intensity values between the isogenic trisomic and disomic cells of the mosaic probands ($r=0.025$, $p=0.900$).

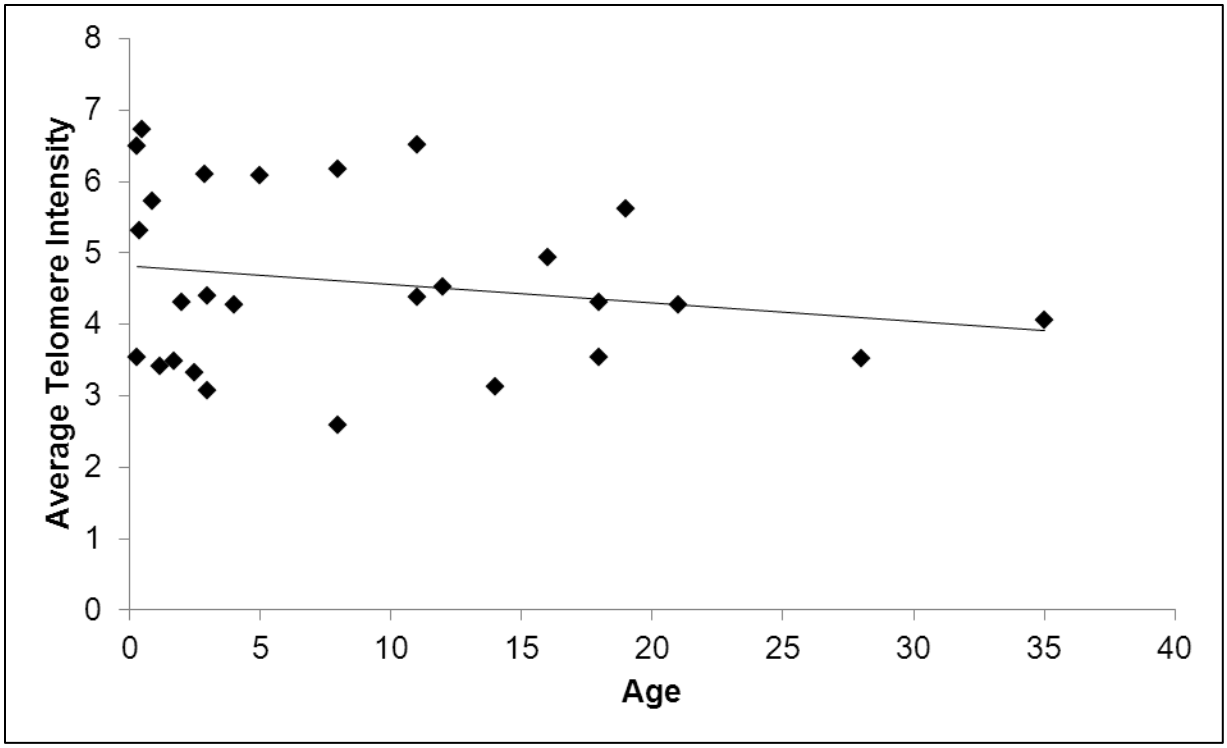

Figure 4. Relationship between age and average telomere intensity value in participants of both cohorts (n=28). Values were averaged across all chromosome arms and all cells. Average telomere intensity values were corrected for batch effects between the two cohorts.

Telomere Intensity Values in Trisomic and Disomic Cells

Cohort 1

The overall average telomere intensity value in the trisomic cells of cohort 1 (average telomere intensity of all chromosomes, averaged again across participants) was 4.305, and in the disomic cells was 4.969. The average difference in telomere intensities (trisomic - disomic) between the trisomic and disomic cells was -0.664. In 5 (50%) of the participants in the new cohort, the average telomere intensities in the trisomic cells were significantly lower than in the disomic cells. In the other 5 (50%), there was no significant difference in telomere intensities between the trisomic and disomic cells (Table 3).

Cohort 2

For cohort 2, the overall average telomere intensity value in the trisomic cells (average telomere intensity of all chromosome arms, averaged across participants) was 3.146, and in the disomic cells was 3.219. The average telomere intensity difference (trisomic - disomic) was -0.073. In this cohort, the average trisomic telomere intensity was lower in 7 participants (39%) and higher in 5 participants (28%). The remaining 6 participants (33%) had no significant difference in telomere intensity values between the trisomic and disomic cells (Table 3).

The differences in telomere intensity values between the isogenic trisomic and disomic cells of each participant are shown in Figure 5. When comparing the 3 categorical assessments of telomere differences between cohorts 1 and 2 (e.g. shorter

in trisomic cells; longer in trisomic cells; or no difference between trisomic and disomic cells), no significant difference was observed ($p=0.178$). Thus, the results from the 2 cohorts were pooled to assess the overall categorical findings for telomere length comparisons between the isogenic cells. When compared to chance expectations using a Chi-Square test, a significant difference in the categorical groups was observed for the overall data set, with the largest group being individuals who had shorter telomeres in their trisomic cells (43%) and the smallest group being individuals who have longer telomere lengths in their trisomic cells (18%) ($p=0.006$) (Figure 6).

When all participants' telomere intensity values were averaged together, for each individual chromosome arm, the telomeres of the trisomic cells had significantly lower intensity values than the telomeres of the disomic cells ($p<0.0001$).

Table 3. Average telomere intensity values in disomic and trisomic cells from people with mosaic Down syndrome.

Age (years)	Sex	Trisomic Cells μ	Trisomic Cells SE	Disomic Cells μ	Disomic Cells SE	Difference (T-D)	p value
				Previous Cases			
0.3	F	2.186	0.056	2.674	0.076	-0.488	*$1.317E^{-11}$
0.3	M	4.181	0.146	4.721	0.163	-0.540	*$4.509E^{-5}$
0.4	F	3.623	0.130	3.668	0.126	-0.045	0.709
0.5	M	4.570	0.149	4.663	0.160	-0.093	0.426
0.9	M	3.747	0.134	4.107	0.132	-0.360	*0.0003
2	F	3.264	0.116	2.658	0.100	0.606	*$3.589E^{-10}$
2.5	F	1.920	0.076	2.632	0.074	-0.712	*$1.218E^{-13}$
2.9	M	4.234	0.146	4.145	0.129	0.089	0.373
3	M	2.202	0.049	2.011	0.045	0.195	*$2.383E^{-5}$
3	F	2.636	0.077	3.394	0.081	-0.757	*$1.427E^{-14}$
4	M	2.664	0.066	3.193	0.103	-0.529	*$1.302E^{-8}$
5	M	4.234	0.146	4.106	0.128	0.128	0.201
11	F	2.659	0.091	3.361	0.110	-0.703	*$6.22E^{-14}$
12	F	3.495	0.081	2.703	0.068	0.793	*$3.66E^{-18}$
14	F	2.144	0.054	2.157	0.074	-0.014	0.805
18	M	3.151	0.138	2.762	0.094	0.390	*0.0008
21	F	3.075	0.089	2.799	0.100	0.275	0.014
28	F	2.641	0.078	2.184	0.055	0.457	*$1.893E^{-7}$
Overall avg.		**3.146**		**3.219**		**-0.073**	
				New Cases			
1.2	F	2.648	0.078	4.170	0.131	-1.522	*$3.432E^{-16}$
1.7	F	2.953	0.089	4.009	0.109	-1.055	*$1.043E^{-10}$
8	F	2.732	0.074	2.455	0.121	0.276	0.005
8	M	5.988	0.128	6.369	0.121	-0.380	0.003
**11	M	6.689	0.133	6.322	0.318	0.367	0.249
***16	M	4.107	0.242	5.773	0.127	-1.666	*$4.463E^{-10}$
18	F	3.397	0.114	3.659	0.072	-0.262	0.048
19	M	4.973	0.154	6.247	0.206	-1.274	*0.00001
35	F	3.844	0.105	4.266	0.109	-0.423	0.004
45	F	5.721	0.181	6.420	0.148	-0.700	*0.0005
Overall avg.		**4.305**		**4.969**		**-0.664**	

*p value is significant; **only one disomic cell scored; ***only one trisomic cell scored

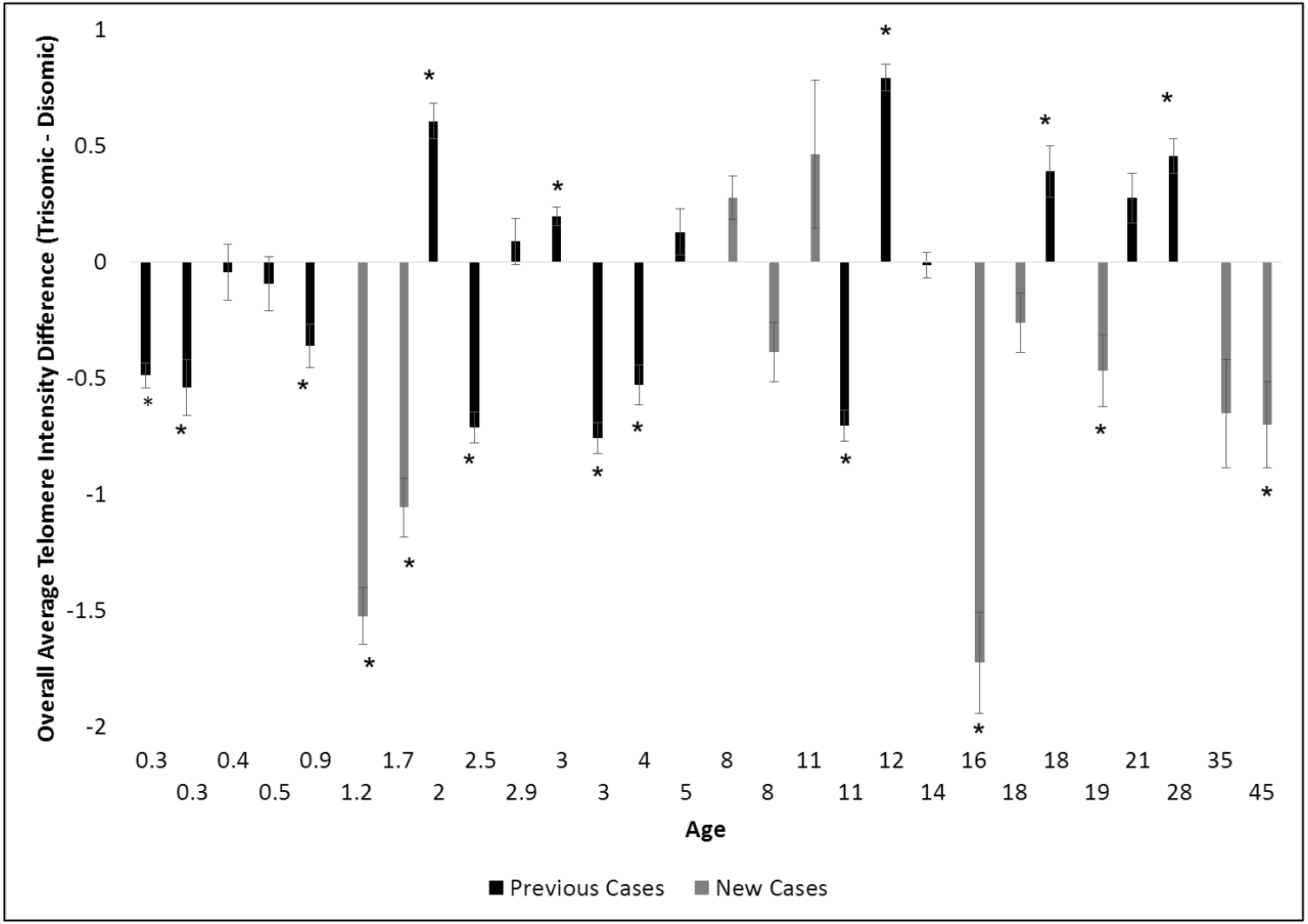

Figure 5. Differences in telomere intensity values between the isogenic trisomic and disomic cells in each participant averaged across all chromosomes (n=28). Each bar represents a participant. Asterisks indicate participants showing significant differences in telomere lengths observed for chromosomes in the trisomic compared to disomic cells.

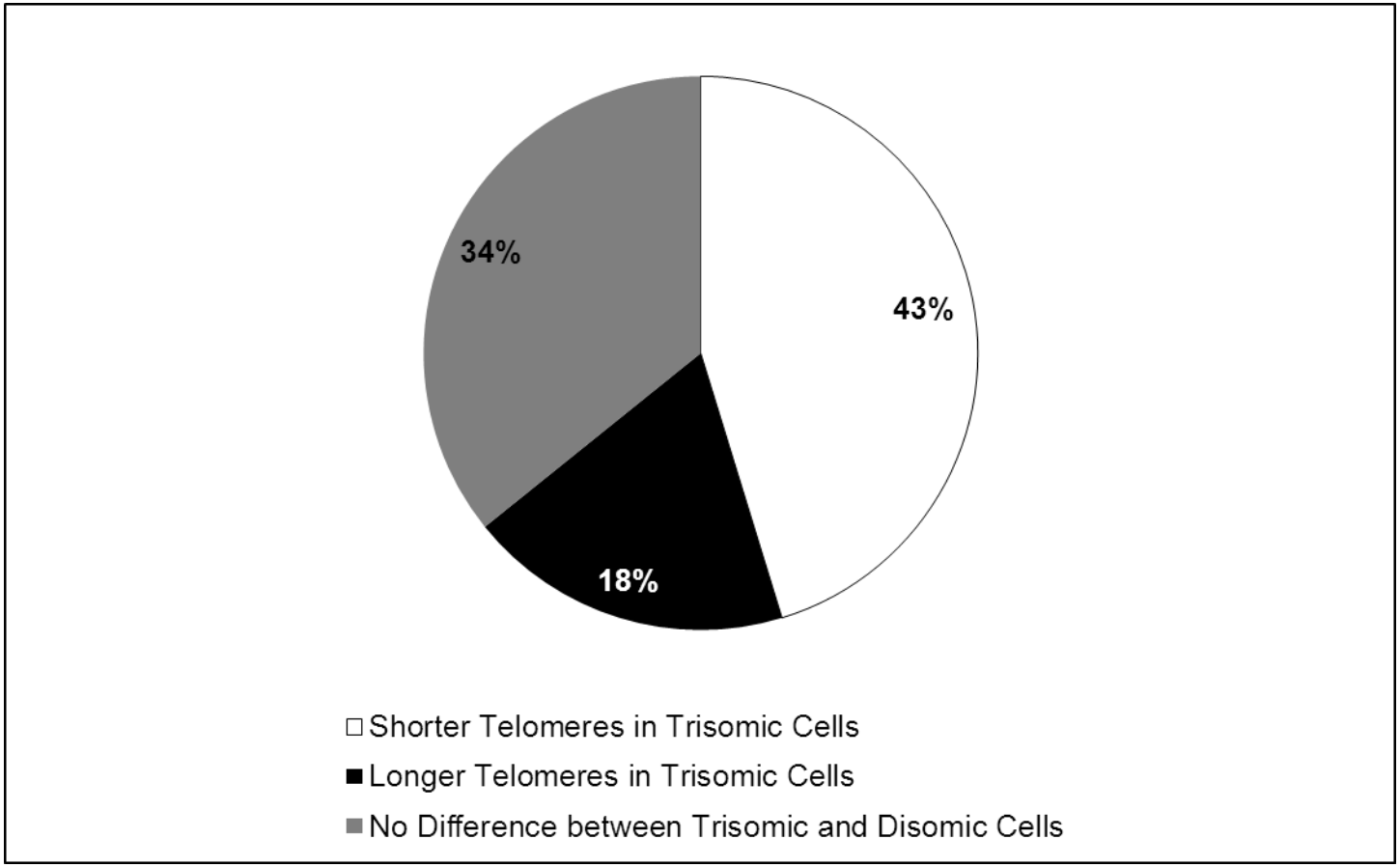

Figure 6. Percentages of cases with significantly decreased telomere intensity values in trisomic cells, significantly increased telomere intensity values in trisomic cells, and no significant difference in telomere intensities between trisomic and disomic cells (n=28).

Chromosome-specific telomere intensity differences in the isogenic trisomic and disomic cells

For each participant, the chromosome-specific telomere intensity differences (Trisomic – Disomic) were assessed (graphically represented in a heat map; Figure 7). Within individual trends were observed for chromosome-arm specific patterns (all telomeres tending to be shorter or all telomeres tending to be longer). Across all individuals, no significant differences in telomere lengths changes were observed for any specific chromosome arms (Figure 7 and Table 4). However, trends toward shorter trisomic cell telomeres were identified for 3q, 4q, 5p, 6q, 9p, 9q, 12q, and 20p (Table 4). To further compare the chromosome-specific patterns of telomere intensity between the trisomic and disomic cells, chromosome-specific telomere intensities were averaged across all participants (Figure 8). In general, the patterns of telomere intensities between the trisomic and disomic cells were parallel to one another, with the trisomic values consistently being lower than the disomic values. For example, the telomeres of the short arms of chromosomes 3, 9 and Y have comparatively greater intensity in both the trisomic and disomic cells, and the telomeres of the long arm of chromosomes 1, 2, and 9 have comparatively lower intensities in both trisomic and disomic cells (Figure 8).

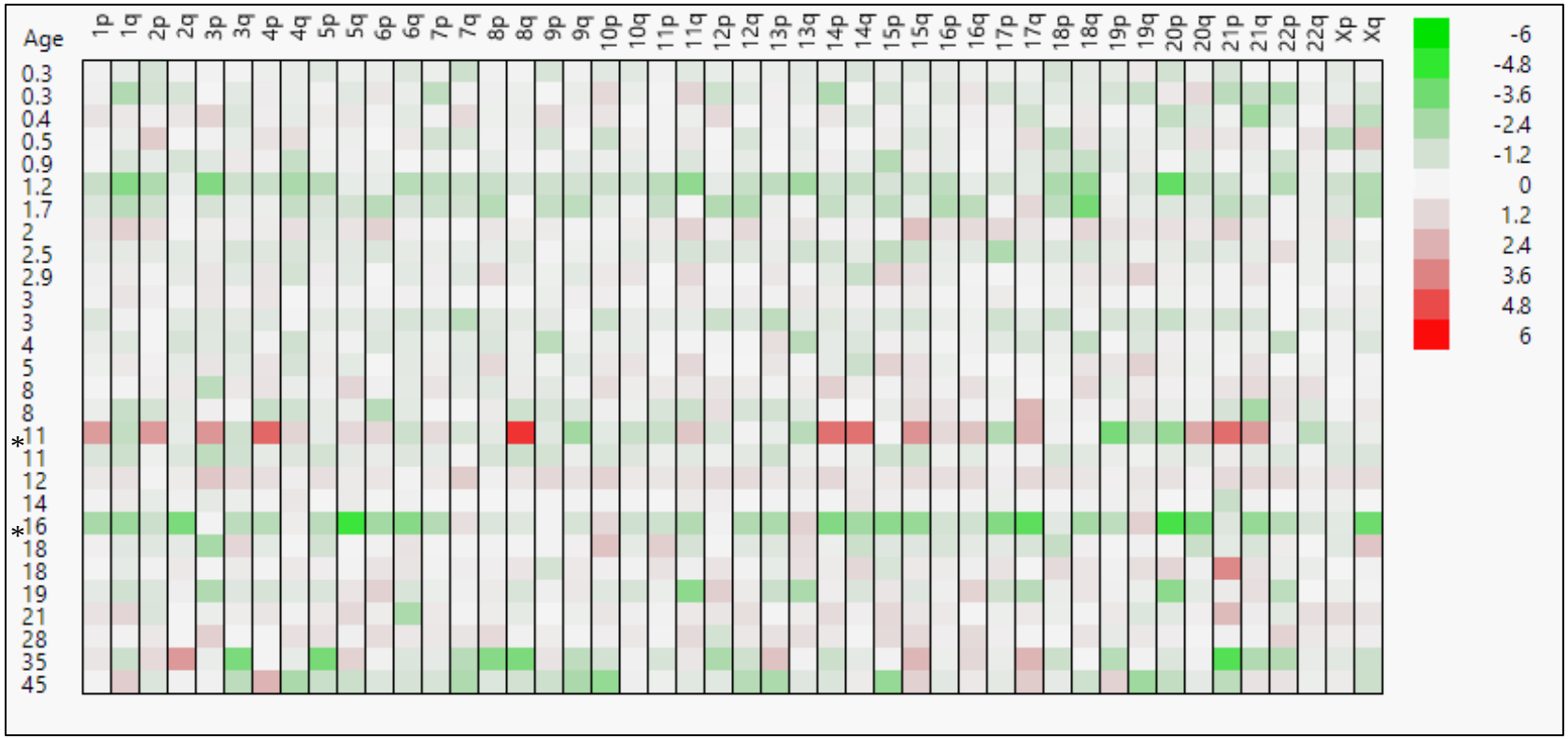

Figure 7. Average telomere intensity differences between trisomic and disomic cells in each chromosome arm for all participants (Difference = Trisomic – Disomic). Negative values (green) indicate chromosome arms in which the telomeres of the trisomic cells are shorter, and positive values (red) indicate chromosome arms in which the telomeres of the trisomic cells are longer. Asterisks denote cases in which only one cell from the “minor cell population” was scored.

Table 4. Chromosome-arm specific telomere intensity values in the trisomic and disomic cells of participants with mosaic Down syndrome (N=28).

Chromosome	Short Arm						Long Arm					
	Trisomic		Disomic		Difference	p value	Trisomic		Disomic		Difference	p value
	Mean	SE	Mean	SE	(T-D)		Mean	SE	Mean	SE	(T-D)	
1	2.568	0.179	2.610	0.168	-0.041	0.816	3.820	0.254	4.304	0.314	-0.484	0.049
2	3.779	0.245	4.010	0.239	-0.231	0.260	2.617	0.226	2.953	0.219	-0.337	0.055
3	4.900	0.377	5.073	0.321	-0.172	0.491	3.632	0.241	4.021	0.290	-0.389	0.020
4	3.236	0.244	3.120	0.284	0.116	0.617	4.135	0.279	4.566	0.303	-0.431	0.028
5	3.473	0.259	3.991	0.324	-0.517	0.005	3.487	0.234	3.807	0.300	-0.320	0.158
6	4.003	0.245	4.205	0.295	-0.203	0.259	3.656	0.259	4.203	0.291	-0.547	0.004
7	3.171	0.229	3.479	0.257	-0.308	0.048	3.726	0.278	4.134	0.312	-0.408	0.024
8	3.933	0.287	4.260	0.310	-0.327	0.064	3.147	0.253	3.295	0.230	-0.148	0.527
9	4.261	0.286	4.783	0.326	-0.522	0.004	2.574	0.202	3.002	0.285	-0.428	0.011
10	4.188	0.289	4.233	0.312	-0.045	0.826	3.309	0.237	3.557	0.296	-0.248	0.092
11	3.274	0.211	3.637	0.303	-0.363	0.126	3.957	0.274	4.127	0.320	-0.170	0.487
12	3.519	0.281	3.759	0.306	-0.240	0.140	3.301	0.221	3.763	0.321	-0.462	0.019
13	3.609	0.241	3.900	0.282	-0.291	0.114	3.932	0.262	4.221	0.310	-0.289	0.139
14	3.416	0.222	3.612	0.279	-0.195	0.445	3.471	0.288	3.731	0.281	-0.260	0.254
15	3.301	0.221	3.794	0.284	-0.493	0.049	4.028	0.295	3.953	0.261	0.076	0.744
16	2.987	0.244	3.359	0.270	-0.372	0.044	3.096	0.216	3.140	0.236	-0.044	0.778
17	3.186	0.264	3.470	0.296	-0.284	0.186	3.228	0.320	3.140	0.222	0.088	0.717
18	3.965	0.271	4.365	0.250	-0.401	0.060	4.054	0.265	4.416	0.277	-0.362	0.126
19	3.102	0.243	3.329	0.282	-0.227	0.199	3.623	0.278	3.661	0.302	-0.038	0.841
20	3.410	0.225	4.161	0.344	-0.751	0.010	2.992	0.243	3.389	0.272	-0.396	0.103
21	4.039	0.287	4.294	0.327	-0.255	0.444	3.284	0.246	3.609	0.292	-0.326	0.216
22	4.032	0.271	4.304	0.297	-0.273	0.147	3.086	0.243	3.259	0.319	-0.173	0.159
X	3.926	0.278	4.229	0.322	-0.304	0.129	3.581	0.295	3.914	0.299	-0.333	0.165
Y	5.318	0.438	5.422	0.437	-0.104	0.772	4.892	0.452	5.053	0.629	-0.162	0.682

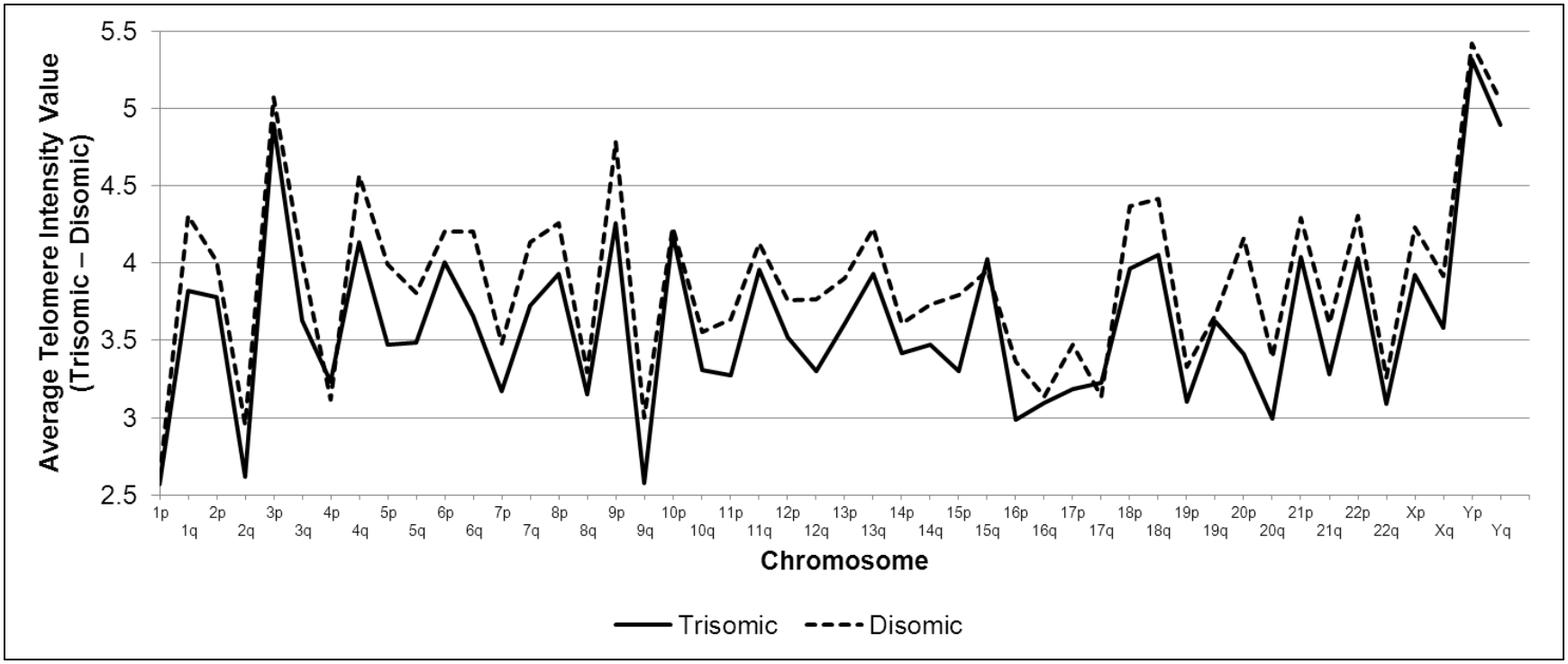

Figure 8. Telomere intensity values of each chromosome arm in the trisomic and disomic cells, averaged across all participants in both cohorts (N=28).

