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Telomere length: implications on the risk for chromosome 21 nondisjunction in oocytes

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Abstract

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By Igor Albizua

Trisomy 21, the chromosomal abnormality responsible for Down syndrome (DS), is a complex condition with many characteristic symptoms as well as an increased risk for numerous congenital anomalies. Previous studies estimated that 93% of DS cases result from a chromosomal error during the generation of the oocyte, with maternal age being the best known risk factor of chromosome 21 nondisjunction. An altered recombination profile, as defined by the number of recombinant events and their location along chromosome 21, has been recognized from detailed analysis of nondisjunction events. Telomeres are specific repetitive DNA sequences formed by 6 nucleotides (TTAGGG), and its complementary sequence (AATCCC), found at the end of chromosomes. The loss of these sequencing and the concomitant shortening of chromosomes are considered a genetic marker for aging.

In our study, we first test the hypothesis that mothers of children with DS have shorter telomeres than mothers of euploid children. In effect, our hypothesis suggests that mothers of children with DS will appear "biologically older" as compare to the mothers of euploid children. We developed and applied a quantitative PCR assay to measure the genome-wide relative telomere length in order to test this hypothesis. We also test the relationship between relative telomere length and absence of recombination, a known risk factor for chromosome 21 nondisjunction during the first meiotic division of the oocyte.

Our data support telomere length as a biomarker of age and hence nondisjunction. We also found a relationship between maternal shortened telomeres and lower levels of recombination in the nondisjoined chromosome. This result suggests that telomere length may also be a biomarker for abnormal telomere functioning during the formation of the oocyte.

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Chapter 1: Introduction

Down Syndrome

The history of Down Syndrome (DS) goes back to the 19th century. In 1866, Dr. John Landon Down published his paper *Observations on an Ethnic Classification of Idiots* where based on the phenotypic characteristics such as broad, flat face, slightly slanted eyes and rough thick tongue (1), he described people with DS as "Mongolian idiots".

Dr. Langdon Down not only described the physical characteristics of these individuals, he was also the first to describe the cognitive aspects of what is nowadays known as DS. Dr. Down also suggested that the syndrome had its origin in early development and never after the baby was born. The amazing part of this hypothesis comes from the fact that he proposed it without any knowledge of the genetic discoveries of Gregor Mendel in 1866.

This hypothesis about the uterine origin of the syndrome was later confirmed by many studies. Dr. Halbertsma (2) studied two identical monozygotic twins and 15 non-identical dyzogotic twins. He observed that while monozygotic twins shared the phenotype, the dizygotic twins did not. This led him to conclude that the DS had a fetal origin and confirmed Dr. Down's hypothesis from 1866.

It was not until 1956 that, thanks to the development of new techniques that allowed researchers to observe condensed chromosomes inside a cell, Tijo and Levan were able to observe that

normal somatic cells contained 23 pairs of chromosomes (3). Using this same technique, in 1959 Lejeune et al. discovered that people with DS had an extra copy of the chromosome 21 in their karyotype confirming for the first type the molecular origin of the DS (4).

Nowadays we know that individuals with DS are affected by symptoms that range from mild to severe. Some are common while others are more variable. Some of the common symptoms are intellectual disabilities, hypotonia, abnormalities of the face, hands and feet. Regarding the variable ones, about half of children with DS are born with heart defects, including atrial septal defect, ventricular septal defect, and atrioventricular septal defects. Children with DS also have an increased risk for certain types of leukemia, which can also cause early death. Adults with DS also have an increased risk for dementia.

Epidemiology of Down syndrome

In 2010, a large population-based study by Parker et al, estimated the prevalence of DS at 14.47 per 10,000 or one in every 691 live births (5) but it is worth mentioning that nearly 80% of trisomy 21 conceptuses are lost prior to term as a result of spontaneous abortion (6).

Canfield et al. (7) discovered different rates of DS incidence between different races or ethnic background in the U.S. For example, non-Hispanic black mothers showed a decreased prevalence of children with DS (OR 0.77, 95% CI 0.69-0.87) while Hispanic mothers showed an increased prevalence of children with DS (OR 1.12, 95% CI 1.03-1.21) when compared to non-Hispanic white mothers. There have been many studies that have tried to explain these

differences based on genetic, environmental, cultural or socioeconomic factors but none of these factors seem to provide conclusive evidence (7-9).

For many years, researchers have been focused in two important aspects of DS. On one side, a lot of research has been done trying to understand the factors that may predispose an embryo to have DS and on the other side, many researchers have focused on understanding the molecular origins of this chromosomal aberration. Nowadays we know that trisomy can arise from 3 different types of chromosomal errors: 1) complete trisomy 21 or three independent chromosomes 21, 2) chromosomal translocations of chromosome 21, and 3) mosaic trisomy 21 where some cells are euploid while others have an extra chromosome 21.

Nowadays we can determine the type of nondisjunction error that gives rise to the trisomy 21. Thanks to the use of polymorphic markers we can see if the error happened during the formation of the gametes or if it was a post-zygotic error. In case the error was during the formation of the gametes, we can also determine if it occurred during the formation of the spermatocyte (paternal error) or during the formation of the egg (maternal error). Results from the National Down Syndrome Project (NDSP) showed a striking distribution of the origin of the trisomy. Out of 800 families genotyped, in 93% of them the error had a maternal origin while only 4% of them had a paternal origin with the rest having a post zygotic origin (11). Among the maternal nondisjunction cases, 75% of cases occur during the meiosis I (MI), while 25% occur during the meiosis II (MII). It is important to mention that some of these MII errors, may have originated during MI, but they look like MII errors (10-12).

A lot of research has been done focusing on the possible risk factor leading to nondisjunction and so far, the most important risk factor identified is maternal age. Already in 1933, Dr. Penrose showed that mothers older than 35 had an increased risk of having a child with DS (13-14). Later research showed that this increased risk is only seen in cases where the trisomy is caused by a maternal error not in cases of paternal origin (15-20). The maternal age influence applies to both MI and MII maternal errors but the increased risk for MI errors starts at around 35 years of age but for MII errors starts a bit later at around 38 (21).

After maternal age, the second most important risk factor involved in maternal chromosome 21 nondisjunction is altered recombination along the nondisjoined chromosome 21. There are three chiasmate configurations where risks were identified. If there is no recombination on chromosome 21, there is an increased risk of nondisjunction during meiosis I (22-23). If there is a single telomeric exchange, there is also an increased risk of nondisjunction during meiosis I while an exchange close to the centromere increases the risk of nondisjunction during meiosis II (24-25).

Female meiosis and human oogenesis

More than 90% of DS cases are due to an error in the formation of oocytes leading to one extra copy of the chromosome 21 present in the egg. As noted above, the age of the egg is an important risk factor for nondisjunction. Based on these findings, it is important to have a clear understanding of the specific characteristics that are present during the formation of female eggs or oogenesis and the female meiosis process that gives rise to them. First, I will review the

meiosis in general, or the method of gamete formation that is common in egg and sperm. Then I will review features of meiosis during oogenesis that differ from meiosis in spermatogenesis.

Meiosis is a special type of cell division that produces gametes (haploid cells called sperm or egg). It is during the first cell division that homologue chromosomes undergo recombination which helps segregate chromosomes properly while giving rise to new combinations of genes on each chromosome.

Meiosis starts with the DNA replication. Subsequently, the cell goes through two different types of divisions that will produce four haploid cells. Meiosis I is where homologous chromosomes separate giving rise to two haploid daughter cells. This division or meiosis I is called reductional division because a diploid cell divides into two haploid cells. The second division or meiosis II is called equational division because in this division, the sister chromatids of each chromosome separate into two daughter cells.

Each meiotic division consist of four different phases: prophase, metaphase, anaphase and telophase but the most important phase of all is the prophase of the first meiotic division. This prophase one is the most important stage because recombination between homologous chromosomes takes place. Oocytes are then arrested at this stage and remain this way until puberty where oocytes start maturing each month. In order to understand prophase I in a better way, we proceed to explain the different sub stages prophase I in a more detailed manner.

During the first stage of prophase I, called leptotene, chromosomes consisting of two sister chromatids condense and proceed into zygotene. In order for it to be successful and by extension, in order for mesiosis to be successful, chromosomes need to have functional (long enough) telomeres. Chromosomes form a structure called bouquet. This name is due to the fact that

chromosomes cluster at one end of the nucleus and attach to the nuclear envelope through their telomeres forming a structure that resembles a bouquet. This is important because proper alignment of homologous chromosomes is essential in order for synapsis to occur. This alignment of homologous chromosomes is also facilitated by the specific characteristics of each chromosome with respect to chromosome length and centromere position.

Once the bouquet formation has taken place and homologous chromosomes have aligned to each other, meiosis proceeds to the pachytene stage. At this point, recombination occurs and non-sister chromatids of homologous chromosomes exchange homologous DNA fragments.

At the next stage, called diplotene, even though the synaptonemal complex that kept the chromosomes together degrades, chromosome homologues remain bound by the chiasmata formed at the exchange sites.

During female meiosis, oocytes remain arrested at this stage until puberty. At that point an egg is recruited for ovulation and starts through the maturation process. Once an egg matures, it goes through the last sub stage of the prophase I which is called diakinesis. At this point, chiasmata formed previously are clearly visible as well as the four chromatids of the homologous chromosomes.

The next step in MI is metaphase I where homologous chromosomes move, or congress, to the metaphase plate. One of the most important characteristics of this stage is the random orientation of chromosome bivalents along the metaphase plate. This process ensures that non homologous chromosomes are pulled to each pole of the cell in a random manner.

During anaphase I, kinetochore microtubules shorten and pull homologous chromosomes apart. This produces 2 haploid sets of chromosomes. Chromosomes are still formed by a pair of sister chromatids that stick together due to the centromere cohesin complex. Telophase is the last stage of prophase I. At this point, chromosomes move to the poles and now each of the two daughter cells contain a haploid set of chromosomes, hence the name reductional division that is given to meiosis I.

Both daughter cells proceed now to meiosis II which is sub divided again into different stages. During prophase II, chromosomes consisting of two sister chromatids, condense and the nuclear envelope disappears. At this point, centrioles play a central role in the next stages. During prophase II, centrioles move to the poles and help to pull the chromatids apart during the next stages. During the metaphase of the meiosis II, centromeres containing two kinetochores attach to the centrioles through spindle fibers. During anaphase II, sister chromatids are pulled apart to the poles. Lastly, during telophase II, new nuclear envelopes and cell wall formed in order to produce 4 haploid daughter cells.

In my thesis, I focused on maternal nondisjunction because it leads to the vast majority of chromosome aneuploidy in humans. To help identify possible risk factors, it will be necessary to further explain female meiosis to provide the context in which these errors are occurring.

Apart from the fact that during oogenesis, oocytes remain stalled at prophase I as we explained before, one of the main characteristic that makes oogenesis unique when compared to spermatogenesis is the fact that females are born with a set number of oocytes. In other words, females cannot increase or maintain their number of oocytes during their adult life unlike males, who produce spermatocytes all through their adult life.

