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GENETIC AND EPIGENETIC CAUSES UNDERLYING RECURRENT PREGNANCY LOSS

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Resumo

A perda recorrente de gravidez é considerada uma doença reprodutiva e afeta, aproximadamente 1 a 5% das mulheres no mundo requerendo especial atenção por parte da comunidade científica uma vez que cerca de 50% dos casos não possuem uma causa associada e são classificados como idiopáticos.

O presente estudo tem como objetivo verificar se existe uma possível relação entre um desvio extremo no padrão de inativação do cromossoma X e o encurtamento dos telómeros com as perdas recorrentes de gravidez que, embora não seja consensual na literatura científica, são consideradas causas potenciais subjacentes à doença.

Com este fim, foram estudados dois grupos de mulheres: um grupo de mulheres com perdas recorrentes de gravidez e um segundo grupo de mulheres com idades emparelhadas e fertilidade comprovada. Nestas amostras foi comparado o padrão de inativação do cromossoma X e tamanho dos telómeros em DNA extraído de sangue periférico.

Não foram obtidas diferenças estatísticas significativas aquando da comparação dos grupos, o que permitiu concluir que os nossos resultados não suportam a hipótese de uma associação entre um desvio extremo no padrão de inativação do cromossoma X ou encurtamento dos telómeros e perdas recorrentes de gravidez.

Adicionalmente, o efeito da idade materna no tamanho dos telómeros e padrão de inativação do cromossoma X foi também investigado, mas não foi obtida uma correlação positiva entre o aumento da idade materna e o aumento do desvio no padrão de inativação do cromossoma X ou encurtamento dos telómeros.

No entanto, nenhuma destas duas hipóteses deverá ser descartada, especialmente considerando algumas limitações do presente estudo e as perspetivas futuras expostas ao longo deste trabalho, e ainda a importância deste tema na comunidade científica e clínica.

Idade Materna; Inativação do Cromossoma X; Perda Recorrente de Gravidez; Tamanho dos Telómeros

Abstract

Recurrent Pregnancy Loss (RPL) is a reproductive disorder affecting about 1 to 5% of women worldwide that requires our attention, especially considering that about 50% of cases are of unknown causes and classified as idiopathic.

The present study is focused on testing a possible association between extreme skewed X chromosome inactivation (ESXCI) patterns and shortened telomeres with RPL cases since both are considered, nonconsensual potential causes underlying RPL in the scientific community.

For this purpose, two groups of women were analyzed and compared: a group of RPL women and a second group of age-matched women with proven fertility, and both X chromosome inactivation patterns and telomere length were measured and compared from extracted DNA of peripheral blood.

Our data obtained no statistically significant differences between groups, suggesting no association between ESXCI and shortened telomeres with RPL.

Additionally, the effect of maternal age on both telomere length and X-Chromosome Inactivation pattern was tested, but no significant correlation was observed between increased maternal age and ESXCI or shortened telomeres.

However, any of the explored hypotheses will be discarded from our line of research, considering the limitations of the present study and future perspectives, and the importance of a better understanding of this subject for the scientific and clinical community.

Maternal Age; Recurrent Pregnancy Loss; Telomere Length; X-chromosome Inactivation

Index

1- Introduction

1.1- Recurrent Pregnancy Loss

The establishment and success of pregnancy require different coordinated mechanisms that altogether result in a live birth. However, risk factors interfering in any pregnancy stage may lead to commitment or loss[1,2]. Miscarriage is a pregnancy loss occurring until 24 weeks of gestation when the fetus is not yet viable. It affects about 15 to 25% of pregnant women, with clinically recognized pregnancies, and is mostly caused by numeric chromosomic abnormalities (aneuploidies)[3,4]. The most frequent aneuploidy in sporadic pregnancy losses is trisomy, from which trisomy 16 has a higher incidence, associated with increased maternal age[2]. It is well-known that female fertility falls because of a decline in successful conceptions and an increasing rate of aneuploid pregnancy losses due to oocyte quantity and quality decline[5,6].