Discussion

Telomere Lengths in Trisomic and Disomic Cells

In this study, we evaluated the telomeres from each chromosomal arm in the isogenic disomic and trisomic cells from people with mosaicism for Down syndrome. Given that the fluorescence intensity values indicate the relative telomere lengths, such that longer telomeres have higher telomere intensity values, significant telomere intensity differences are inferred to be indicative of significant differences in telomere lengths. When comparing chromosome-specific telomere values between the trisomic and disomic cells, we observed deviations from chance expectations, with shorter telomeres in the trisomic cells being seen in 43% of the participants, longer telomeres in the trisomic cells in 18% of the participants, and no significant different between the trisomic and disomic cells in the remaining 39% of individuals. While the largest group of probands had shorter telomeres in their trisomic cells, this finding was not universally observed, suggesting that there may be additional individual factors (other than a trisomic imbalance) that influence or mediate the effect of trisomy 21 on telomere lengths. It could be possible that the downstream effects of trisomy 21, as opposed to the presence of the trisomy itself, contribute to telomere length changes. People with Down syndrome do not develop identical phenotypes. Some individuals have many medical problems, while others have relatively few. It is clear that trisomy 21 does not have equivalent effects on all people with Down syndrome. Given the phenotypic variation that exists in people with Down syndrome, it could be inferred that trisomy 21 also has varying effects on the mechanisms that underlie acquired cellular changes. For example, trisomy 21 could cause different degrees of oxidative stress or mitochondrial

dysfunction amongst individuals based on genetic or environmental modifiers. In those individuals with high levels of trisomy 21-induced oxidative stress, the telomeres in trisomic cells would be expected to be shorter. In contrast, if a person has genetic/environmental modifiers that attenuate the trisomy 21-mediated oxidative stress, there may not be a difference in telomere lengths between cells with and without a trisomy 21 imbalance.

We had hypothesized that cells with a trisomic imbalance would have shorter telomere lengths than those without a trisomic imbalance, and that this difference would be greatest at older ages. Including both cohorts, 43% of the participants had shorter telomere lengths in the trisomic compared to disomic cells. This suggests that in those individuals, the trisomic cells are more susceptible to telomere shortening.

Our observation that a minority of participants had significantly longer telomeres in the trisomic cells compared to disomic cells either reflects a disproportionately accelerated shortening of the telomeres in the disomic cells, or indicates that the telomeres in the trisomic cells have lengthened. Our study did not incorporate a longitudinal component, so we are unable to distinguish between these two possible scenarios. Although age-related shortening of telomeres is the predominant outcome of leukocyte telomere length studies, many researchers have found that the telomeres in a minority of participants lengthen over time (Bateson and Nettle, 2017). Telomere elongation in leukocytes is a controversial concept. Steenstrup et al., (2013) argue that the apparent telomere lengthening in a minority of participants is "*an artefact of measurement error*" (Steenstrup et al., 2013). Bateson and Nettle (2017) created a computational simulation that compares predictions based on many different models of

underlying telomere dynamics, as opposed to using a model that assumes the occurrence of telomere attrition at a constant rate. They tested this model using the empirical data from 10 telomere length studies, and determined that the assumption of constant telomere attrition does provide the “best fit” for the data. This implies that telomere lengthening could have contributed to the differences in telomere lengths between trisomic and disomic cells in the participants with mosaic Down syndrome.

Despite trying to select participants with intermediate ranges of trisomic cells in blood, many of the participants had very low trisomic cell percentages and others had very low disomic cell percentages (Figure 2). In some cases, this resulted in limitations to the numbers of each cell type that were analyzed. In one case, a single trisomic cell was identified for assessment, and in another, a single disomic cell was recognized for assessment (Table 3). It is feasible that the low numbers of the less prevalent cell type in some of the cases may have influenced the telomere length outcomes.

Even though there were person-by-person differences in telomere length outcomes between the trisomic and disomic cells, when averaged across all participants, the telomeres in the trisomic cells were significantly shorter than those in the disomic cells. This demonstrates that with sufficient participants, a trait that is known to be complex, such as telomeres, can be evaluated. Despite the known impact of heritable and environmental variations on person-specific telomere lengths, we were able to show a significant difference in telomere attrition in the trisomic cells compared to disomic cells.

Age-Related Telomere Length Differences in Isogenic Trisomic and Disomic Cells

There was not an apparent association between participant ages and the difference in telomere length between the isogenic cells. Interestingly, both young children and older teens/adults showed significantly shorter telomeres in trisomic cells. In 5 of the 8 children aged 2 and younger, the trisomic-cell telomeres were significantly shorter than the disomic counterparts. It is known that the fastest rate of telomere shortening happens within the first two years of life, so it is possible that cells with trisomy 21 shorten at an especially rapid rate during this potential "vulnerable" time period (Rufer et al., 1999; Rufer et al., 2001).

Chromosome-Specific Telomere Lengths

One of the advantages of the semi-quantitative FISH method for telomere length ascertainment is the ability to identify chromosome-specific patterns of telomere lengths. In this study, we observed chromosome-specific telomere length heterogeneity, a finding that is in agreement with the results of previous investigators who studied lymphocyte chromosomes from chromosomally normal individuals or cell lines (Lansdorp et al., 1996; Henderson et al., 1996; Martens et al., 1998). Analogous to the findings of other investigators, we noted a trend toward longer telomeres for 3p, and shorter telomeres for 2q, among others. However, there were no significant chromosome-specific differences in telomere lengths between trisomic and disomic cells. This indicates that there was not accelerated shortening of particular chromosomes that was caused by the trisomic imbalance. Despite none of the chromosome arms having significant trisomic-disomic cell differences, the genome-wide

patterns of telomere length differences (Figure 8) were largely parallel. This finding suggests that the trend toward telomere shortening in the trisomic cells occurs across all chromosomes, with the variation between chromosomes likely being reflective of innate genetic differences in telomere lengths.

However, chromosome-specific trends toward shorter trisomic cell telomeres were identified for 3q, 4q, 5p, 6q, 9p, 9q, 12q, and 20p. It is possible that the shorter telomeres on these chromosomes in trisomic cells could influence the expression of genes located near the ends of the chromosomes. For example, the methyltransferase EHMT1 is located on the long arm of chromosome 9, which had a trend toward shorter telomeres in trisomic cells (Bock et al., 2016). The shortening of the 9q telomere could therefore possibly affect DNA methylation. The short arm of chromosome 9, which had a trend toward short trisomic cell telomeres, contains the DOCK8 gene which has been previously implicated in intellectual disability in people with 9p subtelomeric deletions (Hauge et al., 2008). It is feasible that the short telomeres could impact the regulation of this gene. Interestingly, the genes encoding the catalytic subunit of telomerase (TERT) and the RNA subunit of telomerase (TERC) are both located near the ends of chromosome arms which had a trend toward shorter trisomic cell telomeres (TERT is on 5p, TERC is on 3q). Shortened telomeres on these chromosome arms could be related to telomerase expression levels.

The primary goal of this study was to use a semi-quantitative assessment of each telomere length. A future study that could also be helpful in recognizing the association(s) between a trisomy 21 imbalance and telomere biology could be to dichotomize the assessment of individual telomeres to those lacking a signal versus

those having a signal that could be visualized since some investigators have shown that a single sentinel “short” telomere is the key trigger for signaling cellular senescence, rather than overall telomere shortening (Hemann et al., 2001).

Overall Conclusions

In this study, we compared the telomere lengths of isogenic trisomic and disomic cells. The results show that there is variability in the potential effect of trisomy 21 on telomere lengths between individuals. The diversity in the trisomic-disomic cell differences in these participants collectively suggest that the relationship between trisomy 21 and telomere lengths is complex and heterogeneous. However, within all of the participants combined, we were able to show a significant difference in telomere attrition in the trisomic cells compared to disomic cells.

Chapter 3. Senescence-associated distension of satellites in isogenic trisomic and disomic cells from people with mosaic Down syndrome

Introduction

The average lifespan of humans has increased, worldwide (United Nations World Population Prospects, 2015; The World Factbook, 2012). As a result, there is an ever-growing need to expand our knowledge of the cellular changes that accompany aging and underlie the development of age-related health problems. One approach for understanding complex traits, such as aging, is to study people with “extreme” or more pronounced forms of the trait. Thus, studies of aging have included research on people with progeroid-like conditions, the latter of which includes (but is not limited to) Down syndrome (also called trisomy 21). People with Down syndrome show an aging phenotype that closely parallels that of chromosomally normal older adults, but they show age-related health conditions at younger ages and also have a more rapid course of progression for these conditions (Zigman, 2013). Thus, it is anticipated that studies targeted to better understand the premature aging phenotype in people with Down syndrome could also help to tease apart the complex cascade of biological changes associated with aging in chromosomally normal people (Nakamur and Tanaka, 1998; Cairney et al., 2009). A robust approach for recognizing trisomy 21-specific cellular alterations of aging is to quantify and compare cellular attributes in the isogenic trisomic and disomic cells from people with mosaicism for Down syndrome. Using this isogenic mosaic Down syndrome model system, we have shown that telomeres tend to be

shorter in trisomic cells (a hallmark of aging) when compared to their genetically (and environmentally) identical (except for the presence/absence of a chromosome 21) disomic cells in individuals with mosaicism. One potential consequence of this accelerated shortening of the telomeres is premature initiation of cellular senescence, with this latter attribute being another hallmark of aging (Swanson, et al., 2013).

Cellular senescence was first described by Hayflick as “the time at which human diploid cell strains can be expected to cease dividing in vitro” (Hayflick, 1965). In their famous work regarding the limited lifetime of human cells, Hayflick and Moorhead surmised that senescence occurs as a function “of the number of potential cell doublings” (Hayflick and Moorhead, 1961). An updated definition, encompassing more than just the replicative form of senescence, states that “senescence describes deteriorative processes that follow development and maturation, and the term is used interchangeably with aging” (Campisi and d’Adda di Fagagna, 2007). This irreversible exit from the cell cycle can be induced by the activation of oncogenes, telomere shortening, oxidative stress, and non-telomeric DNA damage (Aravinthan, 2012). The process of cell cycle arrest in cellular senescence can be mediated through either the p16-Rb or p53-p21 tumor suppressor pathways (Ben-Porath and Weinberg, 2005).

The association between cellular senescence and organismal aging has been established through studies in model organisms and humans. Studies in primates and mice have yielded results suggesting that the frequency of senescent cells in certain tissues is greater in organisms of advanced age (Herbig et al., 2006; Jeyapalan et al., 2007; Jeyapalan and Sedivy, 2008; Wang et al., 2009). Senescent cells have been detected in human tissues affected by age-related pathology in several conditions,

including (but not limited to) arterial lesions, osteoarthritis, benign prostatic hyperplasia, aging skin, and carcinogenesis (reviewed by Jeyapalan 2008).

Redistribution of Heterochromatin in Senescent Cells

Various changes in heterochromatin organization have been implicated in the processes of aging and cellular senescence (Tsurumi and Li, 2012). From the results of studies in yeast and mice, Villeponteau (1997) hypothesized that heterochromatin domains that form during embryonic development deteriorate through aging, resulting in gene expression changes. Heterochromatin loss is involved in the pathogenicity of syndromes associated with premature aging, including Werner Syndrome and Hutchinson-Guilford Progeria syndrome (Shumaker et al., 2006). In fibroblasts from patients with Hutchinson-Guilford Progeria syndrome, mutations in the LMNA gene result in the accumulation of a truncated version of the Lamin A protein in the nucleus, which causes a loss of several marks of facultative heterochromatin (Shumaker et al., 2006). Similarly, in a mouse model of Werner syndrome, Zhang et al. (2015) found that chromatin disorganization resulted in accelerated cellular senescence. By knocking out components of a major histone methyltransferase complex, and thereby disrupting heterochromatin organization, they were able to induce p16-mediated cellular senescence (Zhang et al., 2015).

The diversity in the changes that heterochromatin undergoes during cellular senescence justifies the labelling of this phenomenon as a “redistribution” rather than “loss” of the heterochromatin (Sedivy et al., 2008). There are non-quantitative changes to both the facultative and constitutive heterochromatin that occur during the onset of

cellular senescence. It has been reported that genome-wide DNA methylation patterns vary between cycling and senescent cells (Koch et al., 2012; Franzen et al., 2017). Replicative senescent cells are characterized by global hypomethylation at gene poor regions and lamina-associated domains, which are typically associated with heterochromatin epigenetic signatures (Cruickshanks et al., 2013). Another example of a non-quantitative heterochromatin change in senescent cells is the formation of senescence-associated heterochromatin foci (SAHF) (Narita et al., 2003; Adams, 2007). These foci are comprised of facultative heterochromatin and reduce expression of genes that promote proliferation by sequestering them (Adams, 2007).

Senescence-Associated Distension of Satellites: A Senescence Biomarker

Senescence-associated distension of satellites (SADS) is a recently described biological phenomenon in senescent cells that is characterized by the distension of the pericentromeric alpha satellite (α-sat) and satellite II (sat II) heterochromatic sequences (Swanson et al., 2013). Swanson et al. (2013) first observed SADS in proliferative diploid fibroblast cultures. Only a small proportion of cells in these proliferative cultures had distended satellites. However, in those cells with distension, the majority of centromeres were affected. Senescence was confirmed in these cells through β-galactosidase staining, and failure of BrdU incorporation. They also confirmed that SADS occurs in cells with different types of senescence induction mechanisms, including replicative senescence, SMURF2 overexpression, oxidative stress, and oncogenic Ras. Importantly, SADS were confirmed *in vivo* using β-galactosidase stained tissue from premalignant prostatic intraepithelial neoplasia tumors and in

senescent mouse cells (Swanson, et al., 2013; Farr, et al., 2016). These investigators suggested that SADS represents a large-scale change in the higher order folding of the constitutive heterochromatin and arise as an early-step in a cell's progression toward senescence.

Senescence in Down syndrome

Senescent cells have been quantified in amniocytes, placentas, and mesenchymal stem cell cultures from pregnancies with a diagnosis of trisomy 21 (Amiel et al., 2013; Biron-Shental et al., 2015; Savickiene et al., 2016). In the studies of amniocytes and placental tissues with trisomy 21, there were significantly greater numbers of senescent cells, as determined by cell fragmentation, compared to cells from euploid tissues (Amiel et al., 2013; Biron-Shental et al., 2015). The results of the study of amniotic fluid-derived mesenchymal stem cells showed that trisomy 21 affected pregnancies had increased signs of cellular senescence, including morphologic, molecular, and epigenetic findings (Savickiene et al., 2016). Collectively, the results of these studies in fetal-derived tissues indicate that the cells of people with Down syndrome may be susceptible to senescence earlier in the life span than those of chromosomally normal individuals.

Considering this evidence for the role of cellular senescence in Down syndrome and the reliability of SADS as a senescence marker, we hypothesized that cells with a trisomy 21 imbalance have an increased frequency of SADS. To test this hypothesis, we quantified the frequency of SADS in the isogenic trisomic and disomic cells from people with mosaicism. In this preliminary, "proof of principle" study, we targeted the

pericentromeric region of chromosome 8 for an assessment of SADS because: (1) this chromosome is one of 3 chromosomes (the other 2 chromosomes were 12 and 15) that showed an increased frequency of acquired, primarily whole chromosome copy number changes with aging (Machiela et al., 2015); and (2) evaluations of chromosome 8 would enable us to use our previously collected data on micronucleus frequencies to determine if there was a relationship between SADS and micronuclei in the trisomic and/or disomic cells from the study participants who have mosaicism for Down syndrome. While we also previously determined micronucleus frequencies for chromosome 21, we did not evaluate SADS for chromosome 21 in this pilot study because the alpha satellite pericentromeric sequences for chromosome 21 share homology with those found on chromosome 13, thereby precluding our ability to use the resultant SADS data for direct comparison with the chromosome 21-specific micronucleus frequency values. Also, given that our study of micronucleus frequencies suggested that the observed increase in acquired chromosomal instability was reflective of global chromosomal instability (not limited to chromosome 21), we anticipate that the assessment of satellite sequences from any chromosome would be informative.

Materials and Methods

Study Participants

Participants with mosaic Down syndrome were ascertained through the International Mosaic Down Syndrome Association (IMDSA). A subset of 9 of the 38 total study participants were evaluated in this pilot study. These 9 individuals, which included 5 males and 4 females with a confirmed diagnosis of mosaic trisomy 21, were selected for inclusion in this pilot study to allow for the assessment of SADS frequency at various ages in the lifespan. Additionally, individuals who demonstrated micronucleus frequencies in the upper quartile of our study cohort (see chapter 4) were selected for inclusion in this study. Participants provided informed consent or assent, with their parents/legal guardians providing informed consent for the children or adults who were not cognitively able to provide fully informed consent. Each participant submitted a peripheral blood specimen. Approval for this research was given by the Institutional Review Board at Virginia Commonwealth University (VCU IRB #HM179 CR3).

Lymphocyte Culturing

Leukocytes were collected using Histopaque-1077 (Sigma) according to the manufacturer's protocol. Lymphocytes from this leukocyte culture were stimulated using phytohaemmoglutinin (PHA), with the cells being cultured and harvested according to standard cytogenetic methods (Rooney and Czepulkowski, 1992). Briefly, colcemid was added 20 minutes prior to the cell harvest, the latter of which was completed 72 h after culture initiation. Colcemid was added to the cultures because the resulting cells were also used in studies requiring metaphase-arrested cells. The harvest of cells was

achieved following an incubation in a hypotonic solution ([0.075 M KCl] for 10 minutes), and serial fixation (three times using a 3:1 methanol:acetic acid solution).Slides were made following standard methods.

FISH

As noted previously, a probe targeting the α-satellite, centromeric sequence of chromosome 8 was used to assess SADS (Abbott Molecular). To distinguish between the trisomic and disomic nuclei, a unique sequence probe targeting the RUNX1 locus (21q22) was co-hybridized with the pericentromeric chromosome 8 probe (Abbott Molecular). To allow for an assessment of chromosome 8 chromatin distension in a region not containing pericentromeric heterochromatin, a RUNX1T1 (8q22) unique sequence probe was also present in the probe cocktail (Abbott Molecular). The target DNA (which was affixed to a microscope slide) and the probes were co-denatured at 73°C for 2 minutes. Following denaturation, 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 0.4X SSC/0.3% NP-40 solution at 73 °C for 2 min, followed by a 2 min wash at room temperature (in a 2X SSC/0.1% NP-40 solution). To visualize the cell nuclei, the chromatin was counterstained with DAPI II/antifade (Abbott Molecular).

SADS Scoring

An Axioskop with single- and triple-band pass filters was used to score slides (Zeiss). The chromosome 21 probe signals were used to identify 50 trisomic and 50 disomic interphase cells from the mosaic specimens (metaphase spreads were **not** evaluated). The presence of SADS was defined as the visible distension of one or both of the α-sat probe signals such that the probe signal appeared as a linear, continuously connected signal comprised of intermittent smaller punctate/globular areas and thread-like areas (Figure 9). In contrast, a non-distended signal appears as a compact, rounded area of fluorescence (Figure 9). When scoring each cell, all focal planes were viewed to ensure the detection of all signals in the three-dimensional nuclei. Metaphase nuclei were excluded from the analysis since SADS is an attribute that is defined in relation to the interphase appearance of the chromatin. The probes used in this study have been validated in our CLIA and CAP-approved clinical diagnostic lab and determined to have specificity and sensitivity values of at least a 0.98. FISH images were captured using either a CytoVision Image analysis system (Leica Biosystems) or an Applied Spectral Imaging analysis system.