Lastly, it is important to mention that while the end product of spermatogenesis is four haploid cells or spermatocytes, the end product of oogenesis is only one haploid cell that has the potential to be fertilized and three polar bodies that are discarded. One of the polar bodies is produced at the end of the meiosis I. Once the meiosis I is restarted, the end product of this reductional division is a functional germ cell or secondary oocyte that will go through meiosis II and one polar body that is discarded. This secondary oocyte goes through meiosis II until metaphase II. It remains arrested in this stage until fertilization occurs. If fertilization occurs, this secondary oocyte goes through the rest of meiosis II and produces a functional egg and another polar body.

Telomeres

Background

Between 1975 and 1977, Dr. Elizabeth Blackburn at Yale University discovered the structure of telomeres and its specific repetitive DNA sequence formed by 6 nucleotides (TTAGGG) and its complementary sequence (AATCCC). In 2009, Elizabeth Blackburn, Carol Greider, and Jack Szostak were awarded the Nobel Prize in Physiology or Medicine "for the discovery of how chromosomes are protected by telomeres and the enzyme telomerase."

Function and Structure

As stated before, telomeres are repetitive and noncoding sequences of DNA formed by 6 nucleotides and their complementary bases TTAGGG/CCCTAA. These repeats are located at the end of the linear chromosomes and provide an excellent way to protect the ends of the chromosomes (26) as it is shown by the fact that these sequences are extremely conserved from an evolutionary point of view with little variation in the repeat sequence. While all vertebrates maintain the same six bases, plants (TTTAGGG), insects (TTAGG) or worms (TTAGGC) have a sequence with very small variation.

Also from an evolutionary point of view, it is important to consider the consequences of the transition from circular to linear chromosomes. This change had a huge impact on the genome stability of linear chromosomes due mainly to two reasons. The first one is the "end replication problem" due to the loss of nucleotides at the 5' end of the lagging strand during the

semiconservative DNA replication. The second problem arises from the fact that the end of the linear chromosomes should not be recognized as a double strand break (DSB). Here is where telomeres come into play: their repetitive sequences and protein-DNA complexes work to maintain the chromosome ends and protect them from the DNA repair machinery. We will describe both phenomena in more detail and describe how telomeres help reduce the associated outcomes.

The "end replication problem" is due to characteristics of linear chromosomes and to the enzymes that are involved in their replication. During the DNA replication, DNA polymerase can only add nucleotides in the 5'-3' direction. This creates a leading strand and a lagging strand. In the lagging strand, DNA replication occurs with the formation of the Okazaki fragments. The reduction in telomere sequence occurs with the removal of the RNA primer from the terminal Okazaki fragment on the lagging strand. This results in incomplete copying of the terminus of that strand, consequently leading to a shorter template for copying in the next round of DNA synthesis.

The solution to this problem in humans comes from the presence of a specialized enzyme called telomerase. The efficiency of this system relies on the nature of this reverse transcriptase enzyme. Telomerase synthesizes a G-rich 3' extension composed of TTAGGG at the end of the chromosome thanks to that fact that it carries its own RNA template. The complementary sequence to this extension, AATCCC, is synthesized by DNA polymerases in a normal semi conservative way.

Telomerase elongates telomeres during development in order to allow the cells to go through several rounds of future replications. Telomerase then becomes inactive in somatic cells. With

each cell division, telomeres become shorter until they reach a critical length that leads the cell to apoptosis. In other words, telomeres represent a temporary solution to the end replication problem, but without them to buffer the loss of DNA, chromosomes would get progressively shorter giving rise to chromosome fusions and genomic instability.

The second problem relates to the fact that the ends of linear chromosomes could be recognized as double strand breaks by the DNA repair machinery. Telomeres fix this adopting a structure called "t-loop" with the use of several telomere binding proteins. An unknown nuclease generates a G-rich overhang at the end of the chromosomes after DNA replication. This overhang has a length of 50 to 300 nucleotides (27).

In order to form a closed structure that avoids the problem of being recognized as a double strand break, chromosomes fold back on themselves, the double DNA strand opens up to allow the G-overhang to find its complementary sequence on the C-rich strand, forming the structure called "t-loop".

There are several proteins that collaborate on the formation of the "t-loop". These proteins are part of a complex called "shelterin complex". Six proteins in total take part in the stabilization of this structure: Telomeric Repeat-binding Factors 1 and 2 (TRF1 and TRF2) bind the double strand part of the telomere and TRF1-interacting nuclear protein 2 (TIN2) acts as a link between TRF1 and TRF2. On the other side, protection of telomeres 1 (POT1) binds single stranded DNA and TIN2- and POT1-interacting protein (TPP1) links TIN2 and POT1. Finally, the repressor and activator protein 1 (RAP1), interacts with TRF2 (27).

Telomere shortening

Telomeres get shorter as a consequence of cell division and after several rounds or cell division and DNA replication, telomeric repeat length reaches a point that makes telomeres no longer functional. The consequence of this is genome instability, or replicatve senescence of the cell death, as it can no longer divide. In other words, telomere shortening provides cells with a set of number of division that they can go through after which the cell dies.

There are different theories that try to explain the implications that telomere shortening and replicative senescence have for an organism. While the blocking of cell division due to replicative senescence is important in the prevention of genomic instability and cancer, there are other studies (28) that suggest that shortened telomeres may impair immune function. As a result, rates of cancer may increase because cells become immortalized by telomere extension due to the activation of telomerase or through ALT pathways.

Several authors have also argued about the importance of the telomere length. They suggest that short telomeres are an indicative of poor health and aging (28). Thus, we should expect to see a selection in favor of long telomeres. In 2002, a study suggested (29) that having longer telomeres could cause an increased susceptibility to cancer. However a review in 2011(28) points out that shorter telomeres and the inactivation of the enzyme telomerase are often associated to higher rates of cancer and thus inconsistent with the previous hypothesis. The author points out that this increased risk normally happens late in life and at this point natural selection influence is low.

As we explained before, telomerase is the enzyme responsible for telomere restoration but its activity level is low or undetected in somatic cells. On the other hand, it has a higher level activity in embryonic or adult stem cells and germ line cells (30), though there are differences

between male and female germ cells. For example, Liu et al. (32) established different activity levels for telomerase depending on the type of cell and the moment during the development process. During embryo development, telomerase activity peaks at blastoderm stage and then is almost undetectable at most somatic cells once the embryonic development is complete. For our research is also important to mention that telomerase activity is low in mature oocytes. This makes oocytes susceptible to telomere shortening through oxidative processes, especially if we consider that oocytes do not divide and wait from 13 to 50 years to mature.

Understanding these differences in telomerase activity and telomere dynamics is nowadays a very active field of research. As pointed out by Lansdorpt in 2008, *"the differences in telomere dynamics between germ cells and somatic cells, and among different types of somatic cells, are still poorly understood"* (31).

Summary

In summary, chromosomal aneuploidy is the leading cause of fetal death in humans and thus the leading cause of miscarriages in women. Around 35% of spontaneous abortions have too many or too few chromosomes, with trisomies accounting for 50% of the abnormal abortions (33). Our laboratory focuses on the nondisjunction of chromosome 21 and for the past several years we have focused on several factors that affect the risk of nondisjunction. These factors include maternal age, number and location of recombination events and environmental factors such as maternal folic acid supplementation, smoking, and social economic status.

In 2004, Liu et al. (34) working with telomerase knock out mice showed that shortened telomeres of 4th generation mice had impaired meiotic synapsis and decreased recombination in females. They also observed that male germ cells with dysfunctional telomeres went through apoptosis thanks to a check point on the spermatogenesis process that differentiated viable and non viable germ cells. On the other side, female germ cells go through meiosis until they arrest at prophase I. These data suggest the presence of surveillance mechanism for telomere shortening in males but not in females.

They also showed reduced recombination rates in meiocytes with shortened telomeres from late generation telomerase deficient females as well as impaired chromosome alignment at the metaphase plate. These results lead them to argue that functional telomeres, or in other words telomeres that have a functional length, are important in mammalian meiotic synapsis and recombination. They suggest that this could be the cause of the increased frequency of metaphase chromosome misalignment and spindle disruption that they noted in their studies (35).

In this project, we are interested in the variability of telomere loss and its association with chromosome segregation. As mentioned before, telomeres get shorter with each cell division. Although the loss due to the "end replication problem" is estimated to be less than 10 base pairs per replication in human cells, this loss can be greater with an observed loss between 50–200 base pairs per division in some individuals (36).

With respect to a more specific cell like the oocyte, Keefe et al. in 2007 (37) made a very interesting point. As he mentions, mature oocytes do not divide, so it is intuitive to think that replicative sensescence would not play a role in oocytes. However, we need to consider that female germ cells actually divide during fetal life before entering meiosis. At the same time, according to some studies (38, 39), the first oocytes formed during fetal oogenesis are the first to ovulate in the pubertal female, and the last oocytes formed are the last to ovulate. This is what is called "the production line theory" of oocyte formation and maturation.

Another factor to take into consideration is the telomerase activity in oocytes. It has been shown that telomerase activity is low or undetectable in oocytes and late preimplantation embryos (40). We also know that oxidative processes in the cells due to cellular metabolism contribute to shorten the telomeres and oocytes can suffer these processes for a long time, up to 45-50 years before they mature. These two factors provide an explanation for the "two hit" model of aging on the female reproductive system, one of this two hits occurring during fetal life and the other one during the adult life.

The variation in telomere loss is not well understood. Oxidative stress is thought to be one of the main factors involved (41). This is due to the fact that telomeric DNA is much more susceptible

to oxidative damage than non-telomeric DNA, at least partly due to the high guanine content (42).

We plan to examine telomere length in the context of the other risk factors that we have identified. With our research, we hope to obtain results that will bring insight into the mechanisms that ultimately affect the risk of nondisjunction of chromosome 21.

Chapter 2: Methods development to determine telomere length

Sample Description

Our study examined maternal telomere length as a risk factor for trisomy 21. In order to do this, relative telomere lengths for case and control mothers were obtained from DNA extracted from blood. The majority of blood samples were obtained previously to study the causes and consequences of trisomy 21 (21, 43). Cases were defined as mothers who had an infant with trisomy 21 due to a maternal nondisjunction error (n=404). Controls were defined as mothers who had an infant with trisomy 21 due to a paternal error or a post-zygotic mitotic error (n=24). The assay used to define the type of nondisjunction error is described below. A random sample of mothers who had an infant without trisomy 21 (n=18) were obtained from the general population as controls. For these controls, we do not have data on recombination and environmental exposures. For cases and controls, their blood sample was required to have been drawn within 5 years of the birth of the index case.

Determination of Type of Nondisjunction Error

In order to be able to determine the type of nondisjunction error, our lab genotyped different STR markers on chromosome 21 in the proband, mother and father. In order to read a more detailed description of the method, please see Oliver et al. (44, 45).

To be able to determine the parental origin of the extra chromosome 21 in the proband we looked for at least two informative markers that matched the maternal or paternal alleles. Once the origin of the extra chromosome was determined, the next step was to figure out the type of

meiotic error that gave rise to the trisomy. That is, we wanted to determine if the error had happened during meiosis I or meiosis II. In order to do this, the markers that we used were located in the pericentromeric region of chromosome 21q, more specifically, between the 13,615,252 base and 16,784,299 base. When we analyzed the markers in the proband, if we observe parental heterozygosity, we concluded that the nondisjunction error had happened during meiosis I. If the markers showed homozygosity, we concluded that the error happened during the equational division of the meiotic process.