In its turn, and according to the European Society of Human Reproduction and Embryology (ESHRE), the loss of two or more clinically recognized pregnancy losses, before 24 weeks of gestation, is characterized as a reproductive disorder - Recurrent pregnancy loss (RPL)[1,3]. This definition includes all confirmed pregnancy losses through ultrasonography or histopathology .and excludes ectopic and molar pregnancies, implantation failures, and biochemically confirmed pregnancy losses[3]. Although difficult to estimate with precision, it is expected, based on large-scale studies, that RPL may affect, approximately, 1 to 5% of pregnant women worldwide[1,4]. RPL can be distinguished into a primary or secondary condition corresponding to situations in which all previous pregnancies were lost before 20 to 24 weeks and situations where there is at least one live birth or pregnancy that evolved beyond 20 to 24 weeks, respectively[1,7,8].

RPL is a multifactorial and heterogeneous disorder that has few well-accepted associated etiologies (Figure 1)[1,9,10]. The existence of parental structural chromosomal abnormalities mostly balanced Robertsonian and reciprocal chromosomal translocations is responsible for 2-5% of RPL cases[1,4,9]. In fact, within the known causes other non-genetic causes are more consensual[2,9]. Uterine anomalies, such as congenital Mullerian anatomical defects (unicornuate, didelphys, and septate uterus) or acquired anomalies (uterine myomas, intrauterine adhesions, and endometrial polyps) represent about 10-15% of cases[1,9]. Such anomalies are expected to cause pregnancy losses through the interruption of the endometrium vascularization and promoting an abnormal placentation[11]. Infections causing pregnancy losses may be sporadic

events. However, a higher predisposition for infections caused by any infectious agent, like *Listeria monocytogenes* or herpes simplex virus (HSV), or chronic situations associated with sexually transmitted diseases, are responsible for 0,5-5% of cases[9,11]. These infections may result in pregnancy losses affecting the uterus, fetus, or placenta or associated with chronic endometritis, a chronic endometrium inflammation caused by infection[1,11]. Nevertheless, testing for these infections and treatments with antibiotics are not recommended or supported[4,8]. In its turn, endocrinological disorders such as thyroid dysfunction, polycystic ovarian syndrome (PCOS), luteal phase defect (LPD), and uncontrolled diabetes mellitus are associated with 17-20% of RPL cases[1,9,11]. Finally, autoimmune diseases are responsible for about 20% of cases, from which the antiphospholipid syndrome (APS) has been highlighted as the most usual and treatable autoimmune disorder[1,9]. APS is an acquired thrombophilia associated with antiphospholipid antibodies that may bind to endothelial and placental cells resulting in the activation of inflammatory processes that, in addition to several fetal complications, can result in pregnancy loss[1,11].