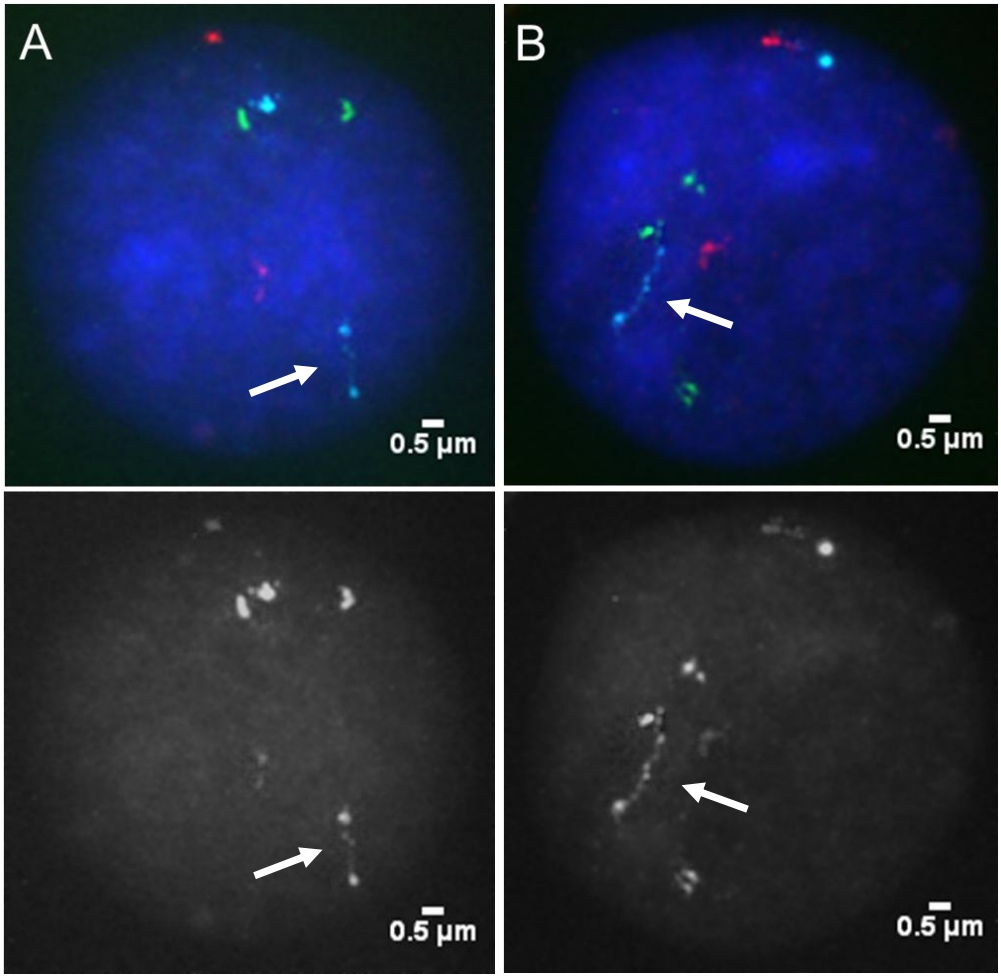

Figure 9. Representative images of SADS+ cells. Cells were categorized as SADS+ based on the presence of visible distension of one (or both) of the chromosome 8 α-satellite (centromeric) probes. The top panels show representative images in color (as viewed at the microscope). To facilitate the visualization of the alpha satellite regions with distension, these representative cells were also converted to black and white images (lower panels), the latter of which allows for enhancement of the contrast between the probe signal and the background nuclear DNA. (A) A disomic cell (two signals for the chromosome 21 probe [green]) has one distended chromosome 8 alpha satellite region (arrow; aqua signal). Neither of the unique sequence regions evaluated from chromosome 8 (red signal)] or chromosome 21 (green) show distention. (B) A trisomic cell (3 green signals) is shown that has one distended alpha satellite region (arrow; aqua signal). None of the unique sequences showed distension. It is important to note that regions with SADS are challenging to document using photographic means. However, these structures are quite clear when viewed through the fluorescent microscope. The investigators who first described SADS (Swenson, et al., 2013) also noted this photographic challenge.

Measurement of Probe Signal Length

In all cells demonstrating at least one chromosome with a SADS, the lengths of the α-satellite probe signals, and unique sequence probe signals (both RUNX1T 1 [8q22] and RUNX1 [21q22]) were measured and recorded. These same measurements were also recorded for the first 5 cells without SADS that were scored in each participant to obtain “control” values for chromatin compaction in cells without SADS.

Calculation of Nuclear Size

To asses if the SADS were associated with technical nuclear “stretching”, the nuclear diameter for each of the cells with SADS was calculated by measuring both the longest (e.g. vertical) and shortest (e.g. horizontal) axes. Human cells vary widely in size and geometry. Human lymphocyte nuclei can have a spherical or polarized, ellipsoid shape (Eisel et al., 1991). When adhered to a slide and visualized with microscopy, the cells appear as a circle or more commonly, an ellipse. The approximation of nuclear size was simplified by considering the cell to be a 2-dimensional structure, as only 2 of the cells’ 3 dimensions are fully discernible when fixed to a microscope slide. Based on the above noted measures, the nuclear size was approximated using the geometric formula for the area of an ellipse:

$$A = \pi \alpha \beta$$

where “α” equals the semi-major axis (half of the largest diameter) and “β” equals the semi-minor axis (half of the smallest diameter of the cell). Although this strategy does not provide us with the true size of the 3 dimensional cells, the resulting value serves

the purpose of ruling out mechanical stretching as an explanation for the distended probe signals.

Statistical Analyses

Statistical analyses were performed using Excel (Microsoft). The association between SADS and age was determined with a Pearson's correlation coefficient and simple linear regression models. A paired t-test was used to determine whether there was a difference in the mean number of SADS between the trisomic and disomic cells in the probands. For all statistical analyses, statistical significance was determined using an α level of 0.05.

Results

SADS Frequency

The percentages of trisomic cells present in peripheral blood cells from these individuals are shown in Figure 10. The mean percentage of trisomic cells in the subset of 9 subjects included in this study (μ=27.2%; range of 11.3% to 90.4%) was not significantly different from the mean (μ=38.0%) and range (10.6% to 90.4%) of the total mosaic cohort of 38 subjects (p=0.281). The overall frequencies of SADS+ cells in the participants with mosaic Down syndrome ranged from 0.07 to 0.21 (μ=0.12, SE +/- 0.02) (Table 5). Over all cells scored (pooled over the 9 study participants [total of 450 trisomic cells and 450 disomic cells]), the frequency of trisomic cells containing SADS (μ=0.15, +/- 0.02) was significantly greater than the frequency of disomic cells with SADS (μ=0.10, +/- 0.01) (p=0.001) (Table 5). The percentage of SADS+ cells showing distended alpha satellite regions for both of the chromosome 8 homologs (as opposed to distension of the satellite region from only 1 homolog) ranged from 0 to 50% (u=22%, SE +/- 5%) (Table 5).

No significant correlation was detected between age and the frequency of SADS in the trisomic cells (r = -0.598, p=0.089), disomic cells (r =-0.494, p=0.176), or overall (pooled disomic and trisomic) cell population (r =0.565, p=0.113) evaluated from the probands with mosaicism for Down syndrome (Table 5).

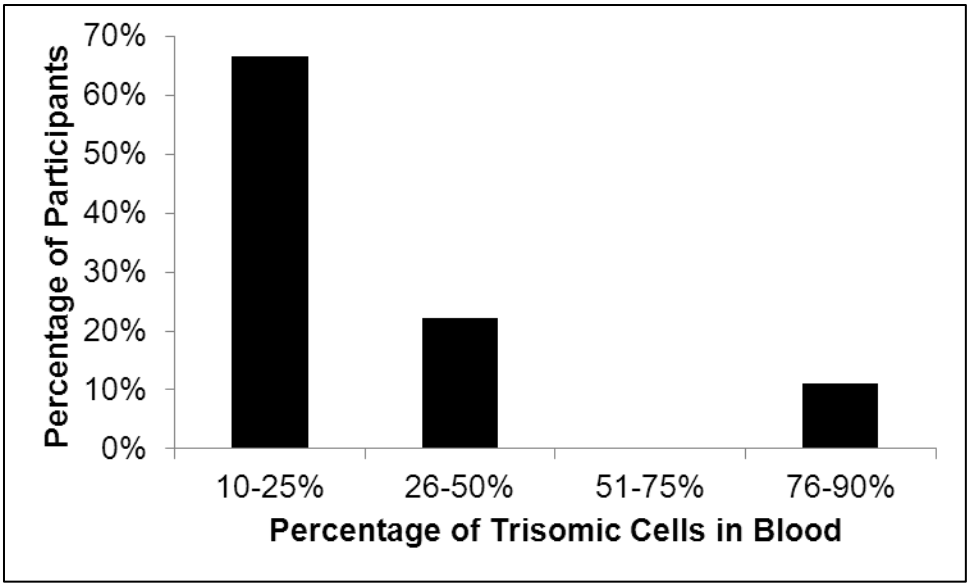

Figure 10. Distribution of the percentages of trisomic cells in blood specimens evaluated from the 9 participants selected for SADS study.

Table 5. SADS frequencies in participants with mosaic Down syndrome.

Age	Sex	Frequency of SADS+ Trisomic Cells	Frequency of SADS+ Disomic Cells	p value (SADS in Tri vs Di Cells)	Overall Frequency of SADS+ Cells	Percentage of SADS+ Cells with Distension in Both Chromosomes 8
1	M	0.26	0.16		0.21	29%
3	F	0.16	0.12		0.14	21%
9	M	0.14	0.10		0.12	25%
16	M	0.12	0.08		0.1	20%
18	F	0.24	0.18		0.21	38%
18	M	0.12	0.08		0.1	50%
24	F	0.10	0.04		0.07	14%
25	M	0.08	0.08		0.08	0%
45	F	0.10	0.08		0.09	0%
				0.001		
Mean		**0.15**	**0.10**		**0.12**	**22%**
SE +/-		**0.02**	**0.01**		**0.02**	**5%**

SADS Length

In each of the participants, the lengths of the distended satellites were not significantly different between the trisomic SADS+ cells and disomic SADS+ cells (Table 6).

Unique Sequence Probe Signal Length

Chromosome 8 and 21 unique sequence probe signal lengths were measured in 6 of the participants with mosaic Down syndrome. In each of these participants, there was no significant difference in the lengths of the chromosome 8 or chromosome 21 unique sequence probe signals between the SADS+ and SADS- cells (Table 7).

Nuclear Area

Nuclear area was measured in 6 of the participants with mosaic Down syndrome (Table 7). In 5 out of 6 probands, the nuclear area of SADS+ cells was not significantly different from that of the SADS- cells. In one participant, the nuclear area of the SADS+ cells (u=1.170, SE +/- 0.016) was significantly smaller than that of the SADS- cells (u=0.245, SE +/- 0.027) (p=0.045).

Table 6. Length of SADS regions present in trisomic and disomic cells (μm).

Participant Age	SADS Length in Trisomic Cells (u, SE)	SADS Length in Disomic Cells (u, SE)	p value
1	1.185, 0.105	1.422, 0.109	0.135
3	0.975, 0.107	1.018, 0.040	0.714
9	1.170, 0.130	1.300, 0.260	0.710
16	0.856, 0.043	1.073, 0.111	0.144
18	1.170, 0.157	1.194, 0.087	0.830
18	1.213, 0.074	1.154, 0.181	0.776
24	1.534, 0.274	1.235, 0.065	0.343
25	1.170, 0.053	1.203, 0.179	0.871
45	0.988, 0.121	1.300, 0.478	0.567

Table 7. Average chromosome 8 α-sat signal length, nuclear area and unique sequence probe signal lengths in SADS+ and SADS- cells of 6 participants (μm).

Age	8 α-sat Signal Length			Nuclear Area			8q22 Signal Length			21q22 Signal Length		
	SADS+ (μ, SE)	SADS- (μ, SE)	p value	SADS+ (μ, SE)	SADS- (μ, SE)	p value	SADS+ (μ, SE)	SADS- (μ, SE)	p value	SADS+ (μ, SE)	SADS- (μ, SE)	p value
1	1.275, 0.079	0.507, 0.021	**<0.001**	9.810, 0.721	12.930, 0.920	0.180	0.337, 0.027	0.442, 0.025	0.121	0.367, 0.021	0.377, 0.031	0.890
3	0.994 0.062	0.390, 0.033	**<0.001**	9.907, 1.025	11.465, 0.722	0.389	0.306, 0.027	0.377, 0.026	0.205	0.319, 0.031	0.277, 0.033	0.530
18	1.158, 0.083	0.416, 0.034	**<0.001**	11.090, 1.632	8.312, 1.327	0.373	0.381, 0.022	0.338, 0.018	0.361	0.332, 0.027	0.325, 0.025	0.704
24	1.449 0.197	0.468, 0.024	**0.002**	16.160, 2.582	15.383, 2.489	0.833	0.371, 0.053	0.585, 0.127	0.176	0.523, 0.063	0.581, 0.092	0.619
24	1.187, 0.087	0.598, 0.164	**0.018**	13.504, 2.136	14.005, 1.742	0.859	0.317, 0.019	0.442, 0.063	0.119	0.501, 0.039	0.507, 0.066	0.940
45	1.127, 0.212	0.429, 0.033	**0.011**	5.427, 0.511	7.838, 0.851	**0.046**	0.274, 0.018	0.260, 0.041	0.759	0.416, 0.042	0.451, 0.041	0.571

Discussion

This study was completed as a pilot, “proof of principle” project and was conceptualized after the majority of study participant specimens were collected. As a result, the methodology used for this pilot study differs from the approach that one would design as an optimal plan for evaluating SADS in the study participants’ trisomic and disomic cells. Indeed, since phytohaemmoglutinin (PHA)-stimulated leukocytes were evaluated, this study design introduced a bias for the inclusion of lymphocytes that are NOT senescent (e.g. enriched for cells that could successfully enter the cell cycle and progress to the mitosis portion of the cell cycle). Despite these methodological challenges, close agreement was observed between the frequencies of cells with at least one SADS region (12% of the overall total of cells evaluated) in the leukocytes evaluated in this study compared to the frequency of SADS (14%) observed in the early passage, non-senescent fibroblasts evaluated by Swanson, et al (2013). While quite preliminary, this result, which to our knowledge is the first study in which leukocytes have been evaluated for the presence of SADS, suggests that the frequency of SADS in non-senescent cells may be similar between leukocytes (with enrichment for lymphocytes) and fibroblasts. The results of this study demonstrate that SADS frequencies can be determined in peripheral blood specimens and serve as a “first” step toward the development of SADS as a potential marker for detecting senescent cells in patient samples.

The primary aim of this study was to determine if there was a difference in SADS in the isogenic trisomic-disomic cells from people with mosaicism for Down syndrome. In this mosaic model system, the disomic cells of each participant function as an ideal “control” group to determine differences attributable to a trisomy 21 imbalance. We observed that the frequency of SADS for satellite sequences from chromosome 8 was significantly greater in the trisomic cells than the disomic cells ($p=0.001$).

Unexpectedly, age was not associated with total SADS frequency (trisomic and disomic pooled), trisomic-cell SADS frequency, or disomic-cell SADS frequency. We had anticipated a positive association between age and SADS frequency, given that SADS is a senescence marker. The absence of an association between age and SADS frequency could be due to a number of reasons. This was a small pilot study with only 9 participants, which limited the ability to detect age-related differences in SADS. It is possible that the use of cultured lymphocytes, which were enriched for cycling cells, impeded the detection of the SADS frequency in senescent cells. Additionally, the premature aging in people with Down syndrome may cause the relationship between age and early-stage cellular senescence to be distinct from that of the normal population. Lastly, our population does not include a large number of older individuals. It is feasible that a larger study, including more older individuals, could reveal associations with age that were not detectable in this small pilot study.

Swanson et al., (2013) used a combination of pan-centromeric and chromosome-specific techniques. Considering that the results of their pan-centromeric experiments in

human fibroblast cell lines indicated that the majority of centromeres in SADS+ cells had α-sat distension, we limited our study to one chromosome instead of using a pan-centromeric approach. This approach allowed us to co-hybridize unique sequence probes to chromosomes 8 and 21 and to have the ability to clearly visualize and measure individual distended satellites (measurements of individual chromosomes cannot be confidently completed in cells using a pan-centromeric probe due to the overlap/entanglement of the multiple pericentromeric signals). Thus, from this dataset, we cannot conclude if there is a genome-wide tendency for increased levels of SADS in cells with a trisomy 21 imbalance. However, we anticipate that we would see SADS for other chromosomes and have identified this as an area for future study.

In addition to the binary categorization of cells as SADS+ or SADS-, we also measured the length of each probe signal to have the potential to assess the distension using continuous variables. For the unique sequence (non-satellite) probe signals evaluated from chromosomes 8 and 21, we saw no significant differences in the average lengths of these probe signals between the SADS+ and SADS- cells. This finding indicates that the distension of the chromosome 8 α-sat heterochromatin in the SADS+ cells did not arise as a result of mechanical stretching or other technical issues, since these factors would also be expected to impact unique sequences. We also measured a two-dimensional approximation of nuclear area in the SADS+ and SADS- cells. In 5 of the 6 participants, there was no significant difference in the size of SADS+ and SADS- nuclei. In one participant, the SADS+ nuclei were significantly smaller than the SADS- nuclei. Collectively, these findings confirm that SADS was not caused by mechanical stretching of the cells.

Only 22% of the SADS+ cells had distension of the α-satellite region in both chromosomes 8. This finding is different from the observation by Swanson et al. (2013) wherein most centromeres from senescent cells showed distension in SADS+ cells. While it is possible that there are chromosome-specific differences in the timing of SADS formation in senescent cells, which have not yet been defined, this observation most likely reflects the fact that the SADS observed in our participants were largely evaluated from non-senescent cells.

Study Limitations and Future Directions

As noted previously, our biggest study limitation was that this pilot project used PHA-stimulated, cycling cells (e.g. non-senescent). A future study could be completed on uncultured cells (possibly from different tissues) to better evaluate the *in vivo* frequency of SADS in trisomic compared to disomic cells. Our decision to evaluate α-satellite sequences only from chromosome 8 (due to budgetary limitations) could be a potential limitation. A future direction for this study could be to utilize a pan-centromeric approach for assessing SADS in the trisomic compared to disomic cells. Alternatively, one could also measure the lengths of the distended satellites from other specific chromosomes and compare those values with micronucleus frequencies for those chromosomes. For example, Swanson et al. (2013) used a probe specific to the chromosome 17 α-satellite region. Chromosome 17 may be an especially promising chromosome in which to detect SADS since it has been shown to have one of the highest rates of acquired, age-related chromosomal loss/instability (Leach, et al., 2004).

Thus, another future extension of the present study could be to quantify SADS in chromosome 17 and compare these results to those of Swanson et al. (2013).

To further characterize the presence of SADS in cells from people with Down syndrome, studies could be completed in participants with specific age-related phenotypes, such as dementia. Additionally, long-term longitudinal studies using repeat samples from the participants would be the optimal method to determine the age distribution of SADS formation in cells with a trisomy 21 imbalance.

In summary, in this study we demonstrated that SADS are present in peripheral blood cells. We also observed that cells with a trisomy 21 imbalance had a higher frequency of SADS than their isogenic cells without a trisomy 21 imbalance. Ultimately, this preliminary finding indicates that trisomy 21 may impact heterochromatin reorganization, and justifies further investigations of heterochromatin changes in people with Down syndrome.

Chapter 4. Somatic cell chromosomal instability in isogenic trisomic and disomic cells from people with mosaic Down syndrome

Introduction

Down syndrome (OMIM 190685) is the most common genetic condition (Sherman et al., 2007). Although the chromosomal etiology of Down syndrome (a trisomic imbalance for chromosome 21) has been known for decades (Lejeune, 1959), the biological basis for how this trisomic imbalance results in the constellation of over 80 phenotypic findings that have been reported in people with this condition remains elusive (Epstein, 1986; Jiang, et al., 2013). In addition to the congenital (also called constitutional) findings that typically lead to the diagnosis of this condition in infancy, adults with Down syndrome also show acquired traits, including premature aging (Head, et al., 2012; Lott, 2012; Zigman, 2013). Some of these age-related health changes include alopecia, immune system abnormalities, osteoporosis, menopause, obstructive sleep apnea, hearing loss, cataracts, and Alzheimer's disease (Zigman, 2013). As people with Down syndrome are now living to older ages (mean life expectancy age of 57.8 years for females; 61.1 years for males [Bittles and Glasson, 2004]), the profile of pathogenic aging related to a trisomy 21 imbalance continues to emerge.

Needs for improving our knowledge of factors contributing to the propensity for premature aging associated with trisomy 21 include expanding our knowledge of

acquired clinical findings and the age at which these traits develop in people with Down syndrome, as well as identification of biological changes that are present in trisomic compared to disomic cells. Recognition of age-related health conditions based on clinical traits in people with Down syndrome can be challenging for several reasons. Firstly, impaired cognitive and communication skills can mask the onset of age-related decline. Secondly, medical problems that are common in Down syndrome (including depression, thyroid abnormalities, and sleep apnea), can mimic the symptoms of age-related decline (Wark et al., 2014; Breslin et al., 2014; Prasher, 1999). For these reasons, identifying cellular or other biomarkers to aid in the assessment of pathological aging in people with Down syndrome is especially important for improving their quality of care. Moreover, the recognition of the cellular changes that arise as a consequence of a trisomic imbalance could aid in our understanding of the etiologies of several of these age-related health conditions.

Acquired chromosomal instability represents one subset of biomarkers associated with/related to aging in the general population, but there is a paucity of studies evaluating acquired somatic chromosomal instability frequencies in people with Down syndrome; especially as this cellular attribute relates to premature aging. In these few previous studies, investigators have observed increased micronucleus frequencies in buccal mucosa cells (Thomas et al., 2008; Ferreira et al., 2009) and decreased spontaneous micronucleus frequencies in lymphocytes (Scarfi et al., 1990) of people with Down syndrome compared to the levels seen in healthy, age-matched controls. Scarfi et al., (1990) found that micronucleus frequencies were elevated in lymphocytes

from people with Down syndrome compared to controls when cells were treated with mitomycin C, which cross-links DNA. Overall, the effect of trisomy 21 on chromosomal instability in lymphocytes is unknown. Genetic and environmental background differences between the participants in the trisomic and control groups have limited the investigators' ability to attribute the observed changes to influences directly reflective of a trisomic imbalance (Jones et al., 2011).

One approach to "unmask" the effects of a trisomic imbalance is to study people with mosaicism since they have both trisomic and normal cells that differ only for the presence (or absence) of an additional chromosome (in this study chromosome 21) (Davidsson, 2014). Importantly, this "mosaic" study design approach not only removes the confounding effects of inter-individual differences due to total genetic make-up, but also controls for the effects of environmental influences, since the trisomic and normal cells in people with mosaicism share identical exposure histories (Figure 1). The primary aims of this current study were to: (1) determine if there are differences in the micronucleus frequencies between the isogenic trisomic and disomic cells obtained from people with mosaicism for trisomy 21; and (2) determine if the micronucleus frequencies in the trisomic and/or disomic cells of people with mosaicism are influenced by age. To our knowledge, this is the first study of micronucleus frequencies in individuals with mosaicism for trisomy 21, and also the first to directly compare micronucleus frequencies in isogenic trisomic and disomic cells within an individual.

Materials and Methods

Ethics Statement

Human subjects' research was approved by the Virginia Commonwealth University IRB (protocol number #HM179 CR3). Written documentation of informed consent/assent was obtained from all research participants, with parental informed consent being obtained for children or adults who demonstrated intellectual disabilities levels that might compromise their ability to provide fully informed consent.

Study Participant Ascertainment

A total of 38 participants with mosaicism for Down syndrome were ascertained through their membership/participation with the International Mosaic Down Syndrome Association. The only selection criterion was that the study member had a chromosomally confirmed diagnosis of mosaicism for Down syndrome. After giving their informed consent/assent (Virginia Commonwealth University IRB Committee, protocol #HM179 CR3), peripheral blood specimens were collected for each study participant using venipuncture.

Quantitation of Chromosomal Instability

Chromosomal instability was quantified using the cytokinesis-blocked micronucleus (CBMN) assay (Fenech and Morley, 1985; Fenech and Morley, 1986). Micronuclei are cellular structures indicative of somatic cell chromosomal instability (Figure 11). A micronucleus can result from acentric chromosomal fragments, a chromatid, or whole

chromosomes that lag behind during the metaphase/anaphase transition (Figure 11). To evaluate micronuclei frequencies, leukocytes from the peripheral blood specimens were collected using Histopaque-1077 (Sigma) and established in culture according to our adaptation of the protocol of Thomas and Fenech (2007). Following mitogenic stimulation using phytohemaglutinin (PHA), lymphocytes were arrested at cytokinesis by adding Cytochalasin B (3.0 µg/ml; Sigma) to the cells 44 hours after culture initiation. At 72 hours, binucleate interphase cells were harvested as previously reported (Leach and Jackson-Cook, 2001). Briefly, this harvest included incubation in a hypotonic solution (0.075 M KCl) for 10 minutes, followed by fixation (three times using a 3:1 methanol:acetic acid solution). Slides were made following standard methods as described previously (Leach and Jackson-Cook, 2001).