One of the caveats of our method to determine the type of meiotic error is that sometimes, we cannot distinguish perfectly some of the meiotic errors. For example, it could happen that even if the error happened during meiosis I when homologous chromosomes do not segregate properly, then they may go through a correct meiotic II division but we would only see the sister chromatids in the oocyte.

Determination of Recombination Profile

Determination of the recombination profile in chromosome 21 was done following Oliver et al. (2008) (44). We know that some specific patterns of recombination, like for example, absence of recombination on chromosome 21, represent a risk factor for nondisjunction. We will analyze the recombination profile along chromosome 21 and telomere length with respect to nondisjunction of chromosome 21. In order to do that, we characterized the recombination profile on the nondisjoined chromosome 21 in the case mothers. We used both STRs and SNPs that are specific of chromosome 21. The details of the genetic markers and the genotyping quality control measures are provided in Oliver et al. (2008) (44). The location of the recombinant event was based on the changes from nonreduction (N) to reduction (R) or vice versa. For example, if we

observed NNNNRRRRRR, we would determine that a single medial recombinant event has occurred located at the change from N to R.

As a quality control, before considering a change from nonreduction to reduction as a definite recombination point, we looked for at least one informative STR or eight informative SNPs surrounding the recombination point. In those cases where the recombinant event was determined by the most proximal or distal marker, we only needed one informative STR or four SNPs in order to validate it.

In cases of double recombinants the same rules applied regarding the numbers of STR or SNPs needed to validate the recombinant events.

Determination of Telomere Length

We used DNA extracted from blood samples to measure relative telomere length according to the method by Cawthon et al. (2002) (46). This qPCR method has several advantages over previous published methods such those using Southern blots. Southern blots generally require large amounts of DNA between 0.5 to 5 μg of DNA per individual. It has also been observed that the terminal restriction fragment length in individuals can vary as much as 5% depending on the restriction enzymes used. This variation can probably be explained by the presence of restriction site polymorphism that could affect the results.

This could affect the result of comparing inter-individual variation in the mean length of the telomeric repeat sequence. On the other hand, quantitative PCR provides a much faster method and more importantly, it is a way that allows us to obtain reproducible estimates of relative

telomere length using a very small amount of DNA as it requires much less DNA, using as little as 30 ng of DNA. This is obviously a great advantage as it allows us to maximize the value of our DNA samples, many of which are impossible to replenish.

We optimized the method outlined in Cawthon 2002 with some small modifications. Briefly, two master mixes of PCR reagents were prepared, one with the telomere (T) primer pair and the other with the single copy gene (S) primer pair.

The choice of the single copy gene (S) is important, as pointed out by Cawthon, as *"the number of copies of S per cell that are effectively PCR-amplified must be the same in all individuals being studied in order for the relative T/S ratios to reflect relative length differences in telomeric DNA."* Similar to Cawthon, we used 36B4 gene as a single copy gene, which encodes acidic ribosomal phosphoprotein PO and is located on chromosome 12 (47). This gene was tested and shown to meet all the requirements (46).

Following Cawthon 2002, the composition of telomere (T) and single copy gene (S) PCRs master mixes were identical except for the oligonucleotide primers. The final concentrations of reagents in the PCR were 0.16× Sybr Green I, 15 mM Tris–HCl pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.16 mM each dNTP, 5 mM DTT, 1% DMSO and 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems). The final telomere primer concentrations were: tel 1, 216 nM; tel 2, 720 nM. The final *36B4* (single copy gene) primer concentrations were: 36B4u, 240 nM; 36B4d, 400 nM. The primer sequences were as follows:

TEL 1: 5' – GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT – 3'

TEL 2: 5' - TCCCGACTATCCCTATCCCTATCCCTATCCCTATCC-CTA – 3'

36B4u: 5’ – CAGCAAGTGGGAAGGTGTAATCC – 3’

36B4d: 5’ – CCCATTCTATCATCAACGGGTACAA – 3’

All PCRs were performed on the Bio-Rad CFX96 real-time PCR detection system. The thermal cycling profile for both PCRs began with a 95°C incubation for 10 min to activate the AmpliTaq Gold DNA polymerase. For telomere PCR, there followed 26 cycles of 95°C for 15 s, 54°C for 2 min. For *36B4* PCR, there followed 32 cycles of 95°C for 15 s, 58°C for 1 min. BIORAD software was used to generate the standard curve for each plate and to determine the dilution factors of standards corresponding to the T and S amounts in each sample.

In more detail, two PCR reactions were performed for each experimental sample, one for a single copy gene and another one to measure the quantity of telomere repeats. The first PCR reaction measured the single copy gene relative quantity. The results for each sample were compared to a standard curve that was generated using a randomly chosen sample. This gave us a relative quantity of the single copy gene for each experimental sample. In the second PCR, the same procedure was followed to measure the relative quantity of telomere repeats for each sample. After this, the ratio of these two PCR results for each experimental sample was defined as the relative telomere to single copy gene (T/S) ratio.

We conducted two replicates on each sample to control for experimental variation. For example, two identical aliquots of the DNA sample were added to plate 1 to assay telomere repeat copy and another two aliquots were added to the same well positions in plate 2 to assay the single copy gene. For each standard curve, one reference DNA sample was diluted serially to produce 5 total amounts of DNA ranging from 20 to 1.25 ng.

For quality control, the efficiency of the PCR reaction and the r-square value of the regression line created by the standard dilutions were examined for each reaction. The efficiency of the PCR reaction measured by the threshold cycles of the standard dilutions used to create the regression line had to be between 90% and 110% and the r-square value of the regression line had to be no less than 0.98 (Fig.1, 2). If any of these parameters were not met, the entire PCR for the plate was repeated. This provided us a measure to control the quality of the PCR reaction as a whole. We also applied a quality control to each of the duplicates run for each sample. The duplicate values for each sample could not have a threshold cycle value of more than 0.3 cycles apart. If this parameter was not met, both the T PCR and the S PCR for that sample were repeated.

Chapter 3: Telomere length as a biomarker for maternal age and nondisjunction

Telomere length and chromosome 21 nondisjuntion

Using the study sample outlined in Table 1, we first sought to confirm the negative relationship between telomere length and maternal age. Indeed our results indicated a statistically significant association of T/S ratio measured by quantitative PCR and maternal age in both controls (N=42, Figure 3) and all maternally-derived nondisjunction cases (N=404) (Table 2). As our further analyses required maternally-derived nondisjunction cases to be defined by stage of origin and recombination profile, we ran the above analysis on the subset of cases that were informative for both variables (N=241) to confirm the association of maternal age on T/S ratio. In this select group, we found the same statistically significant association between T/S ratio and maternal age (Table 2). This suggests the selected cases on which we conducted further analyses were representative of the overall group of maternal errors.

In our next set of analyses, we stratified maternally-derived errors based on the stage of the error, namely MI or MII. We first examined the mean T/S ratio by maternal age group, as defined in Ghosh et al. (2010), to provide a comparison of the pattern of the mean telomere length by age for the two studies (Table 3). We note that a direct comparison cannot be made, as each study used a different measure of telomere length. Irrespective, differences between studies were observed. First, Ghosh et al. did not observe a difference in mean telomere length among younger women from the different meiotic outcome groups whereas we observed shorter

telomere lengths in MI and MII error groups compared with controls. Because our sample sizes were small in the youngest group of women, we collapsed the two younger age groups and compared the means of women less than 35 years. We found that T/S ratio among controls (mean=1.90, SD=0.78) was significantly greater than MI error cases (mean=1.61, SD=0.39, $p<0.01$) and greater than MII error cases (mean=1.51, SD=0.4, $p=0.02$). There was no difference in mean telomere length between MI and MII error groups ($p=0.24$). In addition, we found no difference in the mean T/S ratios among controls vs MI ($p=0.41$) and controls vs MII ($p=57$) in the older age group (≥35 yrs).

We then conducted regression analysis to examine the effect of maternal age on telomere length over the entire maternal age range (continuous variable). We found that there was a significant decrease in T/S ratio by maternal age among controls and MI errors, but not MII errors (Figure 3-6, respectively; Table 2). To determine whether the magnitude of the decrease in T/S ratio by maternal age differed among the meiotic outcome groups, we conducted a joint regression analysis that included an interaction term for maternal age by meiotic outcome group for the following datasets: MI and controls, MII and controls, and both MI and MII. For both MI and MII errors, the influence of maternal age on the T/S ratio differed significantly from controls, as indicated by a statistically significant beta coefficient for the interaction terms (Figure 6, $p=0.03$ and $p=0.02$, respectively). The interaction term was not statistically significant when MI and MII errors were examined together (Figure 6, $p=0.39$).

Telomere length and recombination along the nondisjoined chromosome

We then examined the effect of altered recombination patterns along the nondisjoined chromosomes 21 on the T/S ratio adjusting for maternal age. For MI nondisjunction, absence of recombination is a significant risk factor (44); thus, we defined a predictor variable as chromosomes with no detectable recombination vs those with at least one recombination event (referent group). We found a statistically significant association between the absence of recombination on chromosome 21 and telomere length after adjusting for maternal age ($p=0.02$). Among MI errors, those with no recombination had T/S ratios that decreased more rapidly across maternal age than those with recombination (Figure 7).

In addition to the lack of recombination, having a single telomeric recombinant event is also an established risk factor for MI nondisjunction. It is hypothesized that this single distal exchange leads to a sister chromatid cohesion complex that is too limited to stabilize the bivalent or that the event is too far from the kinetochore to aid in the attachment of chromosomes to the meiotic spindle during MI (44,48-51). To examine whether this pattern is associated with telomere length, we included the predictor variable defined as having a single telomeric recombination vs having a single non-telomeric recombination (referent group). Among MI cases, there was no evidence that this recombination phenotype had an association with the T/S ratio adjusting for maternal age ($p= 0.26$), however our sample size was limited for this analysis (Table 4).

Lastly, recombination near to the centromere is a significant risk factor for MII errors, irrespective of the number of recombinants along the nondisjoined chromosome (45). We examined this pattern vs having non-pericentromeric recombination (referent group) and found

no evidence for an effect on the T/S ratio (p= 0.22). Again, it is important to note the small sample size (Table 4).

Statistical Analysis

As stated above, we defined case mothers as those with maternal errors. Control mothers included mothers of probands whose trisomy 21 was due to paternal errors or post-zygotic mitotic errors and mothers of a child without trisomy 21. We first examined T/S ratios by maternal age using scatterplots stratified by meiotic outcome group (MI errors, MII errors and controls) We then used linear regression models to examine the effect of maternal age, type of nondisjunction error and recombination profile on T/S ratio. We conducted analyses separately for each meiotic outcome group. We conducted joint analyses to test whether the pattern of maternal age on T/S ratio across the age span differed by meiotic outcome group by including an interaction term between meiotic outcome group (MI vs controls, MII vs controls, and MI vs MII) and maternal age. To study the effect of the recombination patterns, we defined the predictor variables based on the known recombination-related risk factors for nondisjunction and conducted separate analyses as follows: 1) among MI errors, we defined those with no detectable recombination as 0 (n=89) and those with at least one recombination as 1 (n=49); 2) among MI errors, we defined those with a single, distal recombinant as 1 (n=20) and those with a single non-telomeric recombinant as 0 (n=20); and 3) among MII errors, we defined those with a pericentromeric recombinant as 1 (n=19) and those with a non-pericentromeric recombinant as 0 (n=8).