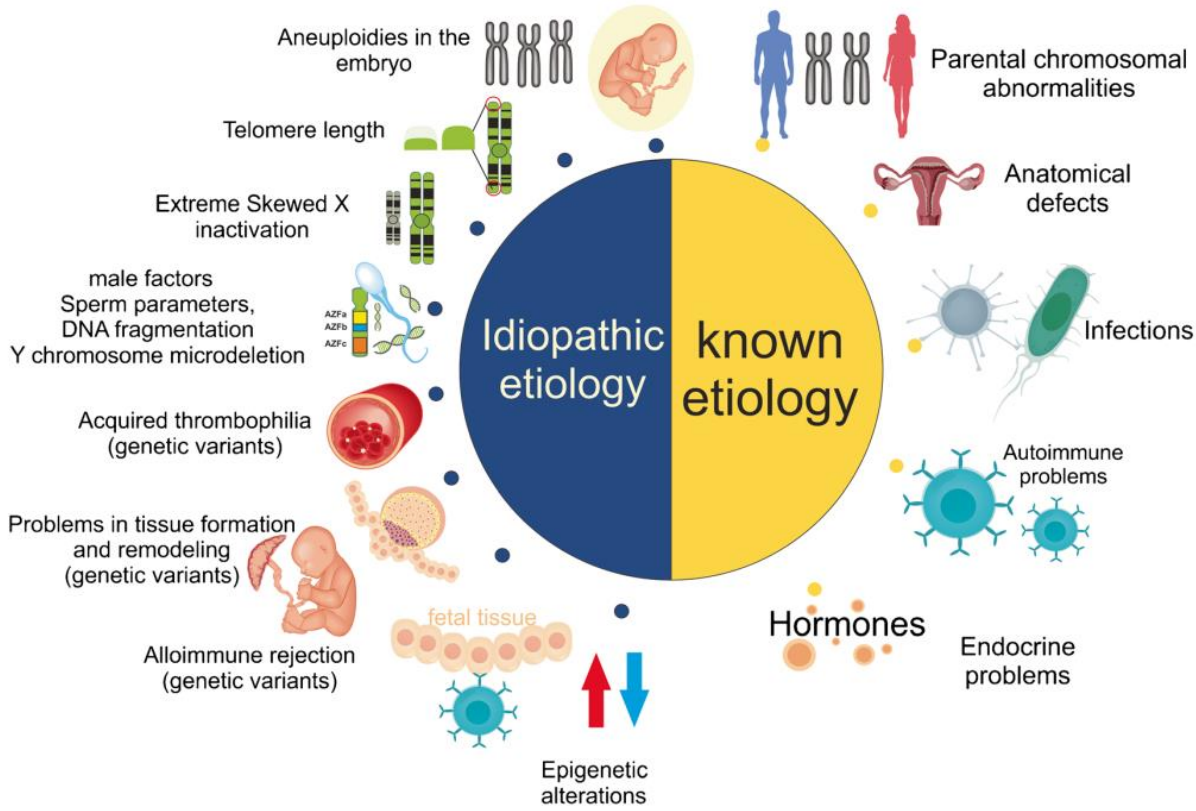

Figure 1- Recurrent Pregnancy Loss: associated etiologies

Idiopathic etiology factors include findings associated with RPL that are not consensual in the scientific community (left side): increased frequency of aneuploidies in the embryo; telomere length; extreme skewed X inactivation; male factors; genetic variants in acquired thrombophilia, tissue formation, and remodeling and alloimmune rejection and epigenetic alterations. Known etiology factors include proven causes of RPL which are consensual among scientists (right side): parental chromosome abnormalities, anatomical defects, infections, and autoimmune and endocrine problems. Adapted from[9].

Although not considered in RPL etiology, there are some risk factors that should be examined. It is well-known that advanced maternal age increases the incidence of pregnancy losses. It was estimated that, while women younger than 35 years old have a risk of pregnancy loss of around 10%, women 40 years or older have a risk of loss of about 50%[1,8,12]. Additionally, the number of previous losses also represents a risk factor, since several studies proved that the higher number of previous losses the higher probability of a subsequent loss[1,13,14]. Other well-accepted RPL risk factors are environmental and lifestyle issues like high caffeine and alcohol intake, smoking, drug consumption, exposure to air pollution, stress, and obesity[1,8,11].

According to the 2017 ESHRE guidelines, RPL management should follow the recommendations and treatments summarized in Figure 2. After the diagnosis of RPL, as the loss of two or more pregnancy losses, the recommended tests include the detection of antiphospholipid antibodies (aPLs), thyroid, and uterine abnormalities[3]. The aPLS identification is key in managing the disorder. The most tested are lupus anticoagulant (LA) and anticardiolipin antibody (ACA) and the standard treatment is low doses of aspirin and heparin[3,4]. If a positive detection of thyroid abnormalities includes overt hypothyroidism, levothyroxine is recommended as the therapeutic solution[3]. Uterine abnormalities are usually detected by ultrasonography and surgical treatment may not always be possible or improve pregnancy outcomes[3,4,13].

Additionally, there are tests that are only recommended in research contexts, or in cases of a previously known family history, which include genetic, immunological antinuclear antibody (ANA), sperm DNA fragmentation tests, and screening of hereditary thrombophilia[3]. If evidence of a possible abnormality in the offspring is detected through genetic testing of parents, especially karyotype, counseling must be provided and options like preimplantation genetic testing (PGT) and amniocentesis may be discussed[3,4,11]. In cases of an immunological positive test of ANA, there is no available treatment and for fragmented DNA sperm, only

lifestyle advice can be provided for men[3]. Other immunological or metabolic hormonal tests are not recommended[3].

In any situation, lifestyle advice and tender loving care (TLC) must be assured and vitamin D recommended[3]. During all management, as previously mentioned, couples should be informed that the prognosis of a future successful pregnancy depends on maternal age, the number of previous losses, and the underlying cause of RPL[3].