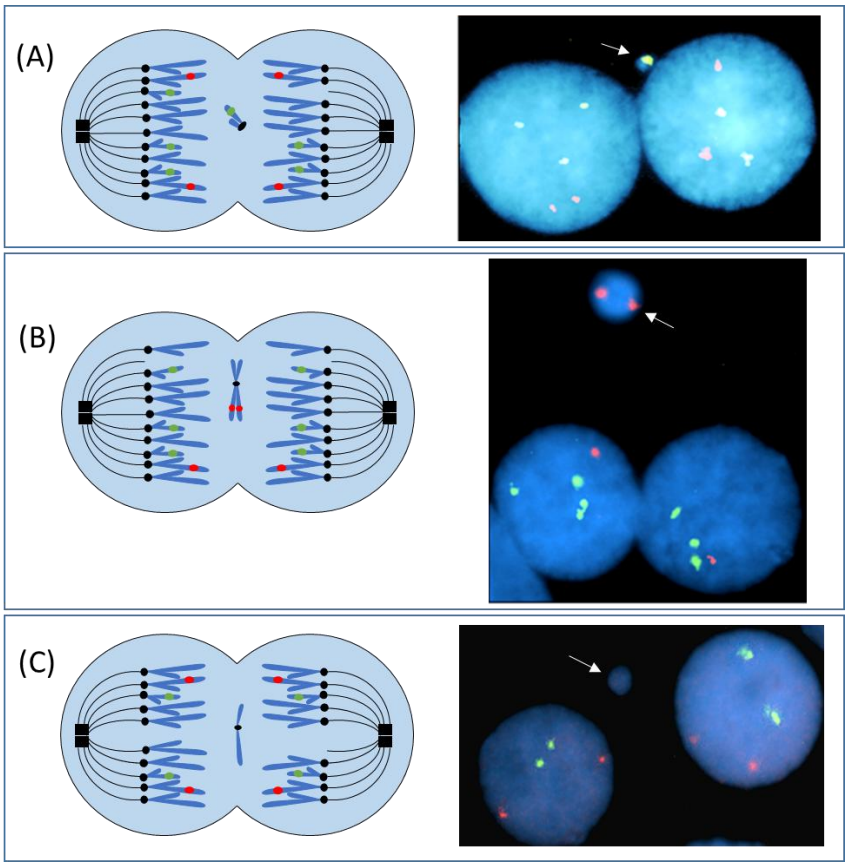

Figure 11. Cytokinesis-blocked micronucleus assay to quantify SCINF in the trisomic compared to disomic cells from people with mosaicism for Down syndrome. This diagram illustrates one mechanism (chromosome or chromatid lagging) whereby micronuclei can form. (A) During the metaphase of a mitotic division one chromatid from chromosome 21 fails to attach to the spindle fibers. As a result, this chromatid lags behind during the anaphase migration and fails to segregate to the spindle poles (left diagram). Following karyokinesis, the laggard chromosome could be excluded from the daughter cell nuclei and become enclosed in a micronucleus. In the right photomicrograph of a trisomic cell, at least a portion of chromatin from chromosome 21 was excluded into a micronucleus (white arrow). Only 2 signals for the chromosome 21 probe are present in the right daughter nucleus (loss of a chromosome 21 signal) compared to 3 signals that are present in the left daughter nucleus (RUNX1 probe [21q22; green]; RUNX1T1 probe (8q22; red)]. In panel (B) trisomic binucleates (3 signals for the chromosome 21 probe) are shown (illustration on left; photomicrograph on right) that had loss of one replicated chromosome 8 (both sister chromatids) into a micronucleus, resulting in daughter cells that each had a monosomic imbalance for chromosome 8. (C) A disomic binucleated cell (both daughter cells have 2 signals for the chromosome 21 and chromosome 8 probes) has a single micronucleus that does not contain chromatin for either the RUNX1 or the RUNX1T1 loci. This micronucleus could contain chromatin from either a chromatid (as shown in the illustration on the left), a replicated chromosome other than chromosome 21 or 8, or a portion of a chromosome that does not encompass the regions present in the probes.

Micronucleus visualization and FISH

To distinguish between trisomic and disomic nuclei, a “test” probe targeting the RUNX1 locus (21q22) was hybridized (Abbott Molecular). A probe for the RUNX1T1 locus (8q22) was also hybridized as an autosomal control probe (Abbott Molecular). FISH was completed according to the manufacturer’s protocol (Abbott Molecular). Briefly, the DNA in the target chromatin (which was affixed to a microscope slide) and the probes were co-denatured at 73°C for 2 minutes. Following denaturation, 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 0.4X SSC/0.3% NP-40 solution at 73 °C for 2 min, followed by a 2 min wash at room temperature (in a 2X SSC/0.1% NP-40 solution). To visualize the binucleated cells and micronuclei, the chromatin in the nuclei and micronuclei was counterstained with DAPI II/antifade (Abbott Molecular). The probes used in this study have been validated for use in our CLIA and CAP approved laboratory and consistently show specificity and sensitivity values of 0.98 or higher.

Micronucleus Scoring

An Axioskop equipped with single- and triple-band pass filters was used to score slides (Zeiss). Micronucleus frequencies were quantified by scoring 1000 binucleated cells. Micronuclei were identified according to the criteria established by Fenech (2007). The number of fluorescent signals present in the daughter nuclei, as well as the micronuclei, 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. Binucleated cells that did not have clear borders or that were overlapping were excluded from the

analysis. The 1000 scored binucleates were categorized as trisomic or disomic according to the chromosome 21 probe signal count. If at least one of the binucleated cells had 3 signals for the chromosome 21 probe, this cell was categorized as a trisomic cell. Each of the micronuclei associated with the binucleates were categorized as follows: (1) positive for chromatin from chromosome 21 (21+ micronuclei); (2) positive for chromatin from chromosome 8 (8+ micronuclei); (3) positive for chromatin from chromosomes 21 and 8 (21+, 8+ micronuclei); or (4) negative for chromatin from chromosomes 21 and 8 (21-, 8- micronuclei). Representative images of micronucleated binucleates are shown in Figure 11.

Statistical Analyses (this section was written by Dr. K Archer)

Total Micronucleus Frequency

The Shapiro Wilk's test for normality for total micronucleus frequency yielded a p-value of 0.078. Because most goodness-of-fit tests have low power, this p-value along with previously conducted studies (Ceppi et al, 2010) indicate that a Gaussian distribution may not be the most suitable for statistical analysis. Therefore, we then examined whether the total micronucleus frequency data would be more appropriately modeled using a negative binomial versus a Poisson distribution by performing a boundary likelihood ratio test. The resulting p-value <0.0001 so the negative binomial distribution is most appropriate for modeling the micronucleus frequency data.

Trisomic vs Disomic Micronucleus Frequency

The percentage of trisomic cells present in these blood specimens was evaluated as part of a previous study, with this data being collected from the same slides that were used for the micronucleus frequency scoring. Because 1,000 binucleated cells were scored for each subject, the expected number of trisomic binucleate cells was obtained by multiplying the proportion of trisomic cells observed in the blood specimen by 1,000; likewise, the expected number of disomic binucleated cells was obtained by the following formula: 1-proportion of trisomic cells)*1,000. These values were used as offsets in the Negative Binomial mixed effect models predicting micronucleus frequency, where subject was considered a random effect, to account for the correlated observations within an individual. Models were fit using the R lme4 package and glmer.nb function; however, the models failed to converge without warning so Poisson mixed effects models were fit using the glmer function instead. To further determine whether there were age-related differences in micronucleus frequency, the participants were divided into two age groups: (1) young children (1-11) and (2) teens/adults (15-44). When examining whether there were differences in micronucleus frequency between trisomic and disomic cells within the young and older age groups, cell type, age group, and the interaction between cell type and age group were included as fixed effects in the Poisson mixed effects model predicting micronucleus frequency considering subject as a random effect.

Micronucleus Contents

To determine if the frequencies of the chromosome 8 and chromosome 21 probes present in the micronuclei reflected a random versus non-random distribution, a chi-square goodness of fit test was performed, which compared the observed to expected frequencies. Expected values were derived based on the assumption that all autosomes in the cell had an equal probability for exclusion into a micronucleus (sex chromosomes were excluded from this analysis since the sex chromosome complement differed between males and females). For example, for chromosome 8, there was a 2 in 44 (4.5%) expected chance for chromosome 8 to be present in a micronucleus associated with a disomic cell (two copies of chromosome 8 were present, with a total of 44 autosomes in the disomic cells) or a 2 in 45 (4.4%) chance for microuclei associated with a trisomic cell (two copies of chromosome 8, with a total number of 45 autosomes in the trisomic cells). For chromosome 21, the disomic cells have the same random expectations for exclusion into micronuclei as chromosome 8 (2 in 44 or 4.5%). However, for the trisomic cells the random expectations were calculated to reflect the fact that there was a 3 in 45 chance (6.7%) for random exclusion of a chromosome 21 into micronuclei (total number of chromosomes 21 compared to the total number of autosomes).

Results

Study Participant Characteristics

The 38 study participants included 17 males and 21 females. The participants ranged in age from 1-44 years with an overall mean of 11.8 years. The percentage of trisomic cells in the participants with mosaicism ranged from 10.6 to 90.4 (Table 8). The percentage of trisomic cells did not differ between children (age ≤11) versus teens/adults (age ≥15) ($p=0.93$). Also, there were no significant differences in constitutional phenotypic traits or acquired physiological health conditions between the younger children and teens/adults cohorts (Table 8).

Table 8. Age, Cytogenetic and Health Characteristics* of the study participants who have mosaicism for Down syndrome.

	Age Group		
	Young children	Teens/Adults	p value
Age range	1 - 11 yo	15 – 44 yo	
Mean (se) age	6 (0.65)	23 (2.25)	
Sex	12 Males; 13 females	5 Males; 8 females	0.734
Mean (se) percentage of trisomic cells	38.23 (5.68)	37.35 (9.22)	0.928
Congenital Heart disease	5/20 (25%)	1/7 (14%)	0.999
Thyroid disease	1/18 (6%)	1/7 (14%)	0.490
Sleep Apnea	4/9 (44%)	4/4 (100%)	0.105
Brachycephaly	11/19 (58%)	5/6 (83%)	0.364
Flat Nasal Bridge	12/20 (60%)	2/6 (33%)	0.365
Epicanthal Folds	14/20 (70%)	3/6 (50%)	0.628
Upslanting Palpebral Fissure	9/20 (45%)	3/6 (50%)	0.999

(P values with Fisher's exact test [2-tailed] using 2x2 contingency table)
*Information about phenotypic traits was not available for all probands

Total Micronucleus Frequency

The mean micronucleus frequency over all cells for the mosaic participants was 17.45. The continuous form of age was significant when fitting a negative binomial model to predict total micronucleus frequency (p= 0.0059). The marginal effect of age from this model reflects an increase in micronucleus frequency by 26.96% for every year increase in age. When dichotomizing age as young (<=11, N=25) versus older (>=12, N=13), age group was still significant when fitting a negative binomial model to predict total micronucleus frequency (p= 0.012) (Table 9). From this model the increase in micronucleus frequency between the young and older age groups is expected to be 5.87.

Trisomic and Disomic Micronucleus Frequencies

In a model that included only cell type (trisomic/disomic) as a fixed effect (that is, with no adjustment for age), there was a significant difference in the number of micronuclei observed in trisomic versus disomic cells overall ($p = 1.00 \times 10^{-4}$). Among younger patients, there was no difference in micronucleus frequency between trisomic (0.17 mean ± 0.02 SE) and disomic (0.14 mean ± 0.02 SE) cells (p=0.091) (Figure 12). However, among older subjects there was a significant difference (trisomic 0.34 ± 0.05; disomic 0.16 ± 0.03) (p=<0.0001) (Figure 12). There was no significant difference when comparing younger children versus teens/adults with respect to disomic micronuclei (p=0.329) (Table 9).

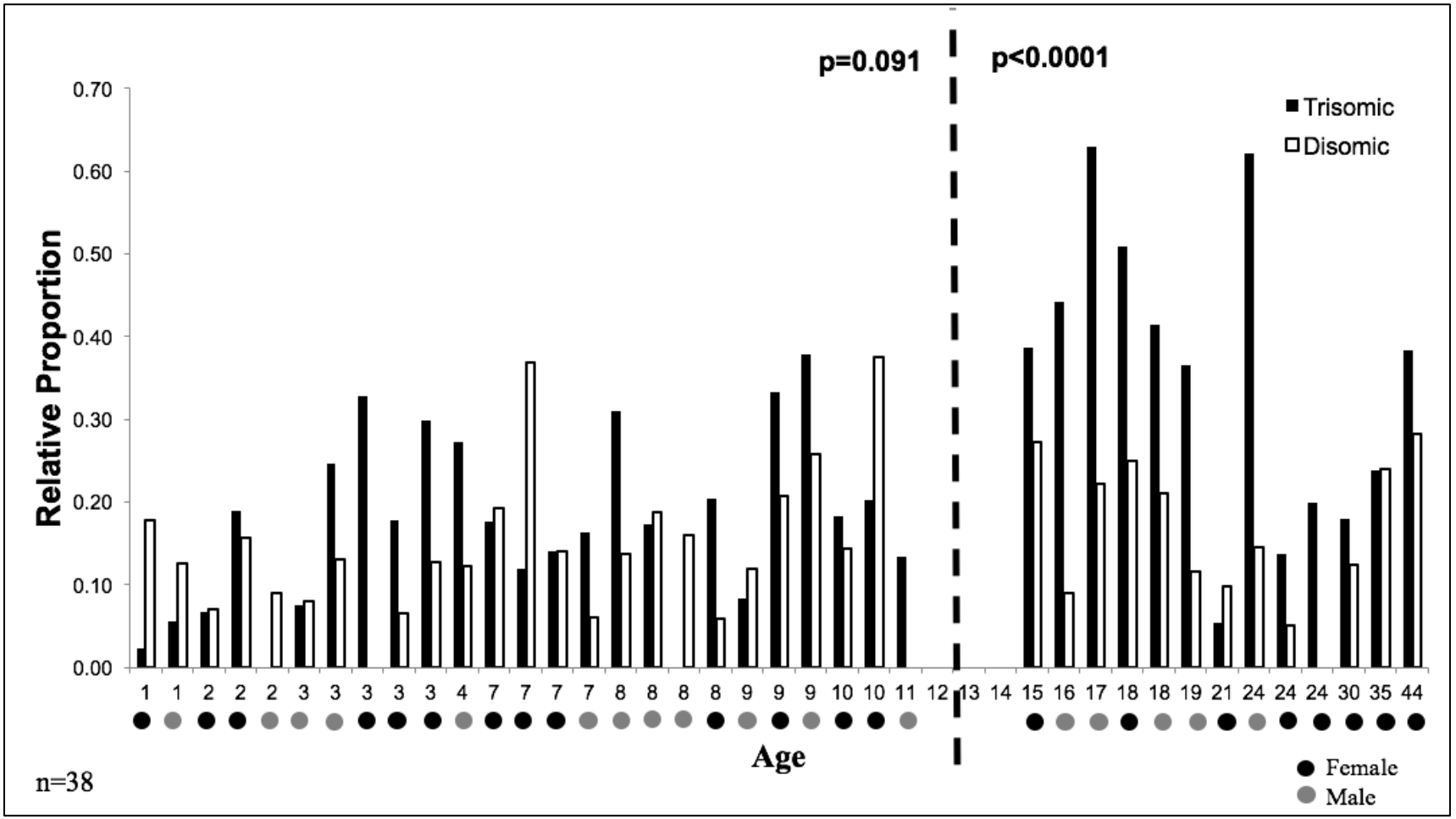

Figure 12. Relative proportions of trisomic (black) and disomic (white) cells containing micronuclei in each participant, ordered by age. In the young children age group (1-11 years of age), there was no significant difference in the relative proportions of micronuclei present in the trisomic and disomic cells (p=0.091), but in the teens/adults group (15-44 yrs) the trisomic cells showed significantly higher levels of micronuclei than the disomic cells (p<0.0001). The sex of each participant is shown below (dark circles represent females; grey circles represent males).

Table 9. Comparison of Micronucleus Frequency in Young Children and Teens/Adults.

	Age Group	Range	Mean	SE (+/-)	p value
Overall Micronucleus Frequency					
	Young Children	0.07-0.29	0.15	1.18	
	Teens/Adults	0.09-0.37	0.21	2.33	
					0.012
Proportion of Micronuclei in Disomic Cells					
	Young Children	0.00-0.38	0.14	0.02	
	Teens/Adults	0.00-0.28	0.16	0.03	
					0.329
Proportion of Micronuclei in Trisomic Cells					
	Young Children	0.00-0.38	0.17	0.02	
	Teens/Adults	0.05-0.63	0.34	0.05	
					0.001

Distribution of Chromosomes in Micronuclei

There was no evidence of non-random chromosomal inclusion for either chromosome 8 or chromosome 21 (p=1 and 1, respectively). However, from a Poisson mixed effect model that included age group, trisomic/disomic cell status, chromosome 8/21 and all possible interactions, there was a significant difference when comparing the following groups with respect to micronuclei from chromosomes 8 versus 21 after accounting for total trisomic/disomic cells scored: (a) micronuclei containing chromosome 8 compared to chromosome 21 in the disomic cells from the young cohort (p=0.014); (b) micronuclei containing chromosome 8 compared to chromosome 21 in the trisomic cells of the young cohort (p=0.0007); (c) the micronuclei containing chromosome 8 compared to chromosome 21 in the disomic cells of the teens/adults (p=0.03); and (d) the micronuclei containing chromosome 8 compared to chromosome 21 in the trisomic cells of the teens/adults (p=0.0004) (Table 10).

Interestingly, the majority of micronuclei (74%) in the trisomic cells contained chromatin that was derived from chromosomes/areas other those interrogated in this study, suggesting that the observed increase in chromosomal instability in the trisomic cells may have a broad effect on multiple chromosomes (genome-wide).

Table 10. Distribution of Chromosomes in Micronuclei.

	Range	Mean	SE	p value	Range	Mean	SE	p value
	Young Children				Teens/Adults			
Proportion of Trisomic Cell Micronuclei Containing								
Chromosome 8	0.00-0.17	0.07	0.03		0.00-0.11	0.06	0.02	
Chromosome 21	0.00-0.33	0.16	0.05		0.09-0.67	0.26	0.08	
				0.0007				0.0004
Proportion of Disomic Cell Micronuclei Containing								
Chromosome 8	0.00-0.13	0.01	0.01		0.00-0.11	0.02	0.02	
Chromosome 21	0.00-1.00	0.12	0.06		0.00-0.50	0.10	0.07	
				0.014				0.03

Telomere length and chromosomal instability

No significant correlation between average telomere length (trisomic and disomic) and chromosomal instability (total micronucleus frequency) was observed ($r = -0.013$, $p=0.972$), but the sample size was small (Figure 13).

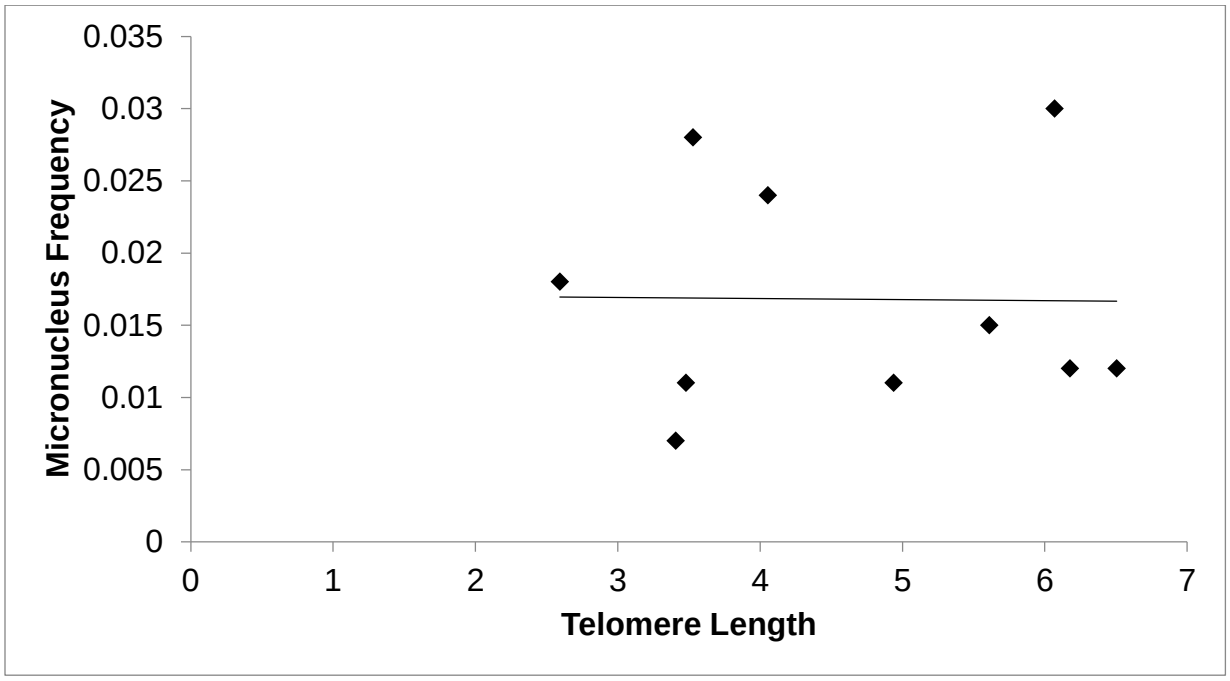

Figure 13. Average telomere lengths and overall micronucleus frequencies in cohort 1 participants (n=10). There was not a significant correlation between average telomere lengths (trisomic + disomic) and micronucleus frequencies.

Comparison of SADS frequencies with micronucleus frequencies

There was not a significant correlation between the SADS and micronucleus frequencies in 9 participants with mosaic Down syndrome (r= -0.171, p=0.660) (Figure 14).

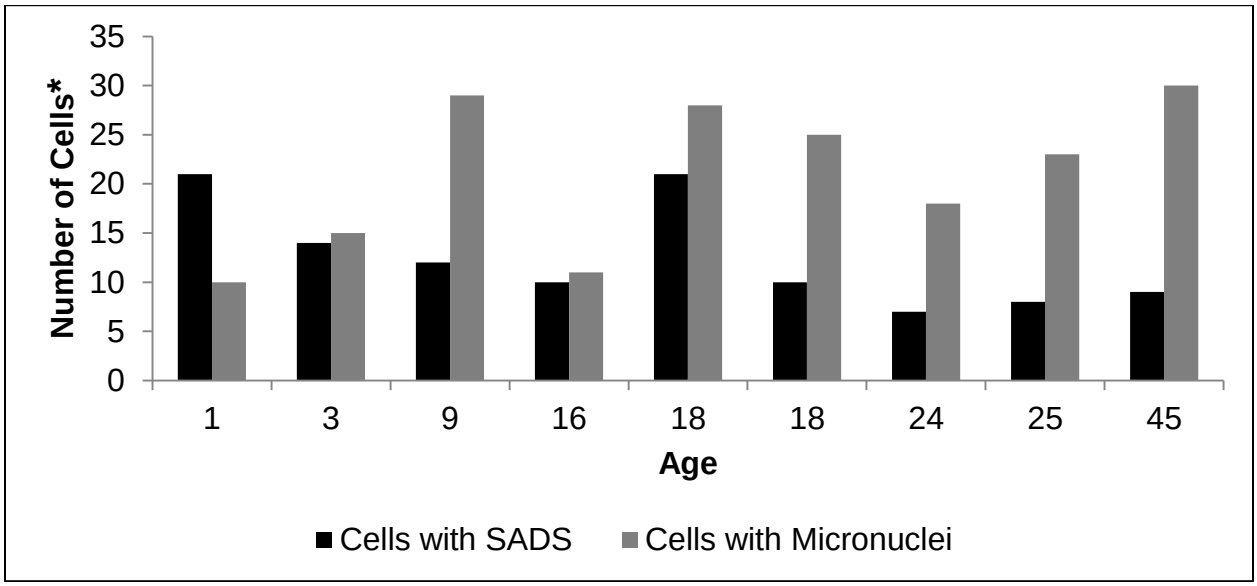

Figure 14. Comparison of SADS and micronucleus frequencies in 9 participants with mosaic Down syndrome. *A total of 100 cells were scored in the SADS assay, and 1000 cells were scored in the micronucleus assay.

Discussion

Our study design, in which we compared micronucleus frequencies between trisomic and disomic cells that had identical genetic backgrounds (isogenic) and environmental histories (since they were both from the same person), allowed us to directly assess the impact of a trisomy 21 imbalance on chromosomal instability. An overall age-related increase in micronuclei was observed in the study participants with mosaicism for Down syndrome, which is a finding that is consistent with data derived from studies of healthy, euploid (chromosomally normal) people (Fenech et al., 1985). Given this observed age effect, we further compared micronucleus frequency values in young children to those seen in teens/adults to establish whether a trisomy-specific age effect was present. Indeed, the trisomic cells in the teens/adults showed significantly greater values of micronuclei than the disomic cells, but this difference was not detected between the trisomic and disomic cells of the younger children. This observation suggests that as people with mosaicism for Down syndrome age, their trisomic cells become increasingly susceptible to chromosomal instability compared to cells with a normal chromosomal complement. To our knowledge, this is the first report of an age-dependent difference in chromosomal instability between trisomic and disomic cells.

The age-dependent nature of the association between trisomy 21 and chromosomal instability is a topic that warrants further study. The increase in chromosomal instability in cells with trisomy 21 could be a key factor in the onset of premature aging in people with Down syndrome and mosaicism for Down syndrome.