Table 2 and 4 shows the different sample size for each of each group of samples that were used in the different linear regression models

Sensitivity analysis:

Our T/S ratios measurements showed four values that were more than two standard deviation above the mean value: two were from cases and two from controls. We repeated the quantitative PCR analysis in all samples but obtained the same results for these four outliers. We had no reason to remove these samples from the analysis but we wanted to address how much these outliers influenced the results. We ran the same regression analyses with these four samples excluded. The only results that were affected were those corresponding to the comparison between controls and MI errors. To determine whether the age relationship with telomere length differs among MI errors and controls, we tested the significance of the interaction term. The p-value for this term was brought from 0.03 to 0.1 when the outliers were excluded. We want to mention that even though the statistical significance of the interaction term was reduced, the conclusions regarding the biological aging of young mothers in the MI group remained the same.

Chapter 4: Conclusions and future studies

The effect of advanced maternal age on the risk of having a child with DS was established nearly 80 years ago (13). Thanks to molecular studies, nowadays we know that there is a clear relationship between increased maternal age and chromosome missegregation specifically in the oocyte (52-54), and, related to our work, this association leads to a high risk of chromosome 21 nondisjunction (14, 21).

At the same time, several studies in mice (34, 35) and in women (37) have shown the importance of the relationship between telomeres and the meiotic machinery. These studies helped to develop the hypothesis that women having a child with DS at a young age may suffered from accelerated genetic aging in comparison to mothers of euploid children. In other words, they were "genetically" or "biologically" older.

Later on, in 2010, a study from Ghosh et al. (55) did not support this hypothesis. Instead they proposed that one possible explanation was that their controls had "decelerated aging" and that they may have a protective mechanism that kept their telomeres long.

Tease et al. (56) conducted a study on human fetal oocytes and observed that recombination is disturbed in oocytes with synaptonemal complex (SC) fragmentation or synaptic abnormalities during meiotic prophase I. They suggested that these errors during recombination may increase the risk of abnormal chromosome segregation in the pool of oocytes that form the mature ovary in a female.

Liu et al. (34) showed that telomere characteristics may be related to recombination outcomes. They showed that shortened telomeres of late-generation (4^{th} generation) telomerase deficient

female mice had impaired meiotic synapsis and decreased recombination. They also observed differences between male germ cells and female germ cells with respect to the outcome of cells with dysfunctional telomeres. While most male germ cells with dysfunctional telomeres undergo apoptosis, female germ cells with shortened telomeres are able to enter meiotic arrest. These led them to think that in order to have a correct synapsis and recombination between homologous chromosomes, functional telomeres of a correct length were needed. In a previous paper by Liu et al in 2002 (35) they also observed that it was not the telomerase that influenced chromosome alignment and spindle integrity but rather, sufficiently long telomeres or in other words, functional telomeres. They got to this conclusion because they did not see any difference in chromosome alignment at the metaphase plates of meiotic spindles in first generation telomerase deficient mice but in fourth generation mice with shortened telomeres the metaphase plates were completely disrupted. Our lab has focused on recombination-related risk factors and nondisjunction. Most recently, Oliver et al. (44) characterized the recombination profile of the extra chromosome 21 in children with DS using chromosome 21 genetic markers (SNPs and STRs) and confirmed that the number of recombination events and their location were associated with the risk of chromosome 21 nondisjunction. These results were based on 615 MI and 253 MII cases stratified by maternal age. For MI errors, they found two associations. First, they found that the absence of recombination represents a risk factors associated with nondisjunction of homologous chromosomes during MI. They hypothesized that this risk factor would have the same influence on homologue segregation, irrespective of the age of the oocyte (i.e., maternal age). If true, they would expect to observe the proportion of the MI errors with no recombination to be highest in the youngest age group of women with such errors. Authors confirmed this hypothesis as they saw an increase in cases with zero recombinant events among the young

versus middle age groups, but they did not find statistical significance when they compared other age groups. Thus these data suggest that absence of an exchange increases the risk for nondisjunction irrespective of age of the oocyte. They also found that a single telomeric exchange increased the risk for nondisjunction at MI and, again, that this risk did not depend on maternal age.

The association of recombination with MII errors differed from that at MI. With respect to meiosis II errors they suggested that the presence of a single exchange within the pericentromeric region of 21q interacts with maternal age-related risk factors. This observation could be explained in two ways:

1) A pericentromeric exchange increases the susceptibility to maternal age risk factors, or

2) A pericentromeric exchange may help homologous chromosomes segregate correctly during meiosis I but it does not help sister chromatids to do the same during meiosis II

In 2010, Ghosh at el. (55) reported the possible relationship between telomere length in mothers who had a child with DS and the risk of nondisjunction. They compared a group of 75 mothers of euploid children to a group of 75 mothers of children with DS. They genotyped the 75 case mothers and were able to separate them into MI and MII errors. They found that younger women among the different meiotic outcome groups did not differ with respect to their telomere length. However they did find that telomeres among both MI and MII case mothers were shorter in the older age groups compared with controls, especially MII case mothers. They concluded that based on these data they could not support previous studies that suggested that younger women who have babies with DS are 'biologically older' than their chronological age. Instead, their

findings suggest that older mothers who have babies with DS are "*biologically older than controls who have euploid babies at the same age"*.

Our study took advantage of a larger sample size of mothers with MI and MII errors in order to confirm or refute the association between telomere length, maternal age and the risk of nondisjunction of chromosome 21. Our first goal was to confirm a negative relationship between telomere length and age, as pointed out by previous studies (57-59). Indeed, our results indicate a statistically significant association of T/S ratio measured by quantitative PCR and maternal age in both controls (N=42) and all maternally-derived nondisjunction cases (N=404)(Table 2). Our results suggest that controls show longer telomere length in younger women compared to younger women with maternally-derived errors. These results support the hypothesis that oocytes with a nondisjoined chromosome 21 may represent a biologically older ovary compared with those resulting in a meiotically normal outcome. Previous studies in mice would support this hypothesis (34, 35, 60).

We further examined the pattern of the association stratified by MI and MII error groups. There was a significant association between telomere length and age in the MI error group, but we did not observe it among the MII error group (Table 2). The MII error group shows the shortest telomere length in young women compared with controls and MI errors (Tables 2, 3). This result suggests that MII mothers are "biologically" older than women of the same age in the control or MI groups. Although the sample size is small for the MII error group, we did not observe the significant decrease in telomere length by age as did Ghosh et al. (55).

We have considered several possible explanations for the different findings between our study and that of Ghosh et al. First of all, our sample sizes for MI and MII cases were larger than that

in Ghosh's study (48 vs 190 MI cases, 27 vs 51 MII cases), although our sample of controls was smaller (42 vs 75). Also it is important to mention that while our control samples size was smaller than that in Ghosh's study, our controls had a continuous decline in telomere length with age as expected. However, the controls in Ghosh's study did not show that same decrease in the oldest age group. Thus, some differences in findings may be due to limited sample sizes. Another technical issue may be the use of different assays to assess telomere length, although both technologies have been shown to provide comparable results (46). Lastly, there are now many studies suggesting that telomere length is affected by environmental exposures (61, 62). Differences in the environment of among women representing the U.S. and those from Kolkata, India may explain the different observed age-related patterns in telomere length.

Assuming that our results are representative of the women in the different meiotic outcome groups, we draw the following conclusions. First, our data show that younger women with MI and MII errors have significantly shorter telomeres than controls. These data suggest that women with nondisjunction errors in this younger age group are "biologically" older than controls, especially for those with MII errors. Thus telomere length may serve as a biomarker of risk for nondisjunction in this chronologically younger age group. As maternal age increases, the difference in telomere length among the meiotic outcome groups becomes smaller (Figure 6). Interestingly, the regression lines for maternal age on T/S ratio all converge around 35-37 years of age. This is the age where the risk for nondisjunction of chromosome 21 changes from a linear increase to an exponential increase (21). Of the two types of errors, the MII mothers appear to be the biologically "oldest" group with a T/S ratio significantly smaller at young ages compared to controls and with no evidence of a decrease with maternal age. It is important to note that we do not have longitudinal data, that is, we do not have several measures on relative telomere length

for each of the women in the study over their life span which makes it difficult to argue that the decline in telomere length was different in control mothers versus case mothers. We also found no statistically significant difference in slope of the regression line for maternal age between MI and MII error groups. Thus, we must be cautious in our speculation that women with MII errors may be the biologically "oldest" meiotic outcome group. A possible explanation for this smaller decline in both MI and MII mothers could be on the fact that telomeres do not shorten linearly across all ages. Several studies have shown that telomeres do not decline in a linear way over the age span (63, 66): from the first years of life until around 30-35 years old, telomere attrition steadily declines, from 35 of age to 60-65 years old telomere length attrition slows and after age 65 the decline is more rapid. In that case, for example, the telomeres of younger mothers with nondisjunction errors may be at the point where decline is reduced because those telomeres are acting as if they are in a biologically older stage, those within the 35 to 60 year range.

Even though we could argue that mothers of children with DS who had a meiotic I or II error may be suffering a smaller decline in telomere length due to the fact that they are biologically older than their chronological age, the lack of longitudinal data in our study is an important factor to take into account. Also, another possible hypothesis that would explain the differences observed between controls and case mothers could be due to the presence of a threshold of biological age, for which telomere length is a biomarker, that vastly increases the risk for DS. To reach this threshold there would be 2 possible options: 1) the mother must be biologically older than the mothers of euploid children at the same chronological age or 2) the mother is actually chronologically older. In our data, what we see is a combination of these two; the younger case mothers are biologically older and hence have an increased risk of having a child with DS while

control mothers reach that threshold when they are chronologically older, at around 37 years of age.

Our data support this hypothesis and explain why the regression lines of control mother and case mothers cross at around 37 years of age when the probability of having a child with DS increases exponentially. Our data supports the idea of telomere length as a biomarker for biological age and fit under the theory of biological aging.

The second part of our research focused on the possible relationship between telomere length and the established recombination-related risk factors for nondisjunction of chromosome 21. We found that the absence of recombination had a significant negative association with the T/S ratio: mothers with MI errors and no observed recombination had shorter T/S ratio compared with those mothers with MI errors and at least one observed recombinant event. Although limited by sample size (Table 4), we found no evidence for an association between the location of recombination and the T/S ratio.

Our results are consistent with the studies of Liu et al (34) in mice. Their studies on telomerase deficient mice showed that telomere shortening was associated with lack of recombination. More exactly, they observed that synapsis and recombination was jeopardized at the pachytene stage of the prophase of MI for cells that had shortened telomeres. Studies in human spermatocytes showed that telomeres could be involved in the synapsis between homologous chromosomes (64) and in a study conducted in mice spermatocytes, authors found that the sequences responsible for the presence of a "hot spot" of meiotic recombination were the telomeric repeats (65).