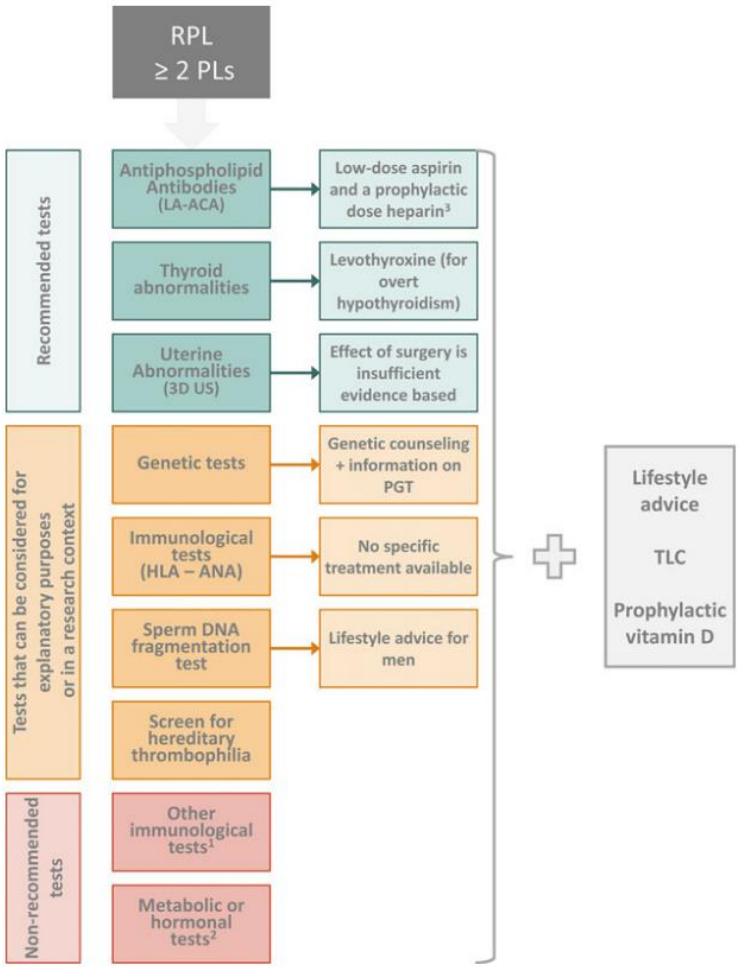

Figure 2 - Recommendations and management of RPL couples (2017 ESHRE guidelines)

After the diagnosis of RPL (≥ 2 Pregnancy losses (PLs)), the guidelines are divided into recommended, recommended under some conditions, and non-recommended tests. (Recommendations and management are described in detail above) 1 - Tests including Natural Killer (NK) cell detection or anti-HLA antibodies; 2 - It includes assessment of polycystic ovary syndrome (PCOS) or luteal phase defect detection and 3 – Treatment recommended after three or more PLs or in clinical trials contexts. Adapted from[3].

Nevertheless, it has been estimated that, approximately, 50% of RPL cases remain unexplained, and are often mentioned as idiopathic[1,9,11]. Considering that idiopathic recurrent pregnancy loss (iRPL) is a distressing condition for couples, who usually develop anxiety and depression, and frustrates physicians due to the difficulty in couple orientation and management, several efforts have been made in the context of scientific research to find potentially related causes (Figure 1)[4,9]. Although commonly associated with maternal advanced age and parental chromosomal abnormalities, the incidence of aneuploidies in RPL has not always been explained[4,9]. Then, it has been hypothesized that such aneuploidies can be caused by genetic variants in parents that may lead to a higher predisposition to meiotic errors during gametogenesis[9]. Another discussed factor is the existence of shorter telomeres in parents, related to aging, which may have consequences during the embryo cell replication resulting in its loss[9]. Extreme skewed X chromosome inactivation (ESXCI) patterns in women have also been associated with RPL[9]. Importantly, male factors have also been investigated especially the genetic influence on sperm. Sperm aneuploidy, DNA fragmentation, and microdeletions on the Y chromosome, associated in previous studies with male infertility, have been highly associated with RPL in some studies[8,9]. Additionally, some authors proposed that the existence of specific genetic variants may lead to acquired thrombophilia, problems in tissue formation and remodeling, and alloimmune rejection that in extreme cases may result in pregnancy loss[8,9]. Epigenetic deregulation, such as differences in methylation patterns of some loci in placenta samples, has also recently been highlighted in RPL women[9].