Given the multi-factorial nature of chromosomal instability, there are several aspects to consider in trying to better understand the underlying basis for our observation. Based on the delineation of our two age groups, it is possible that the onset of puberty marks an initiating period of increased propensity for chromosomal instability in trisomic cells. The completion of puberty has been shown to be associated with increased micronuclei frequencies in the chromosomally normal individuals (Jones et al., 2011). However, in our cohort, the age-related increase in micronucleus frequency was exclusive to the trisomic cells, as there was no significant difference in the relative proportions of disomic cells with micronuclei between the children and teens/adults. It is unclear why the completion of puberty would be associated with increased chromosomal instability solely in the trisomic cells, but one could speculate that this might reflect differential responses of the trisomic cells to hormone levels and/or cellular stress or oxidation levels (Garlet et al., 2013). In healthy adults with euploid chromosomal complements, the greatest increases in micronucleus frequencies are observed when people reach their 50s and 60s (Jones, et al. 2011; Bonassi et al., 2001; Fenech et al., 2011). It is plausible that the trisomic cells showing increases in micronucleus frequencies in the teens/young adults observed in this study have a “biological” age that more closely parallels that of a euploid person in their 40s, 50s or 60s. Indeed, people with Down syndrome begin to show physiological traits associated with aging decades earlier than euploid individuals (Zigman, 2013).

It is interesting to note that increased levels of micronucleus frequencies have been observed in older people with normal chromosomal complements who develop

Alzheimer disease and Parkinson disease (Migliore et al., 2011). In particular, there is evidence that people with Alzheimer's disease acquire cells with a trisomic imbalance for chromosome 21 (Potter, 1991). In 1999, Geller and Potter used FISH in primary fibroblast cultures from people with Alzheimer's disease and unaffected controls to demonstrate that people with Alzheimer's disease have an approximately two-fold increase in the number of cells with trisomy 21. Age-related increases in micronucleus frequencies have also been observed in numerous tissues, including liver, heart, small intestines, spleen, and brain (Schmid and Pfitzer, 1985; Nicholson et al., 2015). Currently, it is not clear if chromosomal instability is a "driver" in the cascade of events leading to these aging phenotypes, or if it is a correlative finding that shares an underlying biological basis with driver elements that lead to the acquisition of age-related traits. Regardless of its "driver" or "passenger" status, our finding that cells with trisomy 21 are susceptible to age-related increases in micronucleus frequency suggests that assessments of somatic cell chromosomal instability have the potential (following more confirmatory studies) to serve as a biomarker to assist with the recognition of atypical aging/health conditions in people with Down syndrome or mosaicism for Down syndrome.

In addition to comparing micronucleus frequencies between the isogenic trisomic and disomic cells, we also assessed if the micronuclei demonstrated a non-random pattern of inclusion of chromatin derived from chromosome 21 (e.g. trisomy rescue or other types of mechanisms). The inclusion of chromatin from chromosome 21 into micronuclei was not significantly different from random expectations. However, in

the trisomic and disomic cells of the young children and teen/adult participants, chromosome 21 was included in micronuclei more often than chromosome 8. There are several factors that one could speculate to be contributory to differential exclusion of chromosomes 21 and 8 into a micronucleus. Chromosome 21 has many late-replicating regions, which could result in an increased likelihood of delay in completing DNA synthesis; the latter of which could contribute to this chromosome lagging behind the other chromosomes in its completion of the S phase, possibly contributing to spindle attachment errors (Watanabe et al., 2002; Woodfine et al., 2004). There are conflicting reports regarding the localization of chromosome 21 within the nucleus. Early studies by Bolzer et al., (2005) and Sun et al., (2000) predicted that chromosome 21 localizes near the center of the nucleus due to its small size. In contrast, Manvelyan et al., (2008) reported that chromosome 21 locates at the periphery of the nucleus because of its comparatively low gene density. Peripheral localization of chromosome 21 could be another mechanism by which chromosome 21 migrates late to the metaphase plate and becomes excluded into a micronucleus. Importantly, the majority of micronuclei from both the trisomic (74%) and disomic (84%) cells of the teens/adults evaluated contained chromatin from regions other than those included in the chromosome 21 and chromosome 8 probes. Thus, the observed increase in micronucleus frequency in the trisomic cells of these teens/adults is most reflective of an apparently global, genome-wide increase in somatic cell chromosomal instability, rather than one that is limited to chromosome 21 (or 21q22).

There was not an association between telomere lengths and chromosomal instability in the cohort 1 participants. This was unexpected, because telomere shortening is known to compromise genomic stability (Murnane, 2012; Gonzalo and Eissenberg, 2016). Possible explanations for the observed lack of an association between telomere length and chromosomal instability are discussed in the summary chapter.

We also compared satellite distension (Chapter 3) to chromosomal instability frequencies to determine if there was association between these cellular attributes. Although the frequencies of both of these acquired chromosomal changes are greater in cells with trisomy 21, we did not detect an association between these attributes. This finding suggests that the cellular changes contributing to micronucleus formation and satellite distension may be different. This finding is also discussed further in the summary chapter.

A potential limitation of this research is that the distribution of study participants is skewed toward younger ages, which reflects the membership of the parent support group (IMDSA) through which they were ascertained. Future studies in which the age range of individuals with mosaicism for Down syndrome could be expanded may help to better understand the continuum of age-related findings contributing to micronucleus frequency. However, since cytogenetic methods to identify mosaicism via clinical testing were not widely available until the 1970s, it is quite challenging to identify people with a confirmed diagnosis of mosaicism who are in their upper 40s to 60s.

In summary, the results of this study suggest that an increase in the frequency of somatic cell chromosomal instability is an age-related, trisomy 21-associated cellular attribute that is not limited to alterations involving chromosome 21. Importantly, the results of this study illustrate the value of the isogenic mosaic Down syndrome model system for teasing apart the impact of a trisomy 21 imbalance on the presentation of complex traits associated with Down syndrome.

Chapter 5. Somatic cell chromosomal instability in isogenic trisomic and disomic cells from people with Down syndrome and regression

Introduction

Down syndrome is the most common genetic condition that results in intellectual disability (Daunhauer et al., 2014). While the chromosomal etiology of Down syndrome has been known for 58 years (Lejeune, et al., 1959), the biological basis for how this trisomic imbalance results in intellectual disability, as well as other health and/or behavioral conditions, remains enigmatic. In addition to the congenital findings that typically lead to the diagnosis of Down syndrome in infancy, adolescents and adults with this condition also show behavioral conditions. One of the most common, but under-studied concerns for adults with Down syndrome is depression (Borthwick-Duffy,1994; Leboyer, et al., 1998; Horovit, et al., 2011). Also, research findings suggest that individuals with Down syndrome have an increased risk for internalizing psychopathology (Hermans and Evenhuis, 2012). Some adolescents and young adults with Down syndrome experience profound behavioral deterioration. This phenomenon has been described using various terms, including developmental regression (Devenny and Mathews, 2011), obsessional slowness (Charlot et al., 2002), Down syndrome disintegrative disorder (Worley et al., 2014), acute neuropsychiatric disorder (Akahoshi et al., 2012), and catatonia (Jap and Ghaziuddin, 2011). Here, we use the term regression. Down syndrome regression is characterized by a decline in cognitive abilities, changes in behavior, impairments in adaptive functioning, motor disturbances,

and mood changes (Ghaziuddin et al., 2015; Jacobs et al., 2016). In these patients, regression results in a loss of previously acquired skills (Prasher, 2002). The cause of the deterioration is unknown and is distinct from the onset of Alzheimer's dementia. General medical explanations have been ruled out with clinical evaluations and laboratory testing (Ghaziuddin et al., 2015). A summary of published case reports is shown in Table 11.

Table 11. Case Reports of People with Down Syndrome Who Demonstrate Regression.

Reference	Cases	Symptoms	Treatment	Outcome
Prasher, 2002	Minority of people with Ds between ages 15-30	-Regression in cognition, language, mobility, adaptive and social skills -Mutism, withdrawal, low mood	Often treated with antidepressants	Some improvement with antidepressants
Charlot et al., 2002	6 females, 5 males, ages 25-42	Slowness in ADL, OCD, tics	SSRIs	-2 improved -9 partially improved
Jap and Ghaziuddin, 2011	-Case 1: female age 16 -Case 2: female age 14	-Case 1: psychomotor slowing, staring, rigidity, obedience, hallucinations, weight loss, withdrawal -Case 2: depression, psychotic symptoms, oppositional defiant disorder, impulse control disorder	-Case 1: lorazepam, bilateral ECT, fluoxetine -Case 2: nortriptyline, fluoxetine, lorazepam	-Case 1: No improvement with lorazepam; improved with ECT, fluoxetine -Case 2: No response to nortriptyline or fluoxetine; some improvement with lorazepam
Akahoshi et al., 2012	7 females, 6 males, ages 10-29	Slowness, OCD, abulia, withdrawal, mutism, insomnia, depression, self-talking/laughing	SSRIs, barbiturates, antipsychotics, benzodiazepenes,	-3 improved -7 partially improved -1 did not improve -2 no data
Stein et al., 2013	Female age 13	Loss of language, social, and toileting skills after move, classroom change, puberty and OSA onset	Antidepressants CPAP, increased psychosocial support	-Returned to baseline function

Garvia and Benejam, 2014	3 females, ages 22, 25, 26	Cognitive decline, withdrawal, psychomotor slowing, rebellion, mutism -Decline after life changes	Antidepressants, antipsychotic	1 case improved with antidepressant, 2 cases improved only slightly with antipsychotics
Torr and D'Abrera, 2014	Female age 23 mosaic Ds	Depression after life events, mutism, psychomotor slowing	Antidepressants, bilateral ECT	-No improvement with antidepressants -Improvement with ECT
Ghaziuddin et al., 2015	2 females ages 15,16 2 males ages 16,18	Unspecified catatonia, psychomotor slowing, stereotyped behaviors, unusual movement, decline in ADL	Antidepressants, antipsychotics, antiepileptics, stimulants, lorazepam, ECT	-No improvement with antidepressants, antipsychotics, antiepileptics, stimulants -Most improved with lorazepam and ECT
Worley et al., 2015	7 females, 4 males, Mean age 14.8	Autistic deterioration, cognitive decline, insomnia	Antidepressants, antipsychotics, cognition-enhancers	No medication was effective in all cases, but each helped some cases
Jacobs et al., 2016	-male, age 19	Psychotic symptoms, staring, loss of speech and written expression, decreased ADL, sad affect	Antidepressants, antipsychotics	Parent reports 85% return to baseline level of functioning with clozapine

ADL- activities of daily living; OCD- obsessive compulsive disorder; SSRI- selective serotonin reuptake inhibitor; ECT- electroconvulsive therapy; OSA- obstructive sleep apnea; CPAP- continuous positive airway pressure.

Behavioral Symptoms

The specific behavioral changes vary amongst those with regression, but there are often similar patterns. In several case studies investigators have noted that people with Down syndrome who develop regression show obsessional slowness in completing activities of daily living. Charlot et al. (2015) described patients who would take hours to complete everyday routines such as eating, bathing, and dressing. Sometimes these obsessive behaviors coincide with a diagnosis of obsessive compulsive disorder (Akahoshi et al., 2012). In addition to slowness, other barriers to completing activities of daily living can include perfectionism and inflexibility in daily routines. Some patients with Down syndrome regression are aggressive. Charlot et al. (2015) described a patient with aggressive tendencies if routines were interrupted, and Ghaziuddin et al. (2015) reported unprovoked aggression in a patient with no prior history of aggressive behaviors. Regression can cause people with Down syndrome to be oppositional and defiant (Garvia and Benejam, 2014) or overly obedient (Jap and Ghaziuddin, 2011). Multiple patients were reported to be socially withdrawn and mute following clinical deterioration (Prasher 2002). Sleep behaviors appear to be altered too, as multiple patients with regression developed insomnia (Akahoshi et al., 2012; Worley et al., 2015).

Cognitive Decline

Various types of cognitive deficits occur in people with Down syndrome and regression. Communication abilities, both oral and written, can deteriorate. Jacobs et al. (2016) described a patient with incoherent speech and one-word responses. Some

patients have episodes of self-talking and self-laughing (Akahoshi et al., 2012). Other cognitive changes that have been reported in patients with regression include new onset autism (Worley et al., 2015) and psychotic episodes (Jap and Ghaziuddin, 2011; Jacobs et al., 2016). Although the cognitive deficits can appear similar to those of people with Alzheimer's disease, this condition has been described as a distinct process, as the skills can be recovered and the onset is earlier than that of dementia.

Mood Changes

Low mood is a common symptom of regression in people with Down syndrome. Patients with regression have been described as depressed, tearful, socially withdrawn, and uninterested in things that they previously enjoyed (Prasher, 2002; Torr and D'Abrera, 2014). Additionally, there are seven documented cases of young adults with Down syndrome who had unspecified catatonia. In these patients, catatonia was successfully treated with electroconvulsive therapy and/or benzodiapines (Jap and Ghaziuddin, 2011; Torr and D'Abrera, 2014; Ghaziuddin et al., 2015).

Motor Disturbances

Psychomotor slowness is one of the most frequent symptoms of regression in people with Down syndrome regression (Prasher 2002; Charlot et al., 2002; Jap and Ghaziuddin, 2011, Akahoshi et al., 2012; Garvia and Benejam, 2014; Torr and D'Abrera, 2014; Ghaziuddin et al., 2015). Other motor disturbances can include tics, unusual posturing, facial grimacing, freezing, and repetitive, stereotyped movements (Jacobs et al., 2016).

Association with Adverse Life Events

In some of the case studies, researchers have noted that a major life change or adverse event occurred prior to the onset of regression. Examples of such adverse life events include a family move and change in classroom placement (Stein et al., 2013), death of friends (Torr and D'Abrera, 2014), self-rejection of Down syndrome (Garvia and Benejam, 2014), and graduation from high school (Jacobs et al., 2016), However, regression is not always preceded by an identifiable life change. It is not yet clear whether an adverse life event can contribute to regression, or whether these events are coincidental and not associated with the deteriorations (Jacobs et al., 2016).

Diagnostic Work-Up

Consensus guidelines have not yet been established for a diagnostic work-up in patients with Down syndrome who have symptoms of regression. Jacobs et al. (2016) proposed a tiered, systematic approach to the clinical evaluation of patients with apparent regressive symptoms. The first tier of evaluations includes tests for medical problems that are prevalent in Down syndrome, such as hypothyroidism, sleep apnea, depression, infections, celiac disease, cataracts, and hearing loss. Lower tiers include evaluations for medical problems that could cause symptoms of regression but are not necessarily more prevalent in people with Down syndrome.

Treatment

The clinicians who have summarized case reports about their patients with Down syndrome regression have taken various treatment approaches. Different classes of antidepressant, antipsychotic, and anticonvulsant medications have been used with varying degrees of success. Other medicine-based approaches have included cognition-enhancing drugs and stimulants. Several clinicians have had positive results with electroconvulsive therapy in catatonic patients. In one patient with OSA and depressed mood, CPAP and increased psychosocial support were successful treatments. Currently, there is not a consensus about the optimal treatment strategy in patients with Down syndrome regression. Considering the diversity in the treatments which have resulted in successful remission, it is likely that there is not a single, ideal treatment for all patients with regression but rather that individualized approaches are best.

Chromosomal Instability in Patients with Down Syndrome Who Demonstrate Regression

The cause of regression in adolescents and young adults with Down syndrome is unknown. This deterioration affects only a minority of people with Down syndrome, and it is not known whether there are risk factors that make certain individuals more likely to develop this condition. If an acquired cellular change is associated with Down syndrome regression, this could be a candidate biomarker for identifying people at increased risk. One potential biological change that might be associated with regression in people with Down syndrome is acquired cell chromosomal instability.

Somatic cell chromosomal instability has been associated with several of the symptoms of Down syndrome regression in the chromosomally typical population. One of the common changes in Down syndrome regression is depression and psychological distress. In women with breast cancer, there is a significant association between chromosomal instability and perceived stress level (Aboalela et al., 2015). Also, adults who experienced childhood stress and adversity events, such as childhood sexual abuse, show significantly increased levels of chromosomal instability (York, et al., 2013). Many people with regression have comorbid sleep apnea. In the chromosomally normal population, sleep apnea has been associated with chromosomal instability (Xie et al., 2014). Insomnia is another frequent symptom of regression, and insufficient sleep has been determined to contribute significantly to chromosomal instability (Huang et al., 2009). Also, in chromosomally normal individuals, the results of several studies have demonstrated an association between cognitive decline and chromosomal instability. Lee, et al. (2015) found that nuclear buds, a chromosomal damage biomarker, are

increased in lymphocytes from people with mild cognitive impairment. Also, chromosomal instability frequencies have been determined to be higher in people with neurodegenerative diseases, including Alzheimer's disease (Migliore et al., 1997) and Parkinson's disease (Petrozzi et al., 2002).

People with Down syndrome have increased chromosomal instability compared to age-matched euploid controls. In the previous chapter, using an isogenic mosaic Down syndrome model system, we demonstrated that micronucleus frequencies are greater in cells with a trisomy 21 imbalance than in isogenic disomic cells. Here, we measured the micronucleus frequencies in lymphocytes from people with Down syndrome who are experiencing regression to determine if micronucleus frequencies are elevated in people with Down syndrome during regression.

Methods

Study Participants

In collaboration with Dr. Brian Skotko, participants were recruited from a cohort of patients who were evaluated for regression symptoms through the Down Syndrome Program at Massachusetts General Hospital. After obtaining participant assent and the informed consent of the individuals' legal guardian(s), peripheral blood specimens were collected (IRB Protocol Number 2013P000361; Partners HealthCare System). To date, specimens have been collected from 3 females and 4 males, with an age range from 13 to 24 years, (μ = 20.86 years). As a control group, we have also initiated the collection of specimens that were obtained using an identical protocol from comparably aged individuals who have (non-mosaic) Down syndrome without regression. To date, we have received 1 control specimen (female; 31 years old). Lastly, we also compared micronucleus values from the specimens obtained in collaboration with Brian Skotko, to those obtained from our individuals with mosaic Down syndrome (VCU IRB HM 179 CR3). To evaluate the potential role of acquired behavioral conditions on micronucleus frequencies, depressive/anxiety symptoms were also assessed for the individuals with mosaic Down syndrome, through collaboration with Ruth Brown (VCU IRB HM15281).

Quantitation of Chromosomal Instability

Micronucleus frequencies were quantified using the cytokinesis-blocked micronucleus (CBMN) assay (Fenech and Morley, 1985; Fenech and Morley, 1986). To evaluate micronucleus frequencies, leukocytes from the peripheral blood specimens were collected using Histopaque-1077 (Sigma) and established in culture according to

our adaptation of the protocol of Thomas and Fenech (2007). Following mitogenic stimulation using phytohemaglutinin (PHA), lymphocytes were arrested at cytokinesis by adding Cytochalasin B (3.0 µg/ml; Sigma) to the cells 44 hours after culture initiation. At 72 hours, binucleate interphase cells were harvested as previously reported (Leach and Jackson-Cook, 2001). Briefly, this harvest included incubation in a hypotonic solution (0.075 M KCl) for 10 minutes, followed by fixation (three times using a 3:1 methanol:acetic acid solution). Slides were made following standard methods as described previously (Leach and Jackson-Cook, 2001).

Micronuclei visualization and fluorescence in situ hybridization (FISH)

For methodological consistency with our determination of the micronucleus frequencies in the probands with mosaic Down syndrome, probes targeting the RUNX1 locus (21q22) and RUNX1T1 locus (8q22) were hybridized (Abbott Molecular). FISH was completed according to the manufacturer's protocol (Abbott Molecular). Briefly, the DNA in the target chromatin (which was affixed to a microscope slide) and probes was co-denatured at 73°C for 2 minutes. Following denaturation, 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 0.4X SSC/0.3% NP-40 solution at 73 °C for 2 min, followed by a 2 min wash at room temperature (in a 2X SSC/0.1% NP-40 solution). To visualize the binucleated cells and micronuclei, the chromatin in the nuclei and micronuclei was counterstained with DAPI II/antifade (Abbott Molecular). The probes used in this study have been validated for use in our CLIA and CAP approved laboratory and consistently show specificity and sensitivity values of 0.98 or higher.

Micronucleus Scoring

An Axioskop equipped with single- and triple-band pass filters was used to score slides (Zeiss). Micronucleus frequency was quantified by scoring 1000 binucleated cells. Micronuclei were identified according to the criteria established by Fenech (2007). Binucleated cells that did not have clear borders or that were overlapping were excluded from the analysis.

Comparison of Micronucleus Frequencies in People with Mosaic Down Syndrome and Depressive Symptoms Compared to those in People with non-mosaic Down Syndrome and Regression

A subset of the individuals with mosaic Down syndrome were evaluated for depressive and anxiety symptoms as described by Brown, et al. (manuscript submitted). Briefly, depressive symptoms were evaluated by proband self-report using the Glasgow Depression Scale for People with a Learning Disability. Additionally, parents of the probands with mosaicism completed the Glasgow Depression Scale – Carer Supplement to obtain their assessment of their child's behavior.

Statistical Analyses

To assess relationships between age and micronucleus frequency, a Pearson's correlation was calculated. To compare micronucleus frequencies in the teens/young adults with Down syndrome and regression to those seen in the trisomic cells of people with mosaic Down syndrome, a two-sample t-test was performed. For all statistics, p values less than 0.05 were designated to be indicative of statistical significance.

Results

In the 7 participants with Down syndrome and regression, micronucleus frequencies ranged from 0.017 to 0.044 (μ=0.025, SE=0.002). The control participant with Down syndrome who does not have regression, had a micronucleus frequency of 0.012 (the lowest in the cohort received in collaboration with B. Skotko). The results for each participant with Down syndrome and regression and the control participant with non-mosaic Down syndrome without regression are shown in Table 12. No significant correlation was observed between age and micronucleus frequency for the 7 participants with Down syndrome and regression (r=0.527, p=0.224).

The micronucleus frequencies in the teens/adults with Down syndrome and regression (μ=0.025, SE=0.004) were not significantly different from the overall micronucleus frequencies (trisomic and disomic cells) in the teens/adults with mosaic Down syndrome (μ=0.021, SE=0.002) (p=0.395). The micronucleus frequencies in the probands with Down syndrome and regression, in comparison to those observed in the trisomic and disomic cells from people with mosaic Down syndrome, as well as the control participant with non-mosaic Down syndrome, are shown in Figure 15.

Given that regression has been strongly associated with depressive symptoms, comparisons of micronucleus frequencies in individuals with Down syndrome/mosaicism for Down syndrome were also categorized according to the presence (or absence) of recognized behavioral conditions in the study participants (e. g. regression; depressive/anxiety symptoms), as shown in Figure 16. In the mosaic cohort, depressive symptoms were identified in 3 out of the 5 probands who were evaluated for depression. The micronucleus frequencies of the 3 probands with mosaic Down syndrome and

depression (μ=0.021, SE=0.005) did not differ significantly from those of the 2 probands with mosaic Down syndrome who did not have depression (μ=0.027, SE=0.004) (p=0.563). The micronucleus frequencies in the probands with Down syndrome and regression (μ=0.025, SE=0.002) did not differ significantly from either the micronucleus frequencies of the probands with mosaic Down syndrome and depression (p=0.559) or those with mosaic Down syndrome who were not depressed (p=0.803).

Table 12. Micronucleus frequencies in lymphocytes from 8 people with Down syndrome and regression, 1 person without regression, and 3 people with mosaic Down syndrome and depression.