As we mentioned before, Liu et al. showed a relationship between dysfunctional telomeres and absence of recombination. They suggested that this result could affect chromosome segregation

and hence lead to higher rates of nondisjunction and aneuploidy. Our data show for the first time this possible relationship between telomere length, recombination profile and chromosome 21 nondisjunction in MI error mothers of children with DS.

Study Limitations

Even though our study is the largest study to date on the influence of telomere length on the risk of maternal nondisjunction leading to DS, there are still some limitations that future studies could overcome. A larger sample size among control mothers would be advisable in order to observe whether the increased decline rate in telomere length is maintained. Also a larger sample size of the MII error group would be advisable even though this is more difficult to achieve as only 25% of maternal errors correspond to this group (10-12). We have the potential to increase our sample size with increased chromosome 21 genotype. In our study, we have a total of 163 cases where we could not detect the stage of the meiotic error or the recombination profile. With better genotyping methods that use smaller amounts of DNA, we will continue to identify markers that help characterize these cases. The addition of these samples will increase our sample size by a 64%. A larger sample size would improve our power to observe possible differences and to confirm our results.

With respect to our analysis on the relationship between telomere length and the recombination profile on chromosome 21, one of the most important limitations is the misclassification of number of true exchanges at the four-strand stage of meiosis along the nondisjoined chromosomes. That is, we can only classify exchanges by inference from the observed number of detectable recombinants. The group of MI cases with no observed recombinants also includes

some cases with one exchange that could not be detected due to the characteristics of the tetrad analysis. Thus, we have a misclassification problem. This problem should not bias our results, but simply decrease the power to detect a difference between groups. As we observed a significant difference, we suggest that the true difference between may be larger.

Lastly, one limitation is the tissue that we sample and the lack of specificity per chromosome. We obtained the T/S ratio to represent a marker of the overall telomere length among all chromosomes in blood. This ratio is an excellent biomarker. However, to further investigate whether telomere length influences meiosis itself, we would need to examine the T/S ratio in oocytes. It would also be interesting to measure the telomere length of chromosome 21 itself to see if it is representative of other chromosomes or its possible differences when it is involved in nondisjunction.

Conclusions

The results in the first part of our study support the hypothesis of the premature "genetic aging" model for younger mothers (37). Based on our T/S ratios measurements, we propose that young mothers of DS babies are "biologically older" than mothers of euploid babies in the same age group. Based on these results, telomere length could be considered a biomarker of age and hence nondisjunction. We hypothesized about the existence of a threshold line for biological age in the form of telomere length that would explain the older biological age of these women and hence their increased risk of nondisjunction.

In the second part of our research we report a significant relationship between the absence of recombination in MI error mothers and telomere length which confirms the observations by Liu

et al. in their telomerase knockout mice model. Here we provide the first evidence that connects two relevant observations. First, it has been previously shown the decreased levels of recombination present in children with DS (25, 44) and second, it has also been shown dysfunctionally short telomeres are associated with lower levels of recombination. Our data provide evidence that shortened telomeres may be suboptimal during meiosis and increase the chance of disrupted recombination and hence of nondisjunction of chromosome 21.

This study provides new insight in the relationship between maternal age, telomere length and the nondisjunction of chromosome 21 and could be the basis of future studies leading to a better understanding of the maternal age risk factor leading to Down Syndrome.

Future studies that focus on the longitudinal analysis of telomere length over the age spam of a person could also provide two key insights.

First, it could be determined if those mothers who had children with DS have an increased rate of telomere length decline with age. Second, these studies using telomeres as a biomarker for maternal age would provide a much better understanding of the risk of nondisjunction associated with maternal age.

Also, studies on environmental factors associated to both the risk of nondisjunction and telomere length, like for example, folate supplementation during pregnancy, will help understand the interaction between known environmental factors on the risk of nondisjunction and their relationship to telomere length.

References

1. Down JL. Observations on an ethnic classification of idiots. 1866. Ment Retard. 1995 Feb;33(1):54-6.
2. Halbertsma, T., Mongolism in One of Twins and the Etiology of Mongolism. Amer J Dis Child, 1923. **25**: p. 350-353.
3. Tjio, H.J. and A. Levan, The Chromosome Number of Man. Hereditas, 1956. **42**: p. 1-6.
4. Lejeune, J., M. Gautier, and R. Turpin, [Study of somatic chromosomes from 9 mongoloid children.]. C R Hebd Seances Acad Sci, 1959. **248**(11): p. 1721-2.
5. Parker SE, Mai CT, Canfield MA, Rickard R, Wang Y, Meyer RE, Anderson P, Mason CA, Collins JS, Kirby RS, Correa A; National Birth Defects Prevention Network. Updated National Birth Prevalence estimates for selected birth defects in the United States, 2004-2006. Birth Defects Res A Clin Mol Teratol. 2010 Dec;88(12):1008-16.
6. Hassold TJ, Jacobs PA. Trisomy in man. Annual review of genetics. 1984;18:69-97.
7. Canfield, M.A., Honein MA, Yuskiv N, Xing J, Mai CT, Collins JS, Devine O, Petrini J, Ramadhani TA, Hobbs CA, Kirby RS. National estimates and race/ethnic-specific variation of selected birth defects in the United States, 1999-2001. Birth Defects Res.A Clin.Mol.Teratol., 2006. 76(11): p. 747-756
8. Coory, M.D., T. Roselli, and H.J. Carroll, Antenatal care implications of population-based trends in Down syndrome birth rates by rurality and antenatal care provider, Queensland, 1990-2004. Med.J.Aust., 2007. 186(5): p. 230-234.
9. Forrester, M.B. and R.D. Merz, Prenatal diagnosis and elective termination of Down syndrome in a racially mixed population in Hawaii, 1987-1996. Prenat.Diagn., 1999. 19(2): p. 136-141.

10. Gómez D, Solsona E, Guitart M, Baena N, Gabau E, Egozcue J, Caballín MR. Origin of trisomy 21 in Down syndrome cases from a Spanish population registry. Ann.Genet, 2000. 43(1): p. 23-28.
11. Freeman SB, Allen EG, Oxford-Wright CL, Tinker SW, Druschel C, Hobbs CA, O'Leary LA, Romitti PA, Royle MH, Torfs CP, Sherman SL. The National Down Syndrome Project: design and implementation. Public Health Rep, 2007. 122(1): p. 62-72. Mikkelsen, M., et al., Epidemiology study of Down's syndrome in Denmark, including family studies of chromosomes and DNA markers. Dev Brain Dysfunct, 1995. 8: p. 4-12.
12. Sherman, S.L., Allen EG, Bean LH, Freeman SB. et al., Epidemiology of Down syndrome. Ment Retard Dev Disabil Res Rev, 2007. 13(3): p. 221-7.
13. Penrose, L.S., The relative effects of paternal and maternal age in Mongolism. Journal of Genetics, 1933. 27: p. 219-224.
14. Yoon, P.W., Freeman SB, Sherman SL, Taft LF, Gu Y, Pettay D, Flanders WD, Khoury MJ, Hassold TJ., Advanced maternal age and the risk of Down syndrome characterized by the meiotic stage of chromosomal error: a population-based study. Am J Hum Genet, 1996. 58(3): p. 628-33.
15. Antonarakis SE, Petersen MB, McInnis MG, Adelsberger PA, Schinzel AA, Binkert F, Pangalos C, Raoul O, Slaugenhaupt SA, Hafez M. The meiotic stage of nondisjunction in trisomy 21: determination by using DNA polymorphisms. American Journal Human Genetic, 1992. 50(3): p. 544-550.
16. Antonarakis SE, Avramopoulos D, Blouin JL, Talbot CC Jr, Schinzel AA. Mitotic errors in somatic cells cause trisomy 21 in about 4.5% of cases and are not associated with advanced maternal age. Nat.Genet, 1993. 3(2): p. 146-150.

17. Ballesta F, Queralt R, Gómez D, Solsona E, Guitart M, Ezquerra M, Moreno J, Oliva R. Parental origin and meiotic stage of non-disjunction in 139 cases of trisomy 21. Ann.Genet, 1999. 42(1): p. 11-15.
18. Muller F, Rebiffé M, Taillandier A, Oury JF, Mornet E. Parental origin of the extra chromosome in prenatally diagnosed fetal trisomy 21. Human Genetics, 2000. 106(3): p. 340-344.
19. Sherman, S.L., Freeman SB, Allen EG, Lamb NE. Risk factors for nondisjunction of trisomy 21. Cytogenet.Genome Res., 2005. 111(3-4): p. 273-280.
20. Hook, E.B., Down syndrome rates and relaxed selection at older maternal ages. American Journal Human Genetic, 1983. 35(6): p. 1307-1313.
21. Allen EG, Freeman SB, Druschel C, Hobbs CA, O'Leary LA, Romitti PA, Royle MH, Torfs CP, Sherman SL. Maternal age and risk for trisomy 21 assessed by the origin of chromosome nondisjunction: a report from the Atlanta and National Down Syndrome Projects. Hum Genet. 2009 Feb;125(1):41-52.
22. Warren AC, Chakravarti A, Wong C, Slaugenhaupt SA, Halloran SL, Watkins PC, Metaxotou C, Antonarakis SE. Evidence for reduced recombination on the nondisjoined chromosome 21 in Down syndrome. Science, 1987. 237: p. 652-654.
23. Lamb, N.E., S.L. Sherman, and T.J. Hassold, Effect of meiotic recombination on the production of aneuploid gametes in humans. Cytogenet.Genome Res., 2005. 111(3-4): p. 250-255.
24. Lamb NE, Freeman SB, Savage-Austin A, Pettay D, Taft L, Hersey J, Gu Y, Shen J, Saker D, May KM, Avramopoulos D, Petersen MB, Hallberg A, Mikkelsen M, Hassold TJ, Sherman SL. Susceptible chiasmate configurations of chromosome 21 predispose to

non-disjunction in both maternal meiosis I and meiosis II. Nat Genet, 1996. 14(4): p. 400 -5.

25. Lamb NE, Feingold E, Savage A, Avramopoulos D, Freeman S, Gu Y, Hallberg A, Hersey J, Karadima G, Pettay D, Saker D, Shen J, Taft L, Mikkelsen M, Petersen MB, Hassold T, Sherman SL. Characterization of susceptible chiasma configurations that increase the risk for maternal nondisjunction of chromosome 21. Hum Mol Genet, 1997. 6(9): p. 1391-9.
26. Cesare AJ, Reddel RR. Alternative lengthening of telomeres: models, mechanisms and implications. Nat Rev Genet. 2010 May; 11(5):319-30.
27. O'Sullivan RJ, Karlseder J. Telomeres: protecting chromosomes against genome instability. Nat Rev Mol Cell Biol. 11:171–81.
28. Eisenberg DTA (2011). "An evolutionary review of human telomere biology: The thrifty telomere hypothesis and notes on potential adaptive paternal effects". *American Journal of Human Biology* **23** (2): 149–167.
29. Weinstein BS, Ciszek D. The reserve-capacity hypothesis: evolutionary origins and modern implications of the trade-off between tumor-suppression and tissue-repair. Exp Gerontol. 2002 May;37(5):615-27.
30. Weng, N.P. 2008. Telomere and adaptive immunity. Mech. Ageing Dev. **129:** 60–66.
31. Lansdorp, P.M. 2008. Telomeres, stem cells, and hematology. Blood **111:** 1759–1766.
32. Liu L, Bailey SM, Okuka M, Muñoz P, Li C, Zhou L, Wu C, Czerwiec E, Sandler L, Seyfang A, Blasco MA, Keefe DL.2007. Telomere lengthening early in development. Nat. Cell Biol. **9:** 1436–1441.