Briefly, none of these hypotheses and studies have reached a consensus yet. While some authors have shown strong associations with RPL, others did not find supportive results and rejected such postulations. Therefore, in the next sections, special attention will be paid to the association of ESXCI and telomere shortening with RPL as the study's main goal. First, the XCI process and telomeres length dynamics will be explained in detail and then associated with RPL to better understand the matter.

1.2 - X-chromosome Inactivation

1.2.1- Historical context

The evolution of sex chromosomes has been considered a paradigm in understanding the possible sources of genetic sexual dimorphism[15]. In mammals, the sex chromosomes evolution, which started 166 million years ago, resulted in sex chromosomes with a different gene content, gene regulation expression, and inheritance from the autosomes[15]. Additionally, sex chromosomes also differed from each other due to the evolution process itself[15].

Mammal sex chromosomes, as for all sex chromosomes, were developed from autosome pairs[15,16]. Its generation is highlighted by the recombination lack of a new sex-determining gene flanking loci, from a previous pair of autosomes[17]. The process started when a testis-determining locus (proto-*SRY*) was acquired on an autosome[16]. During this process, genes were selected in the proto-Y chromosome and accumulated some male-beneficial mutations[15].

Thereby, the actual Y chromosome lost most of the genes from the ancestral autosome pair while the X chromosome largely maintained its gene content[15]. In humans, as in other mammals, the sex chromosome system is defined as XX in females and XY in males[16]. As a result, there is a dosage difference for X-linked genes of 2:1 in females[16]. In short, the evolution of XY testis determination resulted in female mammals carrying two copies of the relatively gene-rich chromosome (XX), representing the homogametic sex, and in male mammals carrying a single copy of the X-chromosome and one of the gene-poor chromosome (XY), representing the heterogametic sex[15].

The existence of heteromorphic sex chromosomes, caused by a natural mammal's evolution, leads to an imbalance in sex-linked genes between females and males[18]. While the Y chromosome genome is about 55Mb, the X chromosome comprises 155Mb, which represents a difference of 100 Mb[19]. Therefore, this discrepancy covers, approximately, 1000 X-linked genes[19].

1.2.2 - First findings

In 1961, Mary Frances Lyon hypothesized, that such genetic imbalance is compensated by the inactivation of one of the two XXs in early female mammals' development[19–21]. She also proposed that this event occurs to prevent female cells from expressing twice as many X-linked gene products as male cells[20]. Her findings helped the scientific community to understand the

genetic basis of many X-linked gene diseases and provided a later discovery of the molecular X-chromosome inactivation process[20].

After her first discoveries, numerous studies have been developed to understand how the X chromosome inactivation works and influences human female development. Susumu Ohno suggested an additional X-dosage compensation process called X-Chromosome upregulation (XUP), preceding the XCI proposed by Mary Lyon (Figure 3) [15,22,23]. He described an X-linked gene upregulation in males to compensate the presence of only one X-chromosome compared with the presence of two copies of each autosome[22]. According to his findings, as males are hemizygous for their X-linked genes, it occurs a product output duplication[23]. While Mary Lyon's hypothesis has always been accepted, Ohno's proposal remains controversial and poorly explained[22].