Age	Sex	Micronucleus Frequency
Down Syndrome and Regression		
13	F	0.017
19	M	0.017
22	M	0.022
22	M	0.029
23	M	0.019
23	F	0.044
24	F	0.028
Down Syndrome Control		
31	F	0.012
Mosaic Down Syndrome and Depression		
18	F	0.028
24	F	0.012
35	F	0.024
Mosaic Down Syndrome without Depression		
24	M	0.023
45	F	0.030

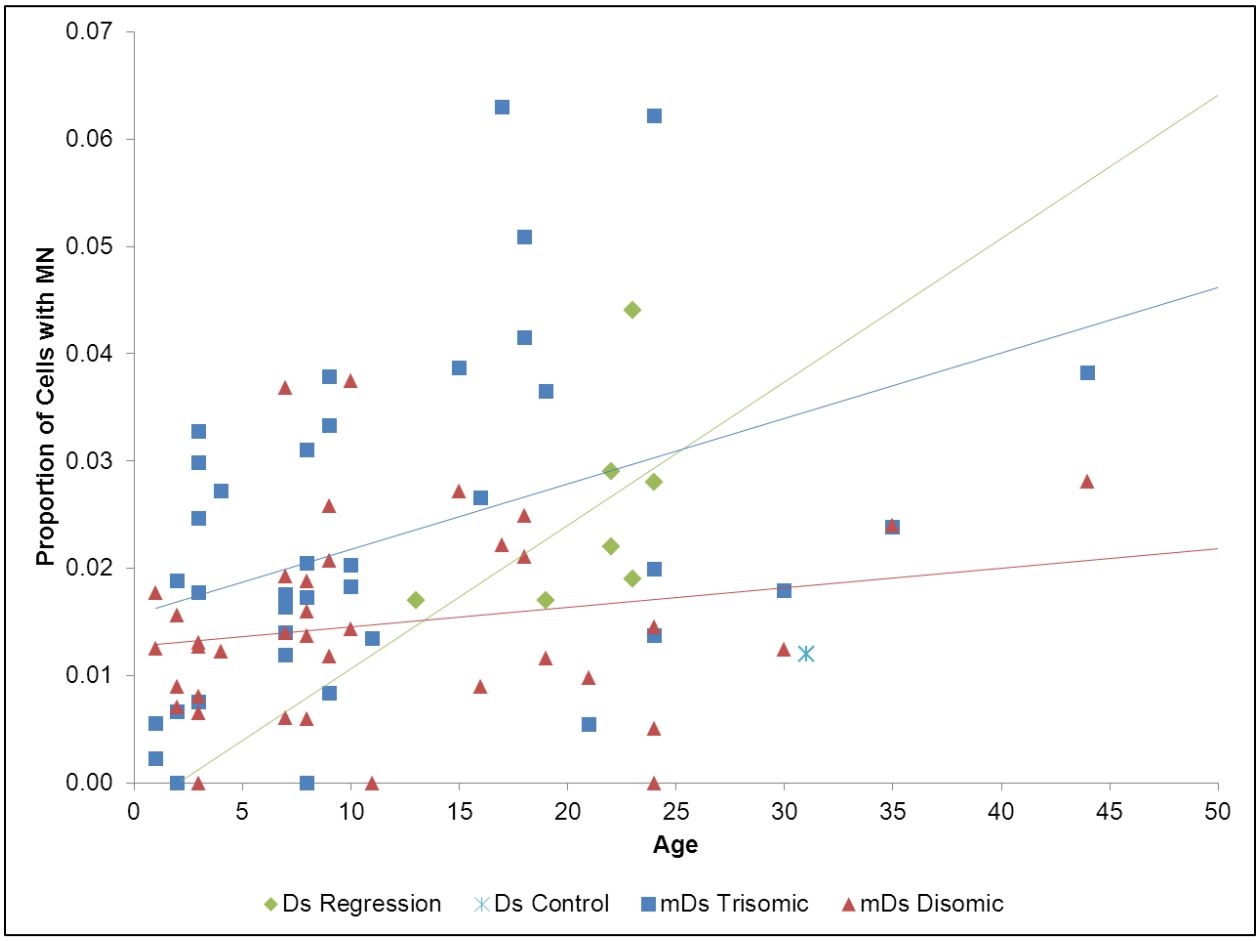

Figure 15. Micronucleus frequencies in probands with Down syndrome and regression (green diamonds), Down syndrome control without regression (blue star), and relative micronucleus frequencies in trisomic (blue square) and disomic (red triangle) cells of probands with mosaic Down syndrome.

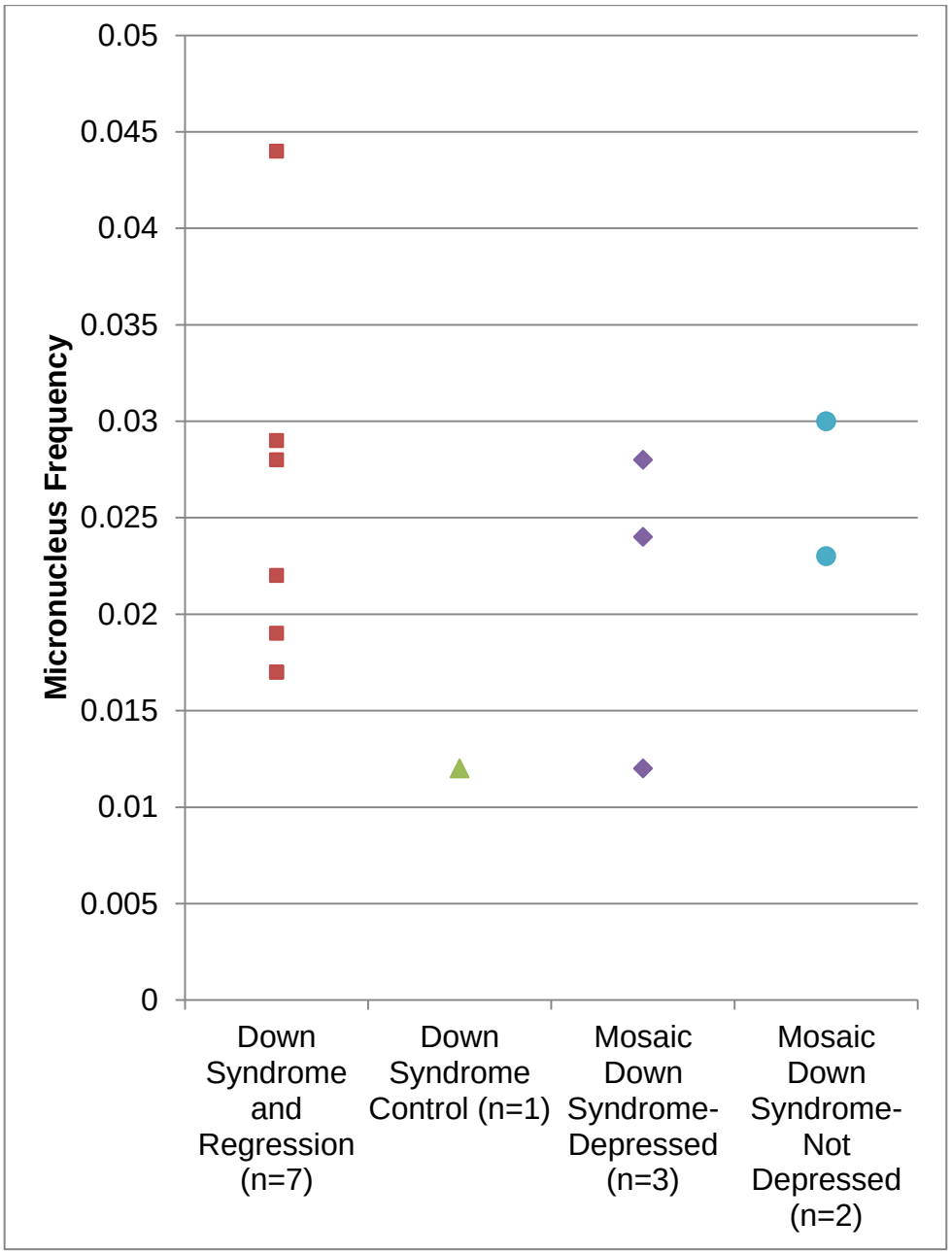

Figure 16. Comparison of the average Micronucleus frequencies in participants with Down syndrome and regression, the control participant with Down syndrome without regression, and the participants with mosaic Down syndrome and depression.

Discussion

The micronucleus frequencies in the cells of teens/young adults with Down syndrome and regression were not significantly different from the overall micronucleus frequencies in the teens/young adults with mosaic Down syndrome. Of the 5 probands with mosaic Down syndrome who were evaluated for depression, 3 (60%) were clinically depressed. There was no significant difference in the micronucleus frequencies of the probands with mosaic Down syndrome according to depression status. Further, the micronucleus frequencies of the probands with Down syndrome and regression did not differ from those of the probands with mosaic Down syndrome who were depressed, or those who were not depressed. Interestingly, the micronucleus frequency of the 31-year-old control participant with Down syndrome without regression (0.012) was not within the range of micronucleus frequencies of the seven 13 to 24-year-olds with regression (0.017-0.044). While this is an interesting observation, the present sample size and inclusion of only one control participant prevents any statistical comparisons between the micronucleus frequencies of non-mosaic trisomic probands with and without regression. There have been few previous publications of spontaneous micronucleus frequencies in lymphocytes from people with Down syndrome, which limits our ability to compare the micronucleus frequencies of people with (non-mosaic) Down syndrome who do and do not have regression. Scarfi et al., (1990) reported spontaneous micronucleus frequencies ranging from 0.005-0.011 in 7 participants with Down syndrome (ages 9 to 55). However, there were some differences in methodology, as Scarfi et al. scored vastly different numbers of binucleated cells in each participant,

ranging from 273 to 1445. Potential differences in methodology prevent the direct comparison of these results with the results of that previous study.

Study Limitations

As noted above, the largest limitation of this pilot study is the small sample size. In future studies, adding additional age-matched control participants without regression may enable us to determine whether regression is associated with a significantly increased micronucleus frequency. Another limitation to this study is the use of blood for the detection of changes related to behavior. There are currently no available techniques for determining micronucleus frequencies in cells with neurological origins. It is unclear whether behavioral or psychological changes can manifest as differences in chromosomal instability in cells from peripheral blood. However, evidence for associations between micronucleus frequency and stress (Aboalela et al., 2015;York et al., 2013) suggest that psychological symptoms can be associated with blood micronucleus frequency. It is possible that behavioral regression in people with Down syndrome is similarly associated with an elevated micronucleus frequency, but additional control participants without regression (or depression) would be required to test this hypothesis.

Chapter 6. Summary

The primary purpose of this study was to determine if a trisomy 21 imbalance is associated with an increase in the acquisition of three somatic chromosomal changes: telomere length; chromosomal instability and SADS. Moreover, we evaluated if these cellular/chromosomal attributes were associated with chronological age. The primary conclusions from our study are discussed below:

1. Telomeres show an overall trend toward faster attrition in people with trisomy 21, but the relationship appears to be a complex association

The results of the semi-quantitative FISH assay of telomere lengths in isogenic trisomic and disomic cells indicated that the relationship between trisomy 21 and telomere length is complex. All possible categorical outcomes of telomere length differences (shorter trisomic-cell telomeres, longer trisomic-cell telomeres, and no difference between trisomic and disomic-cell telomeres) were represented in the results from the 28 participants. The difference in telomere lengths between isogenic cells was not straightforwardly associated with the participants' chronological ages. However, our study results suggest that there are individual factors that influence the effect that trisomy 21 has on telomere length. This observation is consistent with the fact that there is heterogeneity in the severity and number of symptoms in people with Down syndrome. This person-to-person variation may reflect differing effects that trisomy 21

has in the context of an individual's genetic background. Telomere attrition is influenced by inflammation and oxidative stress, which are both affected by a trisomy 21 imbalance. It is then possible that trisomy 21 causes varying degrees of inflammation and oxidative stress in individuals with Down syndrome, and that this, in turn, contributes to the differences in the response of telomere lengths to trisomy 21. Also, given the role of mitochondria in maintaining energy balance, differences in the mitochondrial DNA copy number might contribute to person-specific levels of oxidative damage. Short telomere lengths could then signal an adverse exposure, such as a loss of energy homeostasis.

Although trends were observed for a subset of chromosomes, the chromosome-specific method for telomere length assessment did not result in the identification of any specific chromosome arms that had significantly different telomere lengths in trisomic compared to disomic cells. However, the chromosome-specific method enabled us to show that the telomere lengths in the trisomic and disomic cells were parallel to one another across the individual chromosomes, suggesting that the influence of a trisomy 21 imbalance was constant across all telomeres. Also, we showed that there is considerable variation in telomeres among the different chromosome arms. The results of our study are aligned with the findings of previous investigators who have shown heritable differences in telomere lengths between individual chromosomes (Graakjaer et al., 2006).

In this group of participants, collectively, we were able to demonstrate that the telomeres in the trisomic cells were shorter than the telomeres in the disomic cells.

2. SADS occurred more frequently in cells with trisomy 21, but was not associated with age

We observed a greater frequency of chromosome 8 SADS in the trisomic compared to disomic cells from people with mosaicism for trisomy 21. However, we did not detect an association between a participant's chronological age and the frequency of overall SADS, trisomic-cell SADS, or disomic-cell SADS. This latter finding was unexpected, considering SADS is a marker of early-stage cellular senescence. However, our failure to detect an age-association might be accounted for by small sample size or our use of phytohaemmoglutinin-stimulated leukocyte cultures, which are enriched for cycling cells. Also, it is possible that "chronological" age may not fully reflect "biological" age-related changes, or that the age group we evaluated was too young to capture age-related changes above and beyond those related to a trisomy 21 imbalance. Nonetheless, through this "proof of principle" pilot project, we did demonstrate that SADS can be detected and measured in leukocytes. While we quantified SADS in the centromeric repeats of chromosome 8, it is probable that SADS occurs in other chromosomes as well. In future studies we could interrogate the centromeric region of additional chromosomes to confirm that this acquired cellular attribute is not specific to chromosome 8. Another important future study would be to measure SADS frequencies in uncultured leukocytes from people with trisomy 21, which would be expected to contain a subset of senescent cells. Moreover, since people with Down syndrome have been shown to have a 'Polycomb-opathy' that results in a reduction of the cellular stress response threshold needed to signal the initiation of stem cell senescence and have also been shown to have an age-related reduction in stem

cell proliferation rates, it would be interesting to compare SADS frequencies in stem cells from people with mosaicism for trisomy 21 to determine if associations were present between premature stem cell senescence values and age-related conditions (Cairney, et al., 2009; Adorno, et al., 2013; Souroullas, et al., 2013; Liu, et al., 2015).

Although SADS and telomere shortening are both markers of senescence, Swanson et al., (2013) showed that SADS occurs in response to other types of senescence induction and appeared to be independent of telomere shortening. Interestingly, we did not observe an association between 1) age and SADS frequency; or 2) age and telomere length differences (trisomic - disomic values). Premature aging in Down syndrome may cause the association between age and cellular senescence to differ from the association in chromosomally normal individuals. Our results, along with previous observations of senescent cells derived from trisomy 21 pregnancies (Amiel et al., 2013; Biron-Shental et al., 2015; Savickiene et al., 2016), suggest that cellular senescence may occur at an earlier chronological age in people with Down syndrome.

3. Trisomy 21 is associated with age-related chromosomal instability

Age was associated with total micronucleus frequency in the participants with mosaic Down syndrome. Not accounting for age, the micronucleus frequencies were greater in trisomic compared to disomic cells. In the younger age group, there was no difference in the frequency of trisomic and disomic cells containing micronuclei. In the older group, trisomic cells had increased micronucleus frequencies compared to disomic cells. Overall, these results demonstrate that: 1) micronucleus frequency is associated with age in people with trisomy 21; 2) trisomic cells have more chromosomal instability than disomic cells; and 3) trisomy 21 results in age-related increases in chromosomal instability. Thus, chromosomal instability was the only acquired chromosomal change we evaluated in which we found an age-related increase that was attributable to a trisomy 21 imbalance.

4. Chromosome 21 was excluded into micronuclei more frequently than chromosome 8

Neither chromosome 8 nor chromosome 21 was excluded into micronucleus more often than would be expected by chance. Also, the majority of micronucleus contained chromatin from chromosomes other than the two investigated in our study (chromosomes 8 and 21), suggesting that chromosomal instability in trisomic cells is a genome-wide phenomenon.

5. There was no association between chromosomal instability and telomere length or SADS

Telomere lengths were not associated with micronucleus frequencies in the 10 participants evaluated in both studies. This was unexpected, because telomere shortening has an adverse effect on genomic/chromosomal stability (Murnane, 2012; Gonzalo and Eissenberg, 2016). In normal mammary epithelial cells, chromosomes with short telomeres have been shown to malsegregate more frequently than chromosomes with normal telomeres, resulting in somatic aneuploidy and micronucleus formation (Pampalona et al., 2010). Short telomeres can cause the end-to-end fusions of two chromosomes by non-homologous end joining. The resulting dicentric chromosome may not properly attach to microtubules and lag behind at anaphase, resulting in micronucleus formation (Pampalona et al., 2010). It is possible that the small sample size and young participant ages prevented the detection of an association between short telomeres and micronucleus frequency. Another, although unlikely, contributor to the lack of an association between telomere length and chromosomal instability in this study could be the exclusive measurement of micronucleus frequency, instead of measuring all three abnormalities defined by the cytokinesis-blocked micronucleus cytome assay. In addition to micronuclei, nuclear buds (NBUD) and nucleoplasmic bridges (NPB) can be used as biomarkers of chromosomal instability (Fenech et al., 2011). NPB occur during anaphase when dicentric chromosomes are pulled to opposite poles and the nuclear membrane surrounds them, forming a temporary bridge between the daughter nuclei. Given the tendency of chromosomes with extremely short telomeres to form dicentric chromosomes through end-to-end fusion, it is possible that

there may have been some telomere-associated chromosomal instability that was not detected. However, NPB typically occur only when the telomeres are critically short, and that was not the case in our young cohorts of participants. Also, while not formally scored, NPB were not observed during the microscopic analysis of the cells from the individuals with mosaic or non-mosaic Down syndrome.

In the 9 participants with mosaic Down syndrome who were selected for both the SADS and micronucleus analyses, there was not an association between these two acquired chromosomal changes. The structural integrity of the centromere is important for normal mitotic divisions, so we had considered the possibility that the two measures would be associated. It is probable that the changes underlying SADS result in mitotic checkpoint activation and the prevention of mitosis. Thus, cells with SADS may not contribute to the chromosomal abnormalities causing micronucleus formation.

It is interesting that we observed fewer micronuclei containing chromatin from chromosome 8 compared to chromosome 21 in all cell types across all ages and that we saw no clear age relationship between SADS involving the pericentromeric region of chromosome 8. Also, we did not detect a clear relationship between chromosomal instability frequencies and telomere lengths. Thus, in cells with a trisomy 21 imbalance, it seems that chromosomal instability may be related to cellular factors that are not limited to telomere length and distension of the pericentromeric heterochromatin (as seen in SADS). There are several demonstrated mechanisms underlying the loss of mitotic fidelity that causes chromosomal instability. Abnormalities in centrosome dynamics, such as incorrect timing of centrosome separation, can harm the geometry of the mitotic spindle (Silkworth et al., 2012; Nam et al., 2015). Also, a failure of the

cohesin complex removal can inhibit the separation of sister chromatids, causing anaphase lagging and subsequent micronucleus formation (Wirth et al., 2006). In mouse models, heterozygous mutations in spindle assembly checkpoint genes result in increased chromosomal malsegregation, but there is limited evidence of mutations that alter spindle assembly checkpoint function in humans (Thompson et al., 2010). Merotelic kinetochore orientation, which describes the attachment of the kinetochore to microtubules emanating from both spindle poles, is a frequent cause of mammalian mitotic aneuploidy (Cimini et al., 2003). Cells with merotelic kinetochore attachment pass the spindle assembly checkpoint, because the microtubule occupancy of the kinetochores is numerically the same as it would be in the presence of normal attachments (Cimini et al., 2001). Also, the disruption of cell-cycle regulators has been hypothesized to contribute to chromosomal instability, although this has not been investigated directly (Thompson et al., 2010). It is important to note that chromosomal instability mechanisms have been primarily studied in the context of cancer. People with trisomy 21, despite having factors that predispose to tumorigenesis, like oxidative stress and immunodeficiency, are less likely to develop solid tumors than chromosomally normal individuals (Nižetić and Groet, 2012). Thus, it is possible that the mechanisms underlying chromosomal instability in cells having a trisomy 21 imbalance may differ from the mechanisms that cause chromosomal instability in cancer cells.

6. Chromosomal instability frequencies in cells from people with Down syndrome who are experiencing regression do not appear to be higher than those of cells from people with mosaicism for trisomy 21 who do not demonstrate regression

In addition to measuring micronucleus frequencies in isogenic trisomic and disomic cells from people with mosaicism for trisomy 21, we also determined the micronucleus frequencies of people with non-mosaic Down syndrome who were experiencing regression. We compared these micronucleus frequencies to those of participants with mosaic Down syndrome who were depressed. There was no difference in micronucleus frequencies between participants with Down syndrome and regression and participants with mosaic Down syndrome and depression. There was also no difference in the frequency of micronuclei between participants with mosaic Down syndrome who had depression compared to those who did not have depression. Considering that regression and depression are behavioral phenotypes, it is possible that micronucleus frequencies in blood cells do not capture the biological changes related to the acquisition of regression or depression in people with Down syndrome. Future studies should include more age-matched control participants with Down syndrome who do not have regression/depression.

7. Order of the acquired chromosomal changes in cells with trisomy 21

Our use of a cross-sectional study design and different cells for the measurement of each somatic change precludes a determination of the order in which the acquired chromosomal changes occurred in the cells that we studied. However, based on our knowledge of these acquired changes, we can make inferences about the chronological connections between them. Chromosomal instability and telomere shortening are primary aging hallmarks which initiate damage (López-Otín et al., 2013). The imbalance caused by trisomy 21 contributes to the mitotic errors that result in chromosomal instability that are independent of telomere length. As mitotic errors continue to accumulate, the integrity of the genome becomes compromised. Trisomy 21-related shortening of the telomeres may then contribute to destabilizing the chromosomes, thereby contributing to micronucleus formation (possibly at an age older than the age of the participants evaluated in this study). As the chromosomal instability and telomere shortening progress, these acquired cellular traits may activate mitotic checkpoints that lead to the induction of cellular senescence. Senescence is an antagonistic hallmark of aging, which occurs in response to damage caused by chromosomal instability and telomere shortening. At this stage, SADS would occur. This speculated potential order of the acquired chromosomal changes we investigated in this research is shown in Figure 17.

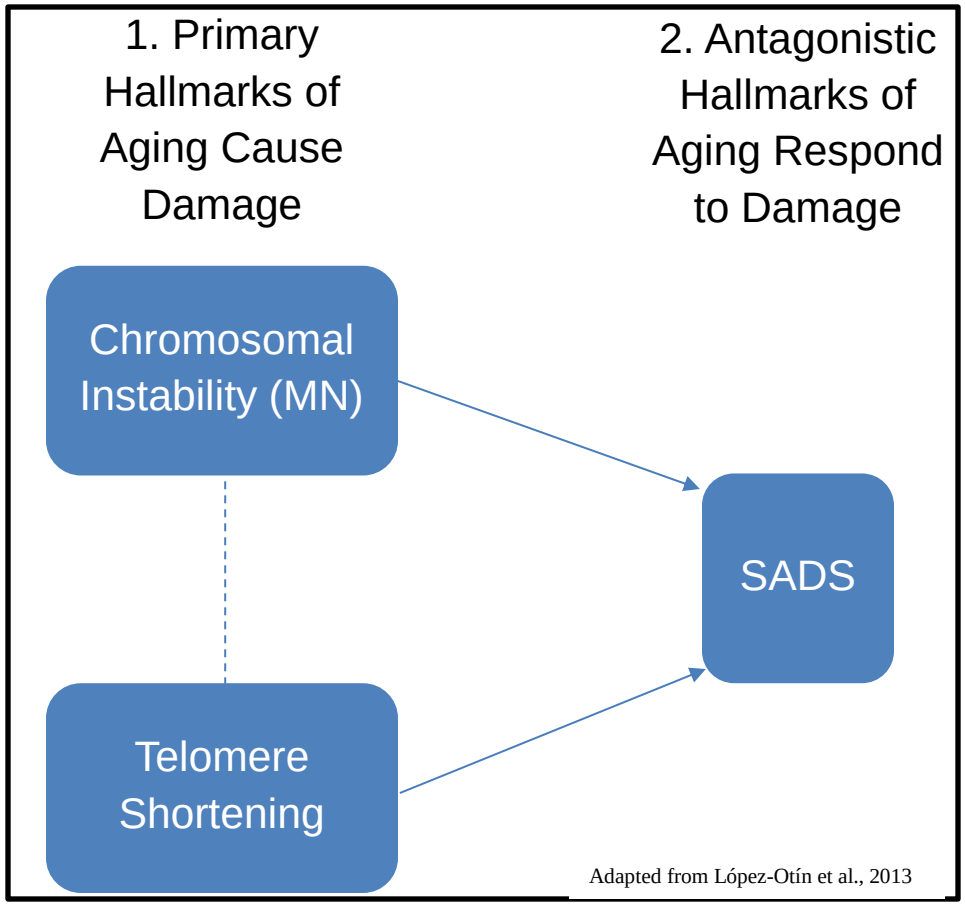

Figure 17. Possible sequence of the acquisition of aging "hallmarks" in cells with trisomy 21. Primary, damage-inducing hallmarks of aging, such as chromosomal instability and telomere shortening, lead to antagonistic hallmarks of aging, like SADS, which respond to the damage. There are additional primary and antagonistic hallmarks of aging which were not investigated in this study (not shown).

8. Of these three acquired chromosomal changes, chromosomal instability is the most promising biomarker for assessing age-related cellular changes in people with Down syndrome

As previously noted, chromosomal instability was the only acquired chromosomal change in which we found an effect of trisomy 21 on age-related increases. In younger participants, there was no difference in the propensity of trisomic and disomic cells to have chromosomal instability. In the teens/adults, the trisomic cells had greater chromosomal instability than the cells without a trisomic imbalance. Thus, of the three acquired cellular changes measured in this study, chromosomal instability has the greatest potential to be developed as a biomarker for premature aging.