33. Hassold T, Chen N, Funkhouser J, Jooss T, Manuel B, Matsuura J, Matsuyama A, Wilson C, Yamane JA, Jacobs PA. A cytogenetic study of 1000 spontaneous abortions.Ann Hum Genet. 1980 Oct;44(Pt 2):151-78.

34. Liu L, Franco S, Spyropoulos B, Moens PB, Blasco MA, Keefe DL (2004) Irregular telomeres impair meiotic synapsis and recombination in mice. Proc Natl Acad Sci USA 101:6496–6501

35. Liu, L., Blasco, M. A. & Keefe, D. L. Requirement of functional telomeres for metaphase chromosome alignments and integrity of meiotic spindles. (2002) *EMBO Rep.* **3,** 230–234.

36. Takai, H., A. Smogorzewska & T. de Lange. 2003.DNA damage foci at dysfunctional telomeres. Curr. Biol. **13:** 1549–1556.

37. D. L. Keefe, L. Liu and K. Marquard. Telomeres and aging-related meiotic dysfunction in women. Cell. Mol. Life Sci. 64 (2007) 139–143

38. Barrit, J. A., Cohen, J., and Brenner, C. A. (2000) Mitochondrial DNA point mutation in human oocytes is associated with maternal age. Reprod. Biomed. Online 1, 96–100.

39. Henderson, S. A. and Edwards, R. G. (1968) Chiasma frequency and maternal age in mammals. Nature 218, 22–28.

40. Wright, W. E., Piatyszek, M. A., Rainey, W. E., Byrd, W. and Shay, J. W. (1996) Telomerase activity in human germline and embryonic tissues and cells. Dev. Genet. 18, 173–179.

41. Houben, J.M.J., H.J.J. Moonen, F.J. van Schooten & G.J. Hageman 2008. Telomere length assessment: biomarker of chronic oxidative stress?. Free Radical Biol.Med. **44:** 235–246.

42. Shinji Oikawa, Shosuke Kawanishi Site-specific DNA damage at GGG sequence by oxidative stress may accelerate telomere shortening (1999) FEBS Letters-453: 365-368
43. Freeman SB, Allen EG, Oxford-Wright CL, Tinker SW, Druschel C, Hobbs CA, O'Leary LA, Romitti PA, Royle MH, Torfs CP, Sherman SL. The National Down Syndrome Project: design and implementation. Public Health Rep. 2007 Jan-Feb;122(1):62-72.
44. Oliver TR, Feingold E, Yu K, Cheung V, Tinker S, Yadav-Shah M, Masse N, Sherman SL. New insights into human nondisjunction of chromosome 21 in oocytes. PLoS Genet. 2008 Mar 14;4(3):e1000033
45. Oliver TR, Tinker SW, Allen EG, Hollis N, Locke AE, Bean LJ, Chowdhury R, Begum F, Marazita M, Cheung V, Feingold E, Sherman SL. Altered patterns of multiple recombinant events are associated with nondisjunction of chromosome 21. Hum Genet. 2012 Jul;131(7):1039-46.
46. Cawthon RM. Telomere measurement by quantitative PCR. Nucleic Acids Res. 2002 May 15;30(10):e47.
47. Boulay JL, Reuter J, Ritschard R, Terracciano L, Herrmann R, Rochlitz C. Gene dosage by quantitative real-time PCR. Biotechniques. 1999 Aug;27(2):228-30, 232.
48. Hawley RS, Frazier JA, Rasooly R (1994) Separation anxiety: the etiology of nondisjunction in Xies and people. Hum Mol Genet 3:1521–1528
49. Koehler KE, Hawley RS, Sherman S, Hassold T (1996) Recombination and nondisjunction in humans and Xies. Hum Mol Genet 5 Spec No: 1495–504
50. Nicklas RB (1974) Chromosome segregation mechanisms. Genetics 78:205–213
51. Ross LO, MaxWeld R, Dawson D (1996) Exchanges are not equally able to enhance meiotic chromosome segregation in yeast. Proc Natl Acad Sci USA 93:4979–4983

52. Morton, N. E., Jacobs, P. A., Hassold, T. & Wu, D. Maternal age in trisomy. Ann. Hum. Genet. **52**, 227–235 (1988).

53. Risch, N., Stein, Z., Kline, J. & Warburton, D. The relationship between maternal age and chromosome size in autosomal trisomy. Am. J. Hum. Genet. **39**, 68–78 (1986).

54. Thomas NS, Ennis S, Sharp AJ, Durkie M, Hassold TJ, Collins AR, Jacobs PA. Maternal sex chromosome non-disjunction: evidence for X chromosome-specific risk factors. *Hum. Mol. Genet.* **10**, 243–250 (2001).

55. Ghosh S, Feingold E, Chakraborty S, Dey SK. Telomere length is associated with types of chromosome 21 nondisjunction: a new insight into the maternal age effect on Down syndrome birth. Hum Genet. 2010 Apr;127(4):403-9.

56. Tease, C., Hartshorne, G. M. & Hulten, M. A. Patterns of meiotic recombination in human fetal oocytes. (2002) Am. J. Hum. Genet. 70, 1469–1479.

57. Takubo, K., Izumiyama-Shimomura, N., Honma, N., Sawabe, M., Arai, T., Kato, M., Oshimura, M., Nakamura, K., 2002. Telomere lengths are characteristic in each human individual. Exp. Gerontol. 37, 523–531.

58. Hoffmann, J., Erben, Y., Zeiher, A.M., Dimmeler, S., Spyridopoulos, I., 2009. Telomere length-heterogeneity among myeloid cells is a predictor for chronological ageing. Exp. Gerontol. 44, 363–366.

59. Ishii, A., Nakamura, K., Kishimoto, H., Honma, N., Aida, J., Sawabe, M., Arai, T., Fujiwara, M., Takeuchi, F., Kato, M., et al., 2006. Telomere shortening with aging in the human pancreas. Exp. Gerontol. 41, 882–886.

60. Liu L, Keefe DL (2002) Ageing-associated aberration in meiosis of oocytes from senescence-accelerated mice. Hum Reprod 17:2678–2685

61. Epel ES. Psychological and metabolic stress: a recipe for accelerated cellular aging? Hormones (Athens). 2009 Jan-Mar;8(1):7-22. Review.

62. Entringer S, Epel ES, Lin J, Buss C, Shahbaba B, Blackburn EH, Simhan HN, Wadhwa PD. Maternal psychosocial **stress** during pregnancy is associated with newborn leukocyte **telomere** length. Am J Obstet Gynecol. 2013 Feb;208(2):134.e1-7. doi: 10.1016/j.ajog.2012.11.033.

63. Baerlocher and Lansdorp, unpublished data

64. Barlow, A. L. & Hulten, M. A. Combined immunocytogenetic and molecular cytogenetic analysis of meiosis I human spermatocytes. (1996) Chromosome Res. 4, 562–573.

65. Ashley, T., Cacheiro, N. L., Russell, L. B. & Ward, D. C. (1993) Molecular characterization of a pericentric inversion in mouse chromosome 8 implicates telomeres as promoters of meiotic recombination. Chromosoma 102, 112–120.

66. Geraldine Aubert and Peter M. Lansdorp. 2008 Telomeres and Aging. Physiol Rev 88: 557–579, 2008; doi:10.1152

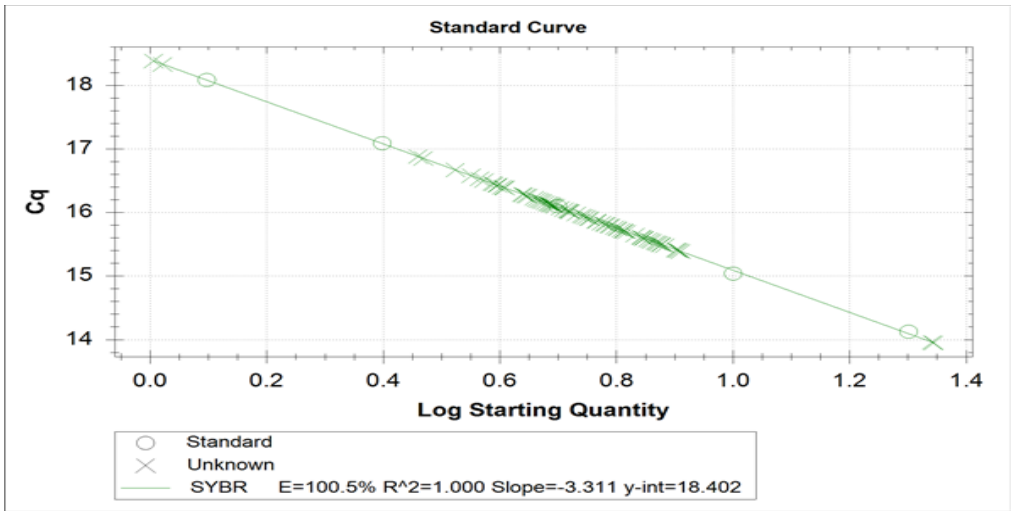

Fig 1. Regression line for the T PCR showing the efficiency of the reaction (E=100.5%) and the R^2 of the regression line (R^2= 1.000).

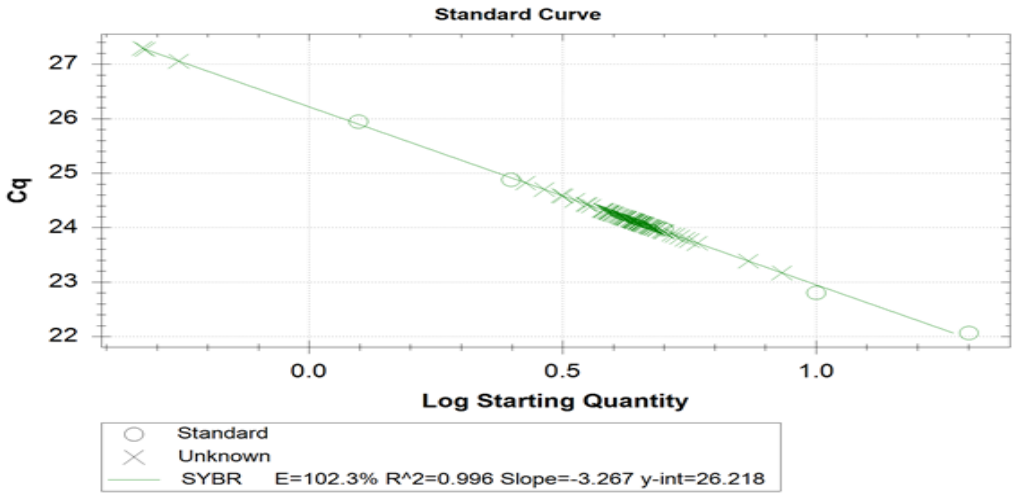

Fig 2. Regression line for the S PCR showing the efficiency of the reaction (E=102.3%) and the R^2 of the regression line (R^2= 0.996).