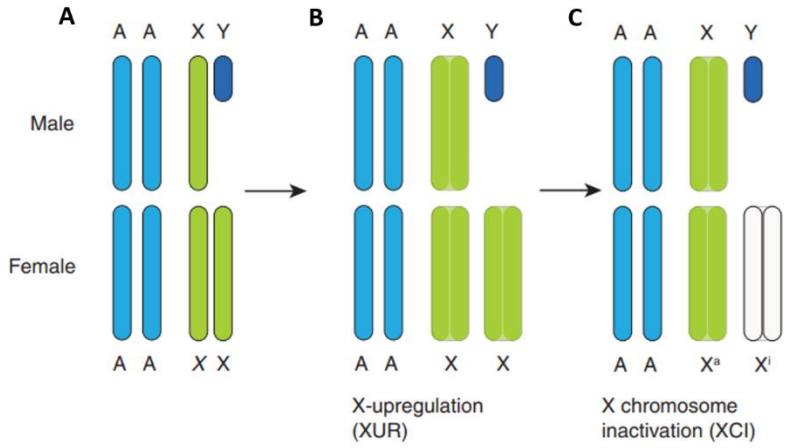

Figure 3 – X-chromosome dosage compensation mechanisms

A – Sex chromosomes (XY- male and XX- female). **B** – During sex chromosome formation, it occurs a surface duplication of the X-Chromosome (X-upregulation - XUR) to equalize the gene dosage between the single X chromosome and autosomes in males (Susumu Ohno proposal). **C** – X chromosome inactivation (XCI) (Mary Lyon proposal). Adapted from [15].

Nowadays, it is well known that the X-chromosome Inactivation (XCI), characterized by a transcriptionally silencing and heterochromatinization of one of the two X chromosomes, starts early in females' embryonic development[24]. It is also expected that the inactivated state, starting *in utero* at the embryo stage, propagates during cell division in female adults [18,25].

In addition, it is speculated that both X-chromosomes, regardless of their origin (paternally inherited (Xp) or maternally inherited (Xm)), have the same probability to be inactivated[22]. As

a result of this random choice, it is expected that women have a 50:50 ratio of cells with the Xm or the Xp chromosome activated, leading to female mosaics, with two cell lines, considering their X-linked allelic expression[22,26,27]. However, as it will be approached later, this does not always take place.

1.2.3 – X-chromosome Inactivation process

The complete understanding of the XCI in human embryos remains elusive, considering the limited availability of embryos and its technical protocols' reproducibility restrictions[28]. Most limitations are due to the process timing since it occurs very early in female embryonic development where there are already cells with a Xi[19]. Following that, most of our knowledge, concerning XCI in human females, is deduced from mice models[29]. Consequently, it is indispensable to summarily compare the process between humans and mice in this section.

One of the main differences is the beginning of the process, since the XCI occurs earlier in mice; in a stage preceding the blastocyst implantation, shortly after fertilization and zygotic genomic activation (ZGA)[30,31]. On the other hand, in humans, the exact stage at which the process begins is not consensual among scientists due to the limited access to human embryos, as previously mentioned[32].

The second, and most relevant, difference, is the presence of two XCI forms in mice: imprinted (iXCI) and random (rXCI) (Figure 4A)[22,24]. The imprinted XCI form is present in all female mouse cells in the preimplanted embryo where the Xp is inactivated and the Xm remains active[24,33]. During the preimplantation development, progress to form the blastocyst, the trophectoderm layers, giving rise to extra-embryonic tissues, maintain their imprinted form[24]. Meanwhile, epiblast cells of the blastocyst in the ICM, which give rise to the embryo proper, reactivate the Xp re-establishing a state with two active X-chromosomes[21,24]. Then, as a second wave, in these cells, both Xs are randomly inactivated after implantation[21,33].

However, this imprinting form has never been observed in rabbit, pig, horse, or human development[24]. In its turn, it was described in human embryos a preceding XCI phenomenon called X-chromosome dampening (XCD), (Figure 4B). XCD is characterized by partial gene silencing, in which there is a mitigation of X-chromosome gene expression[24]. After this phenomenon, it occurs a random XCI like the one observed in mice.

Despite the differences between mice and humans, all forms (imprinted and random) are in both species coordinated by the same mechanism involving the master regulator *Xist* (a long non-coding RNA lncRNA), as will be explained in the next section.