According to the American Federation of Aging Research, there are three requirements of a biomarker for human aging (AFAR, 2011):

1. It must predict a person's physiological, cognitive, and physical function in an age-related way
2. It must be testable and not harmful to test subjects
3. It should work in laboratory animals as well as humans.

We have demonstrated that there are age-related increases in micronucleus frequency in cells with trisomy 21. However, we have not determined whether this increase in micronucleus frequency can predict age-related functioning. In order to do so, we would need to be able to link participants' micronucleus frequency to detailed phenotypic information. Preferably, a longitudinal design in older adults with Down syndrome could enable the comparison of micronucleus frequencies in individuals before and after the onset of age-related declines in phenotypes such as dementia. The

increasing lifespan of people with Down syndrome should enable the inclusion of research participants at older ages than has been previously possible.

Chromosomal instability meets the second criterion for an aging biomarker, because this can be tested in humans without inducing harm. In the present study, peripheral blood samples were obtained through venipuncture, and there were minimal risks to the participants.

The third criterion for an aging biomarker is not met, but future studies could determine whether micronucleus frequency can be tested in an animal model. micronucleus frequency is often measured in cells from rats and mice to study chromosomal instability caused by exposure to genotoxic agents (Kissling et al., 2007; Witt et al., 2008). However, the spontaneous micronucleus frequencies in rodents are unknown, and it is unknown whether they exhibit the same age-related increases as in humans.

Ultimately, future studies would be necessary to determine whether micronucleus frequency can be used to detect the onset of age-related decline.

9. This study demonstrates the utility of the isogenic trisomic-disomic mosaic Down syndrome “model” for determining the effects of trisomy 21 on acquired cellular changes

It is difficult to determine the effect of trisomy 21 on acquired chromosomal changes because of the interpersonal variation in these traits. Our comparison of acquired chromosomal changes in the isogenic trisomic compared to disomic cells from people with mosaic Down syndrome allowed for the elimination of interpersonal variation factors that might contribute to acquired chromosomal changes, to yield a measure of differences that is directly attributable to a trisomic imbalance for chromosome 21. Using this isogenic trisomic-disomic cell model, we were able to determine that trisomy 21 contributes to telomere shortening, SADS, and chromosomal instability. The results of this study demonstrate the utility of an isogenic trisomic-disomic model using cells from people with mosaic Down syndrome for determining the effects of trisomy 21 on cellular traits.

References

Abbeduto L, Murphy MM, Kover ST, et al. Signaling noncomprehension of language: A comparison of fragile X syndrome and down syndrome. *Am J Ment Retard*. 2008;113(3):214-230. doi: 10.1352/0895-8017(2008)113[214:SNOLAC]2.0.CO;2 [doi].

Aboalela N, Lyon D, Elswick RK,Jr, et al. Perceived stress levels, chemotherapy, radiation treatment and tumor characteristics are associated with a persistent increased frequency of somatic chromosomal instability in women diagnosed with breast cancer: A one year longitudinal study. *PLoS One*. 2015;10(7):e0133380. doi: 10.1371/journal.pone.0133380 [doi].

Adams D, Oliver C. The relationship between acquired impairments of executive function and behaviour change in adults with Down syndrome. *J Intellect Disabil Res*. 2010;54(5):393-405. doi: 10.1111/j.1365-2788.2010.01271.x [doi].

Adams PD. Remodeling of chromatin structure in senescent cells and its potential impact on tumor suppression and aging. *Gene*. 2007;397(1-2):84-93. doi: S0378-1119(07)00190-4 [pii].

Adorno M, Sikandar S, Mitra SS, et al. Usp16 contributes to somatic stem-cell defects in Down's syndrome. *Nature*. 2013;501(7467):380-384. doi: 10.1038/nature12530 [doi].

Aghajanova L, Popwell JM, Chetkowski RJ, Herndon CN. Birth of a healthy child after preimplantation genetic screening of embryos from sperm of a man with non-mosaic Down syndrome. *J Assist Reprod Genet*. 2015;32(9):1409-1413. doi: 10.1007/s10815-015-0525-z [doi].

Akahoshi K, Matsuda H, Funahashi M, Hanaoka T, Suzuki Y. Acute neuropsychiatric disorders in adolescents and young adults with Down syndrome: Japanese case reports. *Neuropsychiatr Dis Treat*. 2012;8:339-345. doi: 10.2147/NDT.S32767 [doi].

Albizua I, Rambo-Martin BL, Allen EG, He W, Amin AS, Sherman SL. Association between telomere length and chromosome 21 nondisjunction in the oocyte. *Hum Genet*. 2015;134(11-12):1263-1270. doi: 10.1007/s00439-015-1603-0 [doi].

Ali FE, Al-Bustan MA, Al-Busairi WA, Al-Mulla FA, Esbaita EY. Cervical spine abnormalities associated with Down syndrome. *Int Orthop*. 2006;30(4):284-289. doi: 10.1007/s00264-005-0070-y [doi].

Andrew T, Aviv A, Falchi M, et al. Mapping genetic loci that determine leukocyte telomere length in a large sample of unselected female sibling pairs. *Am J Hum Genet*. 2006;78(3):480-486. doi: S0002-9297(07)62386-6 [pii].

Aravinthan A. Cellular senescence: A hitchhiker's guide. *Hum Cell*. 2015;28(2):51-64. doi: 10.1007/s13577-015-0110-x [doi].

Balkany TJ, Downs MP, Jafek BW, Krajicek MJ. Hearing loss in Down's syndrome. A treatable handicap more common than generally recognized. *Clin Pediatr (Phila)*. 1979;18(2):116-118. doi: 10.1177/000992287901800207 [doi].

Basil JS, Santoro SL, Martin LJ, Healy KW, Chini BA, Saal HM. Retrospective study of obesity in children with Down syndrome. *J Pediatr*. 2016;173:143-148. doi: 10.1016/j.jpeds.2016.02.046 [doi].

Bennet GC, Rang M, Roye DP, Aprin H. Dislocation of the hip in trisomy 21. *J Bone Joint Surg Br*. 1982;64(3):289-294.

Ben-Porath I, Weinberg RA. The signals and pathways activating cellular senescence. *Int J Biochem Cell Biol*. 2005;37(5):961-976. doi: S1357-2725(04)00387-5 [pii].

Bhaumik P, Bhattacharya M, Ghosh P, Ghosh S, Kumar Dey S. Telomere length analysis in Down syndrome birth. *Mech Ageing Dev*. 2017;164:20-26. doi: S0047-6374(16)30160-9 [pii].

Biron-Shental T, Liberman M, Sharvit M, Sukenik-Halevy R, Amiel A. Amniocytes from aneuploidy embryos have enhanced random aneuploidy and signs of senescence - can these findings be related to medical problems? *Gene*. 2015;562(2):232-235. doi: 10.1016/j.gene.2015.02.075 [doi].

Bittles AH, Glasson EJ. Clinical, social, and ethical implications of changing life expectancy in Down syndrome. *Dev Med Child Neurol*. 2004;46(4):282-286.

Blasco MA. Telomeres and human disease: Ageing, cancer and beyond. *Nat Rev Genet*. 2005;6(8):611-622. doi: 10.1038/nrg1656 [doi].

Bock I, Nemeth K, Pentelenyi K, et al. Targeted next generation sequencing of a panel of autism-related genes identifies an EHMT1 mutation in a kleefstra syndrome patient with autism and normal intellectual performance. Gene. 2016;595(2):131-141. doi: S0378-1119(16)30754-5 [pii].

Bolzer A, Kreth G, Solovei I, et al. Three-dimensional maps of all chromosomes in human male fibroblast nuclei and prometaphase rosettes. *PLoS Biol*. 2005;3(5):e157. doi: 04-PLBI-RA-0222R5 [pii].

Bonassi S, Fenech M, Lando C, et al. . HUman MicroNucleus project: international database comparison for results with the cytokinesis-block micronucleus assay in human lymphocytes: I. Effect of laboratory protocol, scoring criteria, and host factors on the frequency of micronuclei, *Environ. Mol. Mutagen*. 2001, vol. 37 (pg. 31-45)

Borthwick-Duffy SA. Epidemiology and prevalence of psychopathology in people with mental retardation. *J Consult Clin Psychol*. 1994;62(1):17-27.

Breslin J, Spano G, Bootzin R, Anand P, Nadel L, Edgin J. Obstructive sleep apnea syndrome and cognition in Down syndrome. *Dev Med Child Neurol*. 2014;56(7):657-664. doi: 10.1111/dmcn.12376 [doi].

Brouilette S, Singh RK, Thompson JR, Goodall AH, Samani NJ. White cell telomere length and risk of premature myocardial infarction. *Arterioscler Thromb Vasc Biol*. 2003;23(5):842-846. doi: 10.1161/01.ATV.0000067426.96344.32 [doi].

Brown RC, Mortensen J, Amstadter AB, Jackson-Cook C. Internalising symptoms and peer relationships of people with mosaicism for Down syndrome. *Submitted for publication*. 2017.

Bruwier A, Chantrain CF. Hematological disorders and leukemia in children with Down syndrome. *Eur J Pediatr*. 2012;171(9):1301-1307. doi: 10.1007/s00431-011-1624-1 [doi].

Burt DB, Loveland KA, Lewis KR. Depression and the onset of dementia in adults with mental retardation. *Am J Ment Retard*. 1992;96(5):502-511.

Cairney CJ, Sanguinetti G, Ranghini E, et al. A systems biology approach to Down syndrome: Identification of Notch/Wnt dysregulation in a model of stem cells aging. *Biochim Biophys Acta*. 2009;1792(4):353-363. doi: 10.1016/j.bbadis.2009.01.015 [doi].

Campisi J, d'Adda di Fagagna F. Cellular senescence: When bad things happen to good cells. *Nat Rev Mol Cell Biol*. 2007;8(9):729-740. doi: nrm2233 [pii].

Capone G, Goyal P, Ares W, Lannigan E. Neurobehavioral disorders in children, adolescents, and young adults with Down syndrome. *Am J Med Genet C Semin Med Genet*. 2006;142C(3):158-172. doi: 10.1002/ajmg.c.30097 [doi].

Capone GT, Aidikoff JM, Taylor K, Rykiel N. Adolescents and young adults with Down syndrome presenting to a medical clinic with depression: Co-morbid obstructive sleep apnea. *Am J Med Genet A*. 2013;161A(9):2188-2196. doi: 10.1002/ajmg.a.36052 [doi].

Catalano RA. Down syndrome. *Surv Ophthalmol*. 1990;34(5):385-398.

Central Intelligence Agency. The world factbook. Updated 2012.

Charlot L, Fox S, Friedlander R. Obsessional slowness in Down's syndrome. *J Intellect Disabil Res*. 2002;46(Pt 6):517-524. doi: 419 [pii].

Chen CC, Spano G, Edgin JO. The impact of sleep disruption on executive function in Down syndrome. *Res Dev Disabil*. 2013;34(6):2033-2039. doi: 10.1016/j.ridd.2013.03.009 [doi].

Cooke HJ, Smith BA. Variability at the telomeres of the human X/Y pseudoautosomal region. *Cold Spring Harb Symp Quant Biol*. 1986;51 Pt 1:213-219.

Coskun PE, Wyrembak J, Derbereva O, et al. Systemic mitochondrial dysfunction and the etiology of alzheimer's disease and Down syndrome dementia. *J Alzheimers Dis*. 2010;20 Suppl 2:S293-310. doi: 10.3233/JAD-2010-100351 [doi].

Costanzo F, Varuzza C, Menghini D, Addona F, Gianesini T, Vicari S. Executive functions in intellectual disabilities: A comparison between williams syndrome and Down syndrome. *Res Dev Disabil*. 2013;34(5):1770-1780. doi: 10.1016/j.ridd.2013.01.024 [doi].

Counter CM, Avilion AA, LeFeuvre CE, et al. Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. *EMBO J*. 1992;11(5):1921-1929.

Couzens D, Cuskelly M, Haynes M. Cognitive development and Down syndrome: Age-related change on the stanford-binet test (fourth edition). *Am J Intellect Dev Disabil*. 2011;116(3):181-204. doi: 10.1352/1944-7558-116.3.181 [doi].

Creavin AL, Brown RD. Ophthalmic abnormalities in children with Down syndrome. *J Pediatr Ophthalmol Strabismus*. 2009;46(2):76-82.

Cruickshanks HA, McBryan T, Nelson DM, et al. Senescent cells harbour features of the cancer epigenome. *Nat Cell Biol*. 2013;15(12):1495-1506. doi: 10.1038/ncb2879 [doi].

Daunhauer LA, Fidler DJ, Hahn L, Will E, Lee NR, Hepburn S. Profiles of everyday executive functioning in young children with Down syndrome. *Am J Intellect Dev Disabil*. 2014;119(4):303-318. doi: 10.1352/1944-7558-119.4.303 [doi].

Davidsson J. The epigenetic landscape of aneuploidy: Constitutional mosaicism leading the way? *Epigenomics*. 2014;6(1):45-58. doi: 10.2217/epi.13.78 [doi].

de Lange T. Protection of mammalian telomeres. *Oncogene*. 2002;21(4):532-540. doi: 10.1038/sj.onc.1205080 [doi].

De Simone R, Puig XS, Gelisse P, Crespel A, Genton P. Senile myoclonic epilepsy: Delineation of a common condition associated with alzheimer's disease in Down syndrome. *Seizure*. 2010;19(7):383-389. doi: 10.1016/j.seizure.2010.04.008 [doi].

Do C, Xing Z, Yu YE, Tycko B. Trans-acting epigenetic effects of chromosomal aneuploidies: Lessons from Down syndrome and mouse models. *Epigenomics*. 2017;9(2):189-207. doi: 10.2217/epi-2016-0138 [doi].

Dykens EM. Psychiatric and behavioral disorders in persons with Down syndrome. *Ment Retard Dev Disabil Res Rev*. 2007;13(3):272-278. doi: 10.1002/mrdd.20159 [doi].

Epstein CJ. Down syndrome (trisomy 21). In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. *The metabolic and molecular bases of inherited disease.* New York: McGraw-Hill; 2001:1223-–1256.

Fenech M. Cytokinesis-block micronucleus cytome assay. *Nat Protoc*. 2007;2(5):1084-1104. doi: nprot.2007.77 [pii].

Fenech M. The in vitro micronucleus technique. *Mutat Res*. 2000;455(1-2):81-95. doi: S0027-5107(00)00065-8 [pii].

Fenech M, Morley AA. Cytokinesis-block micronucleus method in human lymphocytes: Effect of in vivo ageing and low dose X-irradiation. *Mutat Res*. 1986;161(2):193-198. doi: 0027-5107(86)90010-2 [pii].

Fenech M, Morley AA. The effect of donor age on spontaneous and induced micronuclei. *Mutat Res*. 1985;148(1-2):99-105. doi: 0027-5107(85)90212-X [pii].

Ferreira FL, Pra D, Martino-Roth MG, Garcias GL. Buccal micronucleus frequency is associated with age in Down syndrome. *Genet Mol Res*. 2009;8(4):1231-1237. doi: 10.4238/vol8-4gmr636 [doi].

Fitzgerald PH LR. Mosaicism in man involving the autosome associated with mongolism. *Heredity*. 1961;16:509--512.

Foley KR, Bourke J, Einfeld SL, Tonge BJ, Jacoby P, Leonard H. Patterns of depressive symptoms and social relating behaviors differ over time from other behavioral domains for young people with Down syndrome. *Medicine (Baltimore)*. 2015;94(19):e710. doi: 10.1097/MD.0000000000000710 [doi].

Franceschi C, Monti D, Scarfi MR, et al. Genomic instability and aging. studies in centenarians (successful aging) and in patients with Down's syndrome (accelerated aging). *Ann N Y Acad Sci*. 1992;663:4-16.

Franzen J, Zirkel A, Blake J, et al. Senescence-associated DNA methylation is stochastically acquired in subpopulations of mesenchymal stem cells. *Aging Cell*. 2017;16(1):183-191. doi: 10.1111/acel.12544 [doi].

Freeman SB, Bean LH, Allen EG, et al. Ethnicity, sex, and the incidence of congenital heart defects: A report from the national Down syndrome project. *Genet Med*. 2008;10(3):173-180. doi: 10.1097/GIM.0b013e3181634867 [doi].

Freeman SB, Taft LF, Dooley KJ, et al. Population-based study of congenital heart defects in Down syndrome. *Am J Med Genet*. 1998;80(3):213-217. doi: 10.1002/(SICI)1096-8628(19981116)80:3<213::AID-AJMG6>3.0.CO;2-8 [pii].

Frid C, Drott P, Lundell B, Rasmussen F, Anneren G. Mortality in Down's syndrome in relation to congenital malformations. *J Intellect Disabil Res*. 1999;43 (Pt 3)(Pt 3):234-241.

Garlet TR, Parisotto EB, de Medeiros Gda S, et al. Systemic oxidative stress in children and teenagers with Down syndrome. *Life Sci*. 2013;93(16):558-563. doi: 10.1016/j.lfs.2013.08.017 [doi].

Garvia B, Benejam B. Regression in young adults with Down syndrome. A three cases review. *Rev Med Int Sindr Down*. 2014;18(3):43--46.

Geller LN, Potter H. Chromosome missegregation and trisomy 21 mosaicism in alzheimer's disease. *Neurobiol Dis*. 1999;6(3):167-179. doi: S0969-9961(99)90236-X [pii].

Ghaziuddin N, Nassiri A, Miles JH. Catatonia in Down syndrome; a treatable cause of regression. *Neuropsychiatr Dis Treat*. 2015;11:941-949. doi: 10.2147/NDT.S77307 [doi].

Goldberg EL. An epidemiological study of hirschsprung's disease. Int J Epidemiol. 1984;13(4):479-485.

Graakjaer J, Londono-Vallejo JA, Christensen K, Kolvraa S. The pattern of chromosome-specific variations in telomere length in humans shows signs of heritability and is maintained through life. *Ann N Y Acad Sci*. 2006;1067:311-316. doi: 1067/1/311 [pii].

Graakjaer J, Pascoe L, Der-Sarkissian H, et al. The relative lengths of individual telomeres are defined in the zygote and strictly maintained during life. *Aging Cell*.2004;3(3):97-102. doi: 10.1111/j.1474-9728.2004.00093.x [doi].

Gruszecka A, Kopczynski P, Cudzilo D, et al. Telomere shortening in Down syndrome patients--when does it start? *DNA Cell Biol*. 2015;34(6):412-417. doi: 10.1089/dna.2014.2746 [doi].

Guralnick MJ. Involvement with peers: Comparisons between young children with and without Down's syndrome. *J Intellect Disabil Res*. 2002;46(Pt 5):379-393. doi: 405 [pii].

Hamlett ED, Goetzl EJ, Ledreux A, et al. Neuronal exosomes reveal alzheimer's disease biomarkers in Down syndrome. *Alzheimers Dement*. 2016. doi: S1552-5260(16)32892-8 [pii].

Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. *Nature*. 1990;345(6274):458-460. doi: 10.1038/345458a0 [doi].

Hartley D, Blumenthal T, Carrillo M, et al. Down syndrome and alzheimer's disease: Common pathways, common goals. *Alzheimers Dement*. 2015;11(6):700-709. doi: 10.1016/j.jalz.2014.10.007 [doi].

Hasle H, Friedman JM, Olsen JH, Rasmussen SA. Low risk of solid tumors in persons with Down syndrome. *Genet Med*. 2016;18(11):1151-1157. doi: 10.1038/gim.2016.23 [doi].

Hassold T, Hunt P. To err (meiotically) is human: The genesis of human aneuploidy. *Nat Rev Genet*. 2001;2(4):280-291. doi: 10.1038/35066065 [doi].

Hauge X, Raca G, Cooper S, et al. Detailed characterization of, and clinical correlations in, 10 patients with distal deletions of chromosome 9p. Genet Med. 2008;10(8):599-611. doi: 10.1097GIM.0b013e31817e2bde [doi].

Hayflick L. The limited in vitro lifetime of human diploid cell strains. *Exp Cell Res*. 1965;37:614-636.

Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. *Exp Cell Res*. 1961;25:585-621.

Head E, Lott IT, Wilcock DM, Lemere CA. Aging in Down syndrome and the development of alzheimer's disease neuropathology. *Curr Alzheimer Res*. 2016;13(1):18-29. doi: CAR-EPUB-71183 [pii].

Hemann MT, Strong MA, Hao LY, Greider CW. The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability. Cell. 2001;107(1):67-77. doi: S0092-8674(01)00504-9 [pii].

Herbig U, Ferreira M, Condel L, Carey D, Sedivy JM. Cellular senescence in aging primates. *Science*. 2006;311(5765):1257. doi: 1122446 [pii].

Hermans H, Evenhuis HM. Life events and their associations with depression and anxiety in older people with intellectual disabilities: Results of the HA-ID study. *J Affect Disord*. 2012;138(1-2):79-85. doi: 10.1016/j.jad.2011.12.025 [doi].

Holmes G. Gastrointestinal disorders in Down syndrome. *Gastroenterol Hepatol Bed Bench*. 2014;7(1):6-8.

Huang P, Huang B, Weng H, Nakayama K, Morimoto K. Effects of lifestyle on micronuclei frequency in human lymphocytes in japanese hard-metal workers. *Prev Med*. 2009;48(4):383-388. doi: 10.1016/j.ypmed.2008.12.023 [doi].

Hunt PA, Koehler KE, Susiarjo M, et al. Bisphenol a exposure causes meiotic aneuploidy in the female mouse. *Curr Biol*. 2003;13(7):546-553. doi: S0960-9822(03)00189-1 [pii].

Jacobs J, Schwartz A, McDougle CJ, Skotko BG. Rapid clinical deterioration in an individual with Down syndrome. *Am J Med Genet A*. 2016;170(7):1899-1902. doi: 10.1002/ajmg.a.37674 [doi].

Jap SN, Ghaziuddin N. Catatonia among adolescents with Down syndrome: A review and 2 case reports. *J ECT*. 2011;27(4):334-337. doi: 10.1097/YCT.0b013e31821d37c6 [doi].

Jarrold C, Baddeley AD, Phillips CE. Verbal short-term memory in Down syndrome: A problem of memory, audition, or speech? *J Speech Lang Hear Res*. 2002;45(3):531-544.

Jenkins EC, Velinov MT, Ye L, et al. Telomere shortening in T lymphocytes of older individuals with Down syndrome and dementia. *Neurobiol Aging*. 2006;27(7):941-945. doi: S0197-4580(05)00171-5 [pii].

Jeyapalan JC, Ferreira M, Sedivy JM, Herbig U. Accumulation of senescent cells in mitotic tissue of aging primates. *Mech Ageing Dev*. 2007;128(1):36-44. doi: S0047-6374(06)00241-7 [pii].

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(6):745-752.

Jones K. Down syndrome (trisomy 21 syndrome). In: *Smith's recognizable patterns of human malformation.* 6th ed. La Jolla, California, US: Elsevier, Inc.; 2006:7--9.

Jovanovic SV, Clements D, MacLeod K. Biomarkers of oxidative stress are significantly elevated in Down syndrome. *Free Radic Biol Med*. 1998;25(9):1044-1048. doi: S0891-5849(98)00137-3 [pii].

Kappei D, Londono-Vallejo JA. Telomere length inheritance and aging. *Mech Ageing Dev*. 2008;129(1-2):17-26. doi: S0047-6374(07)00170-4 [pii].

Kasari C, Freeman SF. Task-related social behavior in children with Down syndrome. *Am J Ment Retard*. 2001;106(3):253-264. doi: 10.1352/0895-8017(2001)106<0253:TRSBIC>2.0.CO;2 [doi].

Kasari C, Freeman SF, Bass W. Empathy and response to distress in children with Down syndrome. *J Child Psychol Psychiatry*. 2003;44(3):424-431.

Kissling GE, Dertinger SD, Hayashi M, MacGregor JT. Sensitivity of the erythrocyte micronucleus assay: Dependence on number of cells scored and inter-animal variability. *Mutat Res*. 2007;637:235--240.

Kline J, Kinney A, Reuss ML, et al. Trisomic pregnancy and the oocyte pool. *Hum Reprod*. 2004;19(7):1633-1643. doi: 10.1093/humrep/deh310 [doi].

Klusmann JH, Creutzig U, Zimmermann M, et al. Treatment and prognostic impact of transient leukemia in neonates with Down syndrome. *Blood*. 2008;111(6):2991-2998. doi: 10.1182/blood-2007-10-118810 [doi].