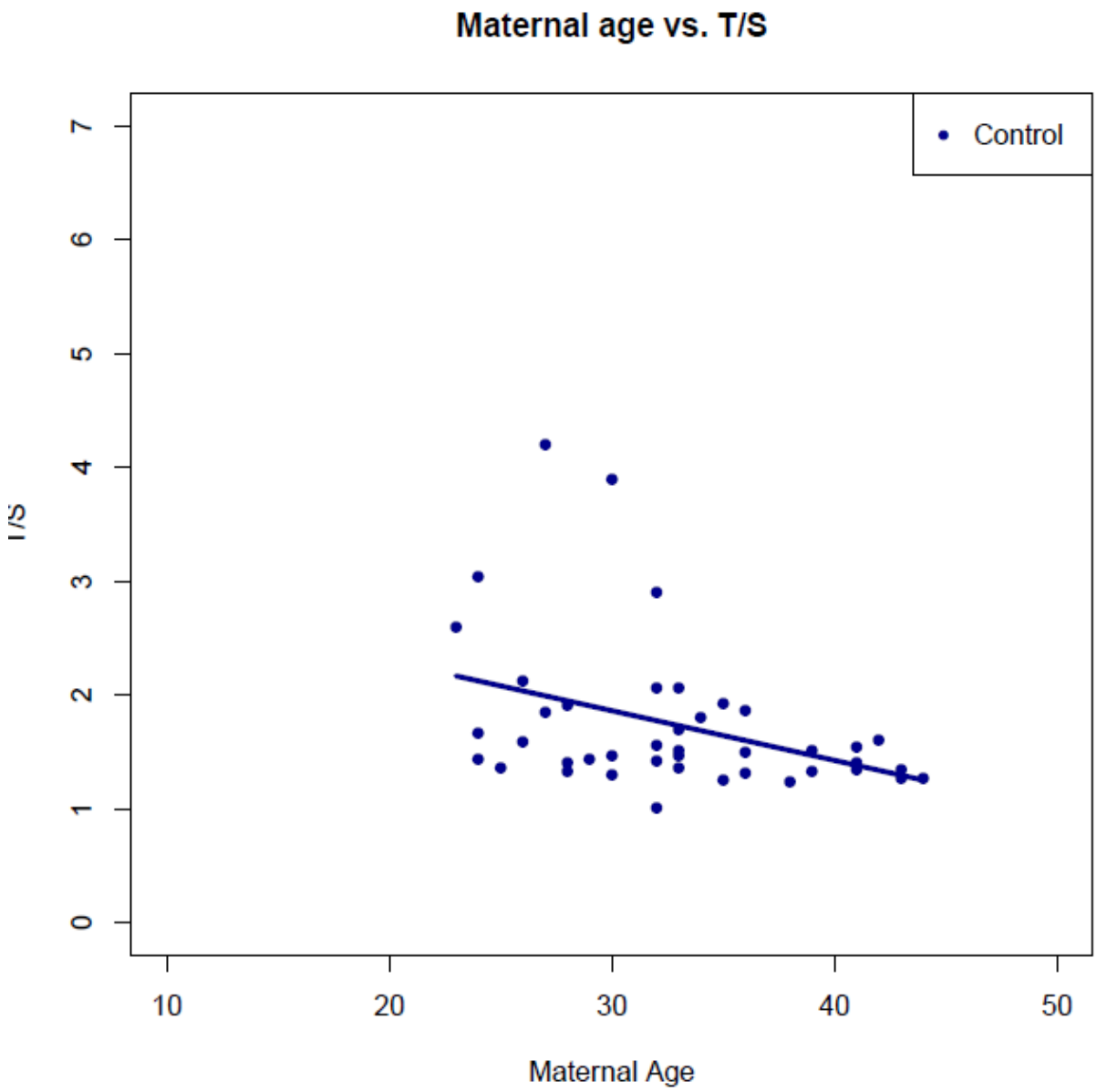

Figure 3. Regression line showing the distribution of T/S ratio as function of maternal age among control mothers.

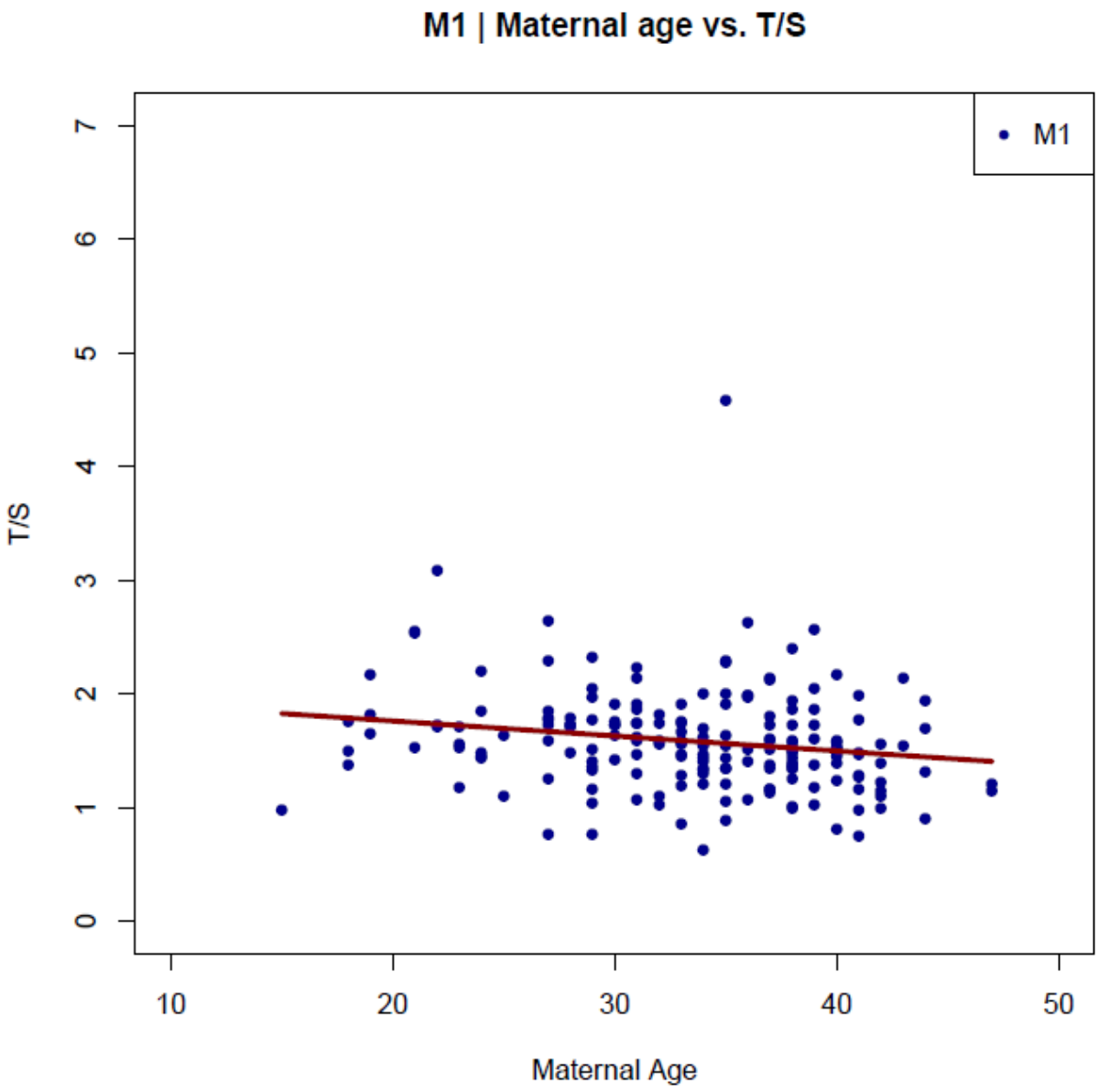

Figure 4. Regression line showing the distribution of T/S ratio as function of maternal age among MI error mothers.

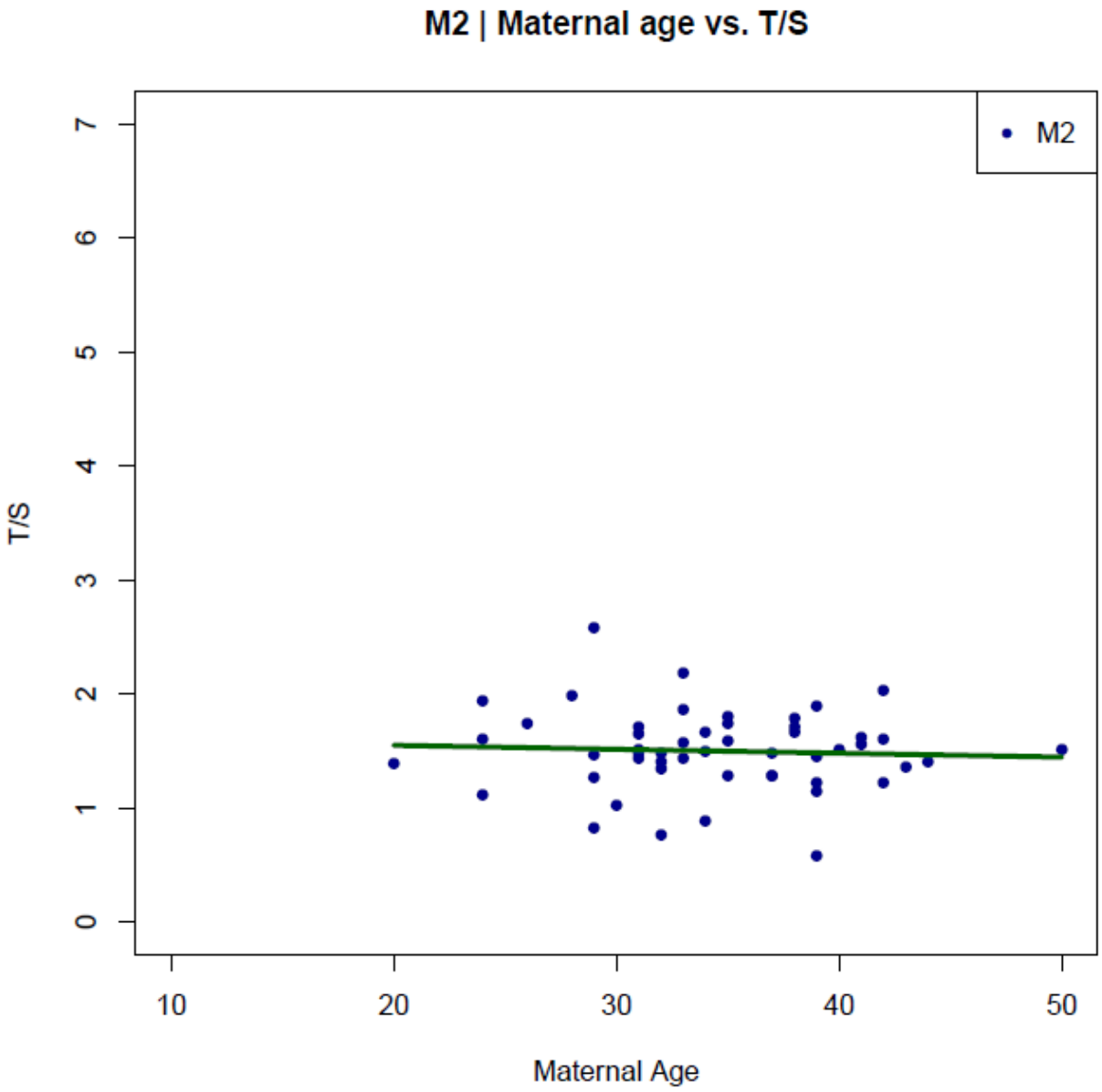

Figure 5. Regression line showing the distribution of T/S ratio as function of maternal age among MII error mothers.