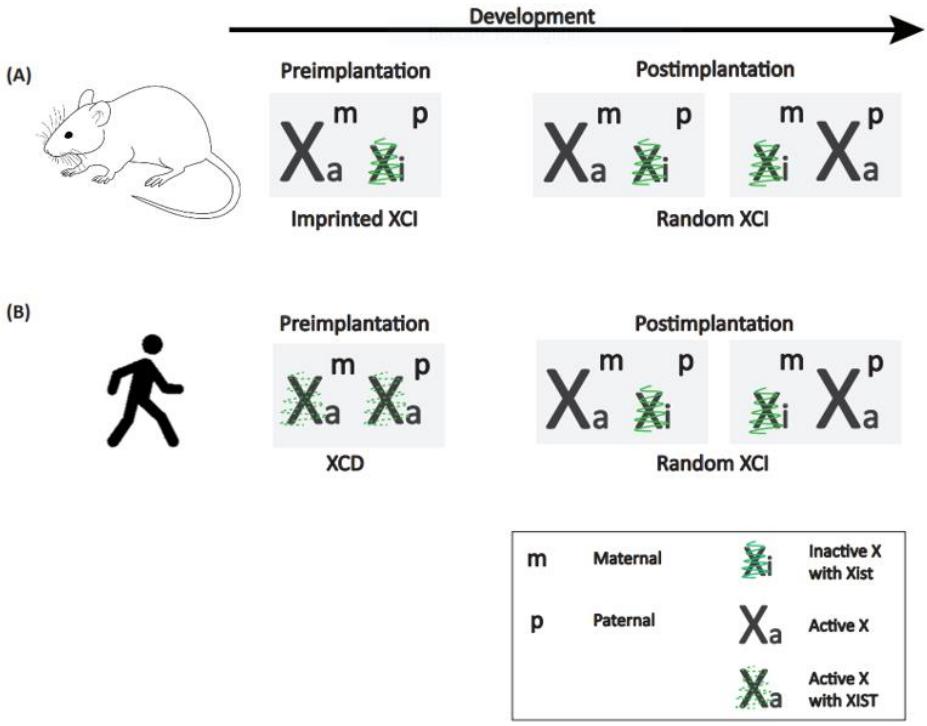

Figure 4 – X-chromosome Inactivation in female mice and humans

A – X-chromosome inactivation in mice females. The process starts in the preimplanted mouse, around the two to the four-cell stage, as an imprinted form where the Xp (paternal X) is inactivated in all cells. This imprinted form is maintained in extra-embryonic tissues, while in cells giving rise to the embryo occurs a reactivation followed by a random inactivation. In both imprinted and random forms, the process is mediated by the long non-coding RNA (lncRNA) *Xist*. **B** – X-Chromosome inactivation in human females. In women, it does not exist as an imprinted form, but it occurs in a phenomenon called X-chromosome dampening (XCD), characterized by a gene expression mitigation that precedes the random XCI. In both dampening and random inactivation, the process seems to be coordinated by *Xist* RNA. Adapted from [24].

1.2.4 – Initiation, establishment, and maintenance

As described by several authors, including Mary Lyon, the XCI comprises three phases: initiation, establishment, and maintenance that correspond to *XIST* regulation, gene silencing, and propagation to daughter cells, respectively[33]. The XCI process is considered a stochastic phenomenon since every XC has the same probability to be inactivated. It has been postulated that, before *XIST* regulation, the homologous XC get closer in an event called X-paring, recognizing the existence of two XC and the necessity to inactivate one of them[33]. A full understanding of the XCI initiation process, considering its timing, steps, and events, was obtained after the discovery of its master regulator gene and locus: *XIS*T (X-inactive specific transcript) and XIC (X-inactivation center), respectively[22].

In both human and mouse females, XCI seems to be coordinated by the XIST long non-coding RNA (lncRNA), expressed by *XIST*, and encoded in the XIC of the X chromosome[24]. The *XIST* gene, known for its role in XCI, is conserved in placental mammals, located in the Xq.13.2[25]. The lncRNA XIST, acting in *cis*, coats the X chromosome to be inactivated and triggers its genetic silencing[24,25,34]. This silencing occurs through protein complexes recruitment that is involved in chromatin remodeling, chromosome structuration, and nuclear organization, as will be described later[31].

1.2.4.1 – Initiation: *XIST* regulation

The existence of XIC represents the critical locus to set off an effective and coordinated XCI initiation, in both humans and mouse[25]. This locus is composed of two domains - topologically associating domains (TADs) - within which there is an increased self-interaction. These TADs encompass all *cis* action elements, between which *XIST* is located[22,29,34]. In fact, cis-transcriptional regulation is done on these TADs in a positive and negative manner.