Koch CM, Joussen S, Schellenberg A, Lin Q, Zenke M, Wagner W. Monitoring of cellular senescence by DNA-methylation at specific CpG sites. *Aging Cell*. 2012;11(2):366-369. doi: 10.1111/j.1474-9726.2011.00784.x [doi].

Krinsky-McHale SJ, Devenny DA, Gu H, et al. Successful aging in a 70-year-old man with Down syndrome: A case study. *Intellect Dev Disabil*. 2008;46(3):215-228. doi: 10.1352/2008.46:215-228 [doi].

Krinsky-McHale SJ, Silverman W. Dementia and mild cognitive impairment in adults with intellectual disability: Issues of diagnosis. *Dev Disabil Res Rev*. 2013;18(1):31-42. doi: 10.1002/ddrr.1126 [doi].

Kumin L, Schoenbrodt L. Employment in adults with Down syndrome in the united states: Results from a national survey. *J Appl Res Intellect Disabil*. 2016;29(4):330-345. doi: 10.1111/jar.12182 [doi].

Kusters MA, Verstegen RH, Gemen EF, de Vries E. Intrinsic defect of the immune system in children with Down syndrome: A review. *Clin Exp Immunol*. 2009;156(2):189-193. doi: 10.1111/j.1365-2249.2009.03890.x [doi].

Lanfranchi S, Jerman O, Dal Pont E, Alberti A, Vianello R. Executive function in adolescents with Down syndrome. *J Intellect Disabil Res*. 2010;54(4):308-319. doi: 10.1111/j.1365-2788.2010.01262.x [doi].

Lansdorp PM, Verwoerd NP, van de Rijke FM, et al. Heterogeneity in telomere length of human chromosomes. *Hum Mol Genet*. 1996;5(5):685-691.

Leach NT, Jackson-Cook C. Micronuclei with multiple copies of the X chromosome: Do chromosomes replicate in micronuclei? *Mutat Res*. 2004;554(1-2):89-94. doi: 10.1016/j.mrfmmm.2004.03.004 [doi].

Leboyer M, Bellivier F, Nosten-Bertrand M, Jouvent R, Pauls D, Mallet J. Psychiatric genetics: Search for phenotypes. *Trends Neurosci*. 1998;21(3):102-105. doi: S0166-2236(97)01187-9 [pii].

Lee SL, Thomas P, Hecker J, Faunt J, Fenech M. Chromosomal DNA damage measured using the cytokinesis-block micronucleus cytome assay is significantly associated with cognitive impairment in south australians. *Environ Mol Mutagen*. 2015;56(1):32-40. doi: 10.1002/em.21890 [doi].

Lemere CA, Blusztajn JK, Yamaguchi H, Wisniewski T, Saido TC, Selkoe DJ. Sequence of deposition of heterogeneous amyloid beta-peptides and APO E in Down syndrome: Implications for initial events in amyloid plaque formation. *Neurobiol Dis*. 1996;3(1):16-32. doi: 10.1006/nbdi.1996.0003 [doi].

Levy MZ, Allsopp RC, Futcher AB, Greider CW, Harley CB. Telomere end-replication problem and cell aging. *J Mol Biol*. 1992;225(4):951-960. doi: 0022-2836(92)90096-3 [pii].

Liu B, Filippi S, Roy A, Roberts I. Stem and progenitor cell dysfunction in human trisomies. EMBO Rep. 2015;16(1):44-62. doi: 10.15252/embr.201439583 [doi].

López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. *Cell*. 2013;153(6):1194-1217. doi: 10.1016/j.cell.2013.05.039 [doi].

Lott IT. Neurological phenotypes for Down syndrome across the life span. *Prog Brain Res*. 2012;197:101-121. doi: 10.1016/B978-0-444-54299-1.00006-6 [doi].

Lott IT. Antioxidants in Down syndrome. *Biochim Biophys Acta*. 2012;1822(5):657-663. doi: 10.1016/j.bbadis.2011.12.010 [doi].

Lott IT, Dierssen M. Cognitive deficits and associated neurological complications in individuals with Down's syndrome. *Lancet Neurol*. 2010;9(6):623-633. doi: 10.1016/S1474-4422(10)70112-5 [doi].

Machiela MJ, Zhou W, Sampson JN, et al. Characterization of large structural genetic mosaicism in human autosomes. *Am J Hum Genet*. 2015;96(3):487-497. doi: 10.1016/j.ajhg.2015.01.011 [doi].

Malinge S, Izraeli S, Crispino JD. Insights into the manifestations, outcomes, and mechanisms of leukemogenesis in Down syndrome. *Blood*. 2009;113(12):2619-2628. doi: 10.1182/blood-2008-11-163501 [doi].

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(1):71-75. doi: S0165-4608(00)00322-8 [pii].

Manvelyan M, Hunstig F, Mrasek K, et al. Position of chromosomes 18, 19, 21 and 22 in 3D-preserved interphase nuclei of human and gorilla and white hand gibbon. *Mol Cytogenet*. 2008;1:9-8166-1-9. doi: 10.1186/1755-8166-1-9 [doi].

Marild K, Stephansson O, Grahnquist L, Cnattingius S, Soderman G, Ludvigsson JF. Down syndrome is associated with elevated risk of celiac disease: A nationwide case-control study. *J Pediatr*. 2013;163(1):237-242. doi: 10.1016/j.jpeds.2012.12.087 [doi].

Maris M, Wojciechowski M, Van de Heyning P, Boudewyns A. A cross-sectional analysis of otitis media with effusion in children with Down syndrome. *Eur J Pediatr*. 2014;173(10):1319-1325. doi: 10.1007/s00431-014-2323-5 [doi].

Massey GV, Zipursky A, Chang MN, et al. A prospective study of the natural history of transient leukemia (TL) in neonates with Down syndrome (DS): Children's oncology group (COG) study POG-9481. *Blood*. 2006;107(12):4606-4613. doi: 2005-06-2448 [pii].

Mather KA, Jorm AF, Parslow RA, Christensen H. Is telomere length a biomarker of aging? A review. *J Gerontol A Biol Sci Med Sci*. 2011;66(2):202-213. doi: 10.1093/gerona/glq180 [doi].

McClintock B. The stability of broken ends of chromosomes in zea mays. *Genetics*. 1941;26:234--282.

Migliore L, Coppede F, Fenech M, Thomas P. Association of micronucleus frequency with neurodegenerative diseases. *Mutagenesis*. 2011;26(1):85-92. doi: 10.1093/mutage/geq067 [doi].

Migliore L, Testa A, Scarpato R, Pavese N, Petrozzi L, Bonuccelli U. Spontaneous and induced aneuploidy in peripheral blood lymphocytes of patients with Alzheimer's disease. *Hum Genet*. 1997;101(3):299-305.

Muller H. The remaking of chromosomes. *Collecting Net*. 1938;13:181--198.

Muramatsu H, Kato K, Watanabe N, et al. Risk factors for early death in neonates with Down syndrome and transient leukaemia. *Br J Haematol*. 2008;142(4):610-615. doi: 10.1111/j.1365-2141.2008.07231.x [doi].

Nakamura E, Tanaka S. Biological ages of adult men and women with Down's syndrome and its changes with aging. *Mech Ageing Dev*. 1998;105(1-2):89-103. doi: S0047-6374(98)00081-5 [pii].

Nam HJ, Naylor RM, van Deursen JM. Centrosome dynamics as a source of chromosomal instability. *Trends Cell Biol*. 2015;25(2):65-73. doi: 10.1016/j.tcb.2014.10.002 [doi].

Nelson L, Johnson JK, Freedman M, et al. Learning and memory as a function of age in Down syndrome: A study using animal-based tasks. *Prog Neuropsychopharmacol Biol Psychiatry*. 2005;29(3):443-453. doi: S0278-5846(05)00012-6 [pii].

Ng DK, Chan CH. Obesity is an important risk factor for sleep disordered breathing in children with Down syndrome. *Sleep*. 2004;27(5):1023-4; author reply 1025.

Nicholson JM, Macedo JC, Mattingly AJ, et al. Chromosome mis-segregation and cytokinesis failure in trisomic human cells. *Elife*. 2015;4:10.7554/eLife.05068. doi: 10.7554/eLife.05068 [doi].

Nizetic D, Groet J. Tumorigenesis in Down's syndrome: Big lessons from a small chromosome. *Nat Rev Cancer*. 2012;12(10):721-732. doi: 10.1038/nrc3355 [doi].

Opresko PL, Shay JW. Telomere-associated aging disorders. *Ageing Res Rev*. 2017;33:52-66. doi: S1568-1637(16)30088-5 [pii].

O'Rafferty C, Kelly J, Storey L, Ryan C, O'Marcaigh A, Smith O. Child and adolescent Down syndrome-associated leukaemia: The irish experience. *Ir J Med Sci*. 2015;184(4):877-882. doi: 10.1007/s11845-014-1212-2 [doi].

O'Sullivan JN, Bronner MP, Brentnall TA, et al. Chromosomal instability in ulcerative colitis is related to telomere shortening. *Nat Genet*. 2002;32(2):280-284. doi: 10.1038/ng989 [doi].

O'Sullivan RJ, Karlseder J. Telomeres: Protecting chromosomes against genome instability. *Nat Rev Mol Cell Biol*. 2010;11(3):171-181. doi: 10.1038/nrm2848 [doi].

Pangalos C, Avramopoulos D, Blouin JL, et al. Understanding the mechanism(s) of mosaic trisomy 21 by using DNA polymorphism analysis. *Am J Hum Genet*. 1994;54(3):473-481.

Panossian LA, Porter VR, Valenzuela HF, et al. Telomere shortening in T cells correlates with alzheimer's disease status. *Neurobiol Aging*. 2003;24(1):77-84. doi: S019745800200043X [pii].

Parker SE, Mai CT, Canfield MA, et al. Updated national birth prevalence estimates for selected birth defects in the united states, 2004-2006. *Birth Defects Res A Clin Mol Teratol*. 2010;88(12):1008-1016. doi: 10.1002/bdra.20735 [doi].

Pennington BF, Moon J, Edgin J, Stedron J, Nadel L. The neuropsychology of Down syndrome: Evidence for hippocampal dysfunction. *Child Dev*. 2003;74(1):75-93.

Perl DP. Neuropathology of alzheimer's disease. *Mt Sinai J Med*. 2010;77(1):32-42. doi: 10.1002/msj.20157 [doi].

Petrozzi L, Lucetti C, Scarpato R, et al. Cytogenetic alterations in lymphocytes of alzheimer's disease and parkinson's disease patients. *Neurol Sci*. 2002;23 Suppl 2:S97-8. doi: 10.1007/s100720200087 [doi].

Potter H. Review and hypothesis: Alzheimer disease and Down syndrome--chromosome 21 nondisjunction may underlie both disorders. *Am J Hum Genet*. 1991;48(6):1192-1200.

Pradhan M, Dalal A, Khan F, Agrawal S. Fertility in men with Down syndrome: A case report. *Fertil Steril*. 2006;86(6):1765.e1-1765.e3. doi: S0015-0282(06)03067-6 [pii].

Prasher V. Disintegrative syndrome in young adults. *Ir J Psych Med*. 2002;19(3):101--102.

Pueschel SM, Scola FH. Atlantoaxial instability in individuals with Down syndrome: Epidemiologic, radiographic, and clinical studies. *Pediatrics*. 1987;80(4):555-560.

Ram G, Chinen J. Infections and immunodeficiency in Down syndrome. *Clin Exp Immunol*. 2011;164(1):9-16. doi: 10.1111/j.1365-2249.2011.04335.x [doi].

Rode L, Nordestgaard BG, Bojesen SE. Peripheral blood leukocyte telomere length and mortality among 64,637 individuals from the general population. *J Natl Cancer Inst*. 2015;107(6):djv074. doi: 10.1093/jnci/djv074 [doi].

Roizen NJ, Patterson D. Down's syndrome. *Lancet*. 2003;361(9365):1281-1289. doi: S0140-6736(03)12987-X [pii].

Roizen NJ, Wolters C, Nicol T, Blondis TA. Hearing loss in children with Down syndrome. *J Pediatr*. 1993;123(1):S9-12.

Samani NJ, Boultby R, Butler R, Thompson JR, Goodall AH. Telomere shortening in atherosclerosis. *Lancet*. 2001;358(9280):472-473. doi: S0140-6736(01)05633-1 [pii].

Samassekou O, Gadji M, Drouin R, Yan J. Sizing the ends: Normal length of human telomeres. *Ann Anat*. 2010;192(5):284-291. doi: 10.1016/j.aanat.2010.07.005 [doi].

Savickiene J, Baronaite S, Zentelyte A, Treigyte G, Navakauskiene R. Senescence-associated molecular and epigenetic alterations in mesenchymal stem cell cultures from amniotic fluid of normal and fetus-affected pregnancy. *Stem Cells Int*. 2016;2016:2019498. doi: 10.1155/2016/2019498 [doi].

Scarfi MR, Cossarizza A, Monti D, et al. Age-related increase of mitomycin C-induced micronuclei in lymphocytes from Down's syndrome subjects. *Mutat Res*. 1990;237(5-6):247-252. doi: 0921-8734(90)90006-D [pii].

Schmid G, Pfitzer P. Mitoses and binucleated cells in perinatal human hearts. *Virchows Arch B Cell Pathol Incl Mol Pathol*. 1985;48(1):59-67.

Sedivy JM, Banumathy G, Adams PD. Aging by epigenetics--a consequence of chromatin damage? *Exp Cell Res*. 2008;314(9):1909-1917. doi: 10.1016/j.yexcr.2008.02.023 [doi].

Sharr C, Lavigne J, Elsharkawi IM, et al. Detecting celiac disease in patients with Down syndrome. *Am J Med Genet A*. 2016;170(12):3098-3105. doi: 10.1002/ajmg.a.37879 [doi].

Sheridan R, Llerena J,Jr, Matkins S, Debenham P, Cawood A, Bobrow M. Fertility in a male with trisomy 21. *J Med Genet*. 1989;26(5):294-298.

Sherman SL, Allen EG, Bean LH, Freeman SB. Epidemiology of Down syndrome. *Ment Retard Dev Disabil Res Rev*. 2007;13(3):221-227. doi: 10.1002/mrdd.20157 [doi].

Sherman SL, Takaesu N, Freeman SB, et al. Trisomy 21: Association between reduced recombination and nondisjunction. *Am J Hum Genet*. 1991;49(3):608-620.

Shumaker DK, Dechat T, Kohlmaier A, et al. Mutant nuclear lamin A leads to progressive alterations of epigenetic control in premature aging. *Proc Natl Acad Sci U S A*. 2006;103(23):8703-8708. doi: 0602569103 [pii].

Siegel MS, Smith WE. Psychiatric features in children with genetic syndromes: Toward functional phenotypes. *Child Adolesc Psychiatr Clin N Am*. 2010;19(2):229-61, viii. doi: 10.1016/j.chc.2010.02.001 [doi].

Silkworth WT, Nardi IK, Paul R, Mogilner A, Cimini D. Timing of centrosome separation is important for accurate chromosome segregation. *Mol Biol Cell*. 2012;23(3):401-411. doi: 10.1091/mbc.E11-02-0095 [doi].

Silverman W. Down syndrome: Cognitive phenotype. *Ment Retard Dev Disabil Res Rev*. 2007;13(3):228-236. doi: 10.1002/mrdd.20156 [doi].

Skotko BG, Levine SP, Goldstein R. Having a son or daughter with Down syndrome: Perspectives from mothers and fathers. *Am J Med Genet A*. 2011;155A(10):2335-2347. doi: 10.1002/ajmg.a.34293 [doi].

Skotko BG, Levine SP, Goldstein R. Self-perceptions from people with Down syndrome. *Am J Med Genet A*. 2011;155A(10):2360-2369. doi: 10.1002/ajmg.a.34235 [doi].

Slagboom PE, Droog S, Boomsma DI. Genetic determination of telomere size in humans: A twin study of three age groups. *Am J Hum Genet*. 1994;55(5):876-882.

Smith DS. Health care management of adults with Down syndrome. *Am Fam Physician*. 2001;64(6):1031-1038.

Souroullas GP, Sharpless NE. Stem cells: Down's syndrome link to ageing. Nature. 2013;501(7467):325-326. doi: 10.1038/nature12558 [doi].

Starkweather AR, Alhaeeri AA, Montpetit A, et al. An integrative review of factors associated with telomere length and implications for biobehavioral research. *Nurs Res*. 2014;63(1):36-50. doi: 10.1097/NNR.0000000000000009 [doi].

Stein DS, Munir KM, Karweck AJ, Davidson EJ, Stein MT. Developmental regression, depression, and psychosocial stress in an adolescent with Down syndrome. *J Dev Behav Pediatr*. 2013;34(3):216-218. doi: 10.1097/DBP.0b013e31828b2b42 [doi].

Stoll C, Alembik Y, Dott B, Roth MP. Study of Down syndrome in 238,942 consecutive births. *Ann Genet*. 1998;41(1):44-51.

Sukenik-Halevy R, Biron-Shental T, Sharony R, Fejgin MD, Amiel A. Telomeres in trisomy 21 amniocytes. *Cytogenet Genome Res*. 2011;135(1):12-18. doi: 10.1159/000329714 [doi].

Sun HB, Shen J, Yokota H. Size-dependent positioning of human chromosomes in interphase nuclei. *Biophys J*. 2000;79(1):184-190. doi: S0006-3495(00)76282-5 [pii].

Swanson EC, Manning B, Zhang H, Lawrence JB. Higher-order unfolding of satellite heterochromatin is a consistent and early event in cell senescence. *J Cell Biol*. 2013;203(6):929-942. doi: jcb.201306073 [pii].

Tedone E, Arosio B, Gussago C, et al. Leukocyte telomere length and prevalence of age-related diseases in semisupercentenarians, centenarians and centenarians' offspring. *Exp Gerontol*. 2014;58:90-95. doi: 10.1016/j.exger.2014.06.018 [doi].

Thomas P, Harvey S, Gruner T, Fenech M. The buccal cytome and micronucleus frequency is substantially altered in Down's syndrome and normal ageing compared to young healthy controls. *Mutat Res*. 2008;638(1-2):37-47. doi: S0027-5107(07)00324-7 [pii].

Thompson SL, Bakhoum SF, Compton DA. Mechanisms of chromosomal instability. *Curr Biol*. 2010;20(6):R285-95. doi: 10.1016/j.cub.2010.01.034 [doi].

Torr J, D'Abrera JC. Maintenance electroconvulsive therapy for depression with catatonia in a young woman with Down syndrome. *J ECT*. 2014;30(4):332-336. doi: 10.1097/YCT.0000000000000116 [doi].

Tsurumi A, Li WX. Global heterochromatin loss: A unifying theory of aging? *Epigenetics*. 2012;7(7):680-688. doi: 10.4161/epi.20540 [doi].

United Nations Department of Economic and Social Affairs. United nations world population prospects: 2015 revision. 2015.

Urv TK, Zigman WB, Silverman W. Psychiatric symptoms in adults with Down syndrome and alzheimer's disease. *Am J Intellect Dev Disabil*. 2010;115(4):265-276. doi: 10.1352/1944-7558-115.4.265 [doi].

Vasa-Nicotera M, Brouilette S, Mangino M, et al. Mapping of a major locus that determines telomere length in humans. *Am J Hum Genet*. 2005;76(1):147-151. doi: S0002-9297(07)62551-8 [pii].

Vaziri H, Schachter F, Uchida I, et al. Loss of telomeric DNA during aging of normal and trisomy 21 human lymphocytes. *Am J Hum Genet*. 1993;52(4):661-667.

Vicari S. Memory development and intellectual disabilities. *Acta Paediatr Suppl*. 2004;93(445):60-3; discussion 63-4.

Villeponteau B. The heterochromatin loss model of aging. *Exp Gerontol*. 1997;32(4-5):383-394. doi: S0531-5565(96)00155-6 [pii].

Walker JC, Dosen A, Buitelaar JK, Janzing JG. Depression in Down syndrome: A review of the literature. *Res Dev Disabil*. 2011;32(5):1432-1440. doi: 10.1016/j.ridd.2011.02.010 [doi].

Wang C, Jurk D, Maddick M, Nelson G, Martin-Ruiz C, von Zglinicki T. DNA damage response and cellular senescence in tissues of aging mice. *Aging Cell*. 2009;8(3):311-323. doi: 10.1111/j.1474-9726.2009.00481.x [doi].

Warburton D. Biological aging and the etiology of aneuploidy. *Cytogenet Genome Res*. 2005;111(3-4):266-272. doi: 86899 [pii].

Wark S, Hussain R, Parmenter T. Down syndrome and dementia: Is depression a confounder for accurate diagnosis and treatment? *J Intellect Disabil*. 2014;18(4):305-314. doi: 10.1177/1744629514552152 [doi].

Watanabe Y, Fujiyama A, Ichiba Y, et al. Chromosome-wide assessment of replication timing for human chromosomes 11q and 21q: Disease-related genes in timing-switch regions. *Hum Mol Genet*. 2002;11(1):13-21.

Wilcock DM, Griffin WS. Down's syndrome, neuroinflammation, and alzheimer neuropathogenesis. *J Neuroinflammation*. 2013;10:84-2094-10-84. doi: 10.1186/1742-2094-10-84 [doi].

Wirth KG, Wutz G, Kudo NR, et al. Separase: A universal trigger for sister chromatid disjunction but not chromosome cycle progression. *J Cell Biol*. 2006;172(6):847-860. doi: jcb.200506119 [pii].

Witt KL, Livanos E, Kissling GE, et al. Comparison of flow cytometry- and microscopy-based methods for measuring micronucleated reticulocyte frequencies in rodents treated with nongenotoxic and genotoxic chemicals. *Mutat Res*. 2008;649:101--113.

Woodfine K, Fiegler H, Beare DM, et al. Replication timing of the human genome. *Hum Mol Genet*. 2004;13(2):191-202. doi: 10.1093/hmg/ddh016 [doi].

Worley G, Crissman BG, Cadogan E, Milleson C, Adkins DW, Kishnani PS. Down syndrome disintegrative disorder: New-onset autistic regression, dementia, and insomnia in older children and adolescents with Down syndrome. *J Child Neurol*. 2015;30(9):1147-1152. doi: 10.1177/0883073814554654 [doi].

Xavier AC, Ge Y, Taub J. Unique clinical and biological features of leukemia in Down syndrome children. *Expert Rev Hematol*. 2010;3(2):175-186. doi: 10.1586/ehm.10.14 [doi].

Xie J, Jiang J, Shi K, et al. DNA damage in peripheral blood lymphocytes from patients with OSAHS. *Sleep Breath*. 2014;18(4):775-780. doi: 10.1007/s11325-014-0942-8 [doi].

Yang Y, Conners FA, Merrill EC. Visuo-spatial ability in individuals with Down syndrome: Is it really a strength? *Res Dev Disabil*. 2014;35(7):1473-1500. doi: 10.1016/j.ridd.2014.04.002 [doi].

Zemel BS, Pipan M, Stallings VA, et al. Growth charts for children with Down syndrome in the united states. *Pediatrics*. 2015;136(5):e1204-11. doi: 10.1542/peds.2015-1652 [doi].

Zhang R, Zhao J, Xu J, Liu F. Association of peripheral leukocyte telomere length and its variation with pancreatic cancer and colorectal cancer risk in chinese population. *Oncotarget*. 2016;7(25):38579-38585. doi: 10.18632/oncotarget.9536 [doi].

Zhang W, Li J, Suzuki K, et al. Aging stem cells. A werner syndrome stem cell model unveils heterochromatin alterations as a driver of human aging. *Science*. 2015;348(6239):1160-1163. doi: 10.1126/science.aaa1356 [doi].

Zhu JL, Obel C, Hasle H, Rasmussen SA, Li J, Olsen J. Social conditions for people with Down syndrome: A register-based cohort study in denmark. *Am J Med Genet A*. 2014;164A(1):36-41. doi: 10.1002/ajmg.a.36272 [doi].

Zigman WB. Atypical aging in Down syndrome. *Dev Disabil Res Rev*. 2013;18(1):51-67. doi: 10.1002/ddrr.1128 [doi].

Zou Y, Sfeir A, Gryaznov SM, Shay JW, Wright WE. Does a sentinel or a subset of short telomeres determine replicative senescence? Mol Biol Cell. 2004;15(8):3709-3718. doi: 10.1091/mbc.E04-03-0207 [doi].

Zuhlke C, Thies U, Braulke I, Reis A, Schirren C. Down syndrome and male fertility: PCR-derived fingerprinting, serological and andrological investigations. *Clin Genet*. 1994;46(4):324-326.

Zwaan CM, Reinhardt D, Hitzler J, Vyas P. Acute leukemias in children with Down syndrome. *Hematol Oncol Clin North Am*. 2010;24(1):

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