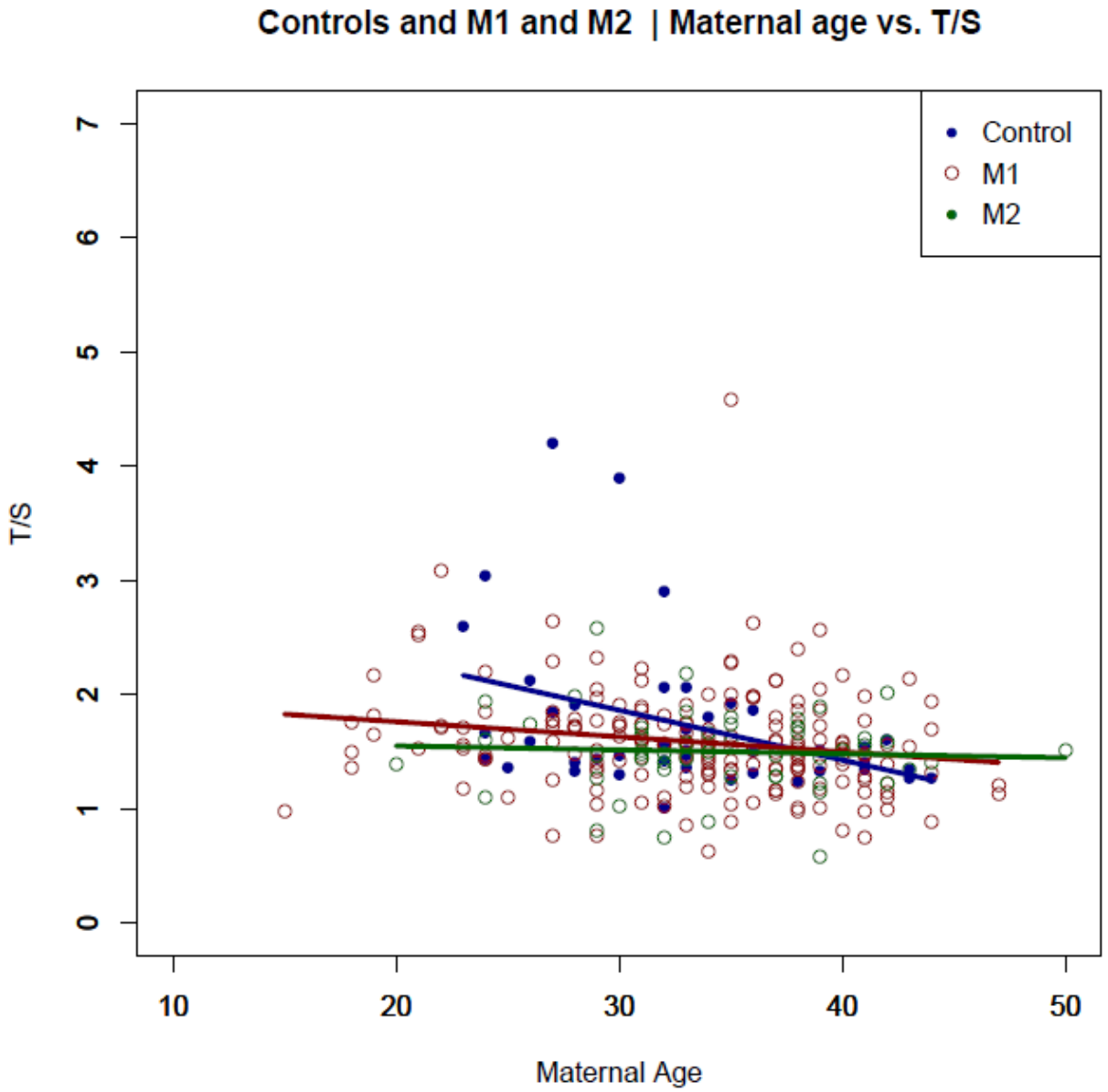

Figure 6. Regression line showing the distribution of T/S ratio as function of maternal age among control, MI error and MII error mothers.

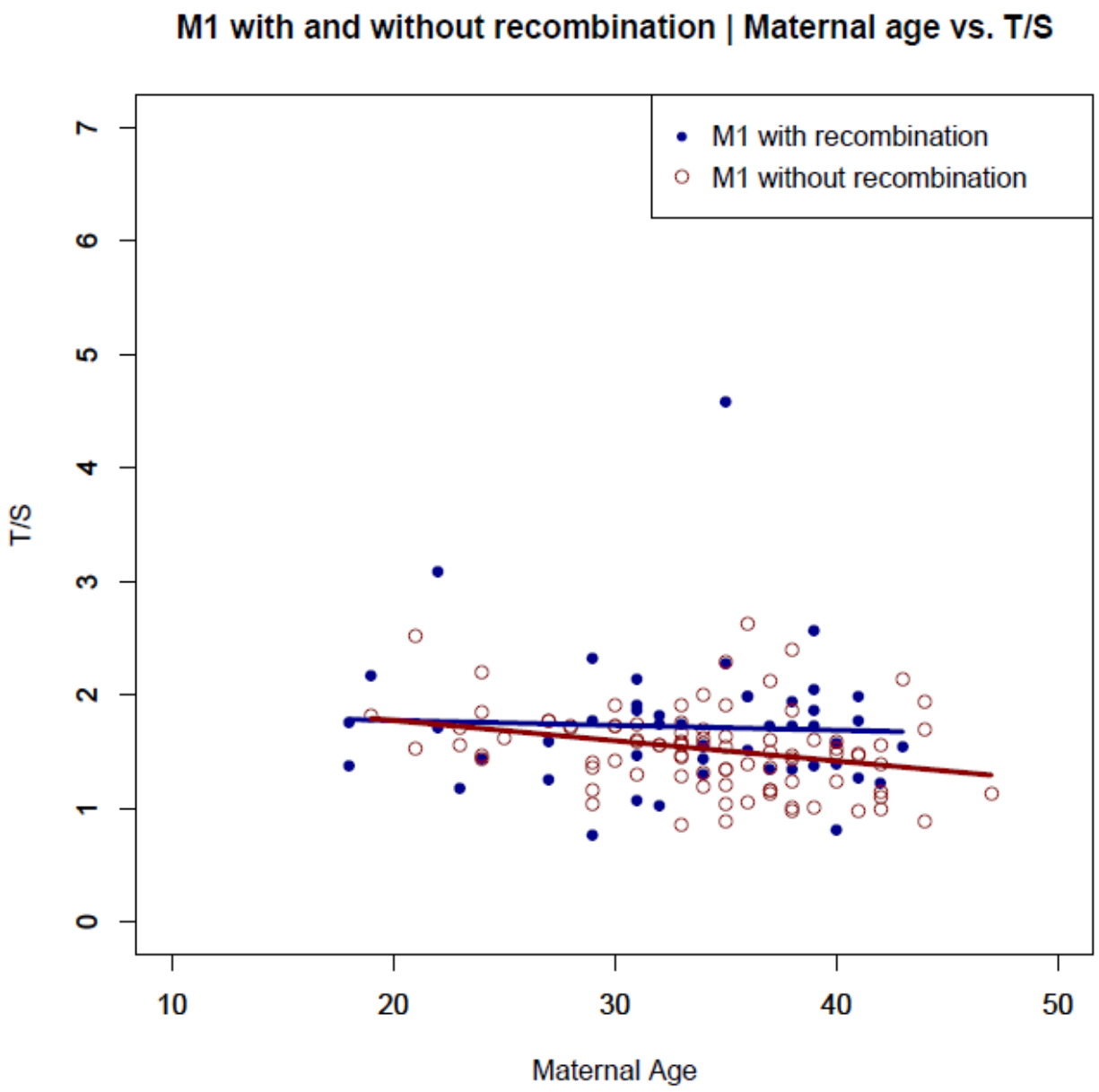

Figure 7. Regression lines showing the distribution of T/S ratio as function of age among MI error mothers in the presence or absence of recombination

Sample size/Mean Age	Controls	MI	MII
18-34 years	27 (29.2)	100 (28.7)	27 (29.9)
≥35 years	15(39.3)	90(38.7)	24(39.4)

Table 1. Sample size for controls, MI errors and MII errors divided by age group.

Meiotic outcome group	N	Intercept (se)	β for age (se)	p-value	R^2
Controls	42	3.170 (0.555)	– 0.044 (0.016)	0.013	0.146
Maternally-derived NDJ Cases	404	2.027 (0.097)	– 0.013 (0.002)	<0.0001	0.054
Selected maternally-derived NDJ	241	1.959 (0.151)	–0.012(0.004)	0.009	0.028
MI errors	190	2.025 (0.173)	– 0.013 (0.005)	0.011	0.034
MII errors	51	1.619 (0.299)	– 0.003(0.008)	0.689	0.003

se= standard error

Table 2. Mathematical models predicting T/S ratio for controls, all maternally-derived nondisjunction (NDJ) cases, those informative for stage and recombination traits (Selected) and MI and MII separately as a function of age

Mean(SD)/Age	Controls	MI	MII
18-34 years	1.90 (0.78)	1.61 (0.39)	1.51 (0.4)
≥35 years	1.44 (0.21)	1.55 (0.51)	1.49 (0.30)

Table 3. T/S ratio mean and Standard deviation (SD) for controls, MI errors and MII errors divided by age group.

	Recombination trait comparison (n)		Intercept	Maternal age		Recombination trait		Overall R^2
				β coef (se)	p-value	β coef (se)	p-value	
MI	**0 recom (89)**	**1 recom (49)**	2.12 (0.21)	-0.012 (0.006)	0.05	-0.18 (0.08)	0.02	0.07
MI	**1 telomeric (20)**	**1 non telomeric (20)**	2.17 (0.54)	-0.009 (0.01)	0.58	-0.23 (0.2)	0.26	0.04
MII	**Centromeric (19)**	**Non-centromeric (8)**	1.77 (0.41)	-0.01 (0.01)	0.37	0.17 (0.13)	0.22	0.104

se= standard error

Table 4. Regression analysis results to determine the influence of recombination on telomere length, adjusting for maternal age.