Then, there is a TAD of 500-550kb that contains the *XIST* promoter and harbors most of its activators (also designed as TAD-E), and an adjacent TAD of 250-300k harboring its known *cis* repressors (also designed as TAD-D)[22,29,34]. On the TAD-E *Xist* contains its positive regulators: *Jpx, Ftx,* and *Rnfl12*. On the other hand, the TAD-D harbors all negative *Xist* regulators: *Tsix* (antisense non-coding transcription unit *x*) and *Linx* (the non-coding RNA locus large intervening transcript)[22].

As mentioned in the previous section, female human preimplantation blastocysts studies showed that XIST may also have a role in dampening mediation involving both X chromosomes (Figure 5). However, this hypothesis remains poorly explained[24,28]. In humans, *TSIX* appears not to be involved in *XIST* repression and the role of *JPX* and *FTX* is not entirely understood. It was predicted that TSIX, although present in humans, is associated with the imprinted XCI in mice[18]. Despite that, an additional lncRNA was described, XACT (X active-specific transcript), which is unique to human pluripotent stem cells (hPSCs), (Figure 5). *XACT* does not exist in XIC and is responsible to coat the Xa maintaining its transcriptional activity through a *XIST* neutralization[24]. Some study results have been demonstrating that *XACT* expression tends to decrease during the blastocyst development progress, which can be indicative of an XCI initiation. Moreover, during the pre-XCI state, XIST and XACT showed a co-accumulation on the Xa of human female blastocysts, (Figure 5).

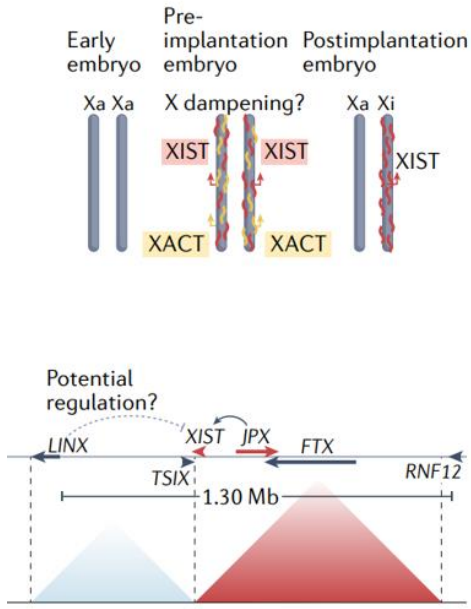

Figure 5 – Random X-chromosome Inactivation in human

Initially, in pre-implanted embryos, it occurs an *XIST* biallelic expression that might lead to a X dampening. Then, there is a *XIST* and *XACT* co-expression that might interfere with XIST accumulation and silencing function. Later in development, *XIST* expression is restricted to the X that would be inactivated. The *XIST TAD*, also known as TAD-E, includes *JPX* and *FTX*, which products are putative *XIST* activators, while the adjacent TAD contains *TSIX* and *LINX*. (On top it is represented the main XCI events where genes are represented by arrows and RNAs by wavelets. At the bottom, it is represented the XIC, where product genes that are *XIST* activators are in red and repressors in blue.

Black arrows between genes represent direct or indirect (dashed lines) regulation. The red triangles are representative of *XIST* TAD (TAD-E), while the blue triangles represent *TSIX* TAD-D). Adapted from [22].

After *Xist* regulation from only one XC, its lncRNA starts to accumulate in cis. It is estimated that about 2000 Xist RNA molecules are in each nucleus of a Xi. Xist RNA coats the XC to be inactivated and promotes striking changes[25].

1.2.4.2 – Establishment and maintenance: molecular mechanism

Following this accumulation and spreading, the second phase takes place – XCI establishment - characterized by events like chromosome-wide gene silencing and chromatin changes[25,33]. At this stage, different events occur - repressive nuclear compartment formation, early gene silencing, and late epigenetic changes[22].

The formation of a repressive compartment, which includes the recruitment of genes that are supposed to be silenced, was reported as the first event. This compartment is mostly composed of repeat-rich regions that are reorganized after *Xist* RNA coating[22,25]. After that, it is time for the XIST RNA to act since its repeat domains (A-F) are all together crucial and promote XC coating, gene silencing, chromatin changes, and chromosomal 3D organization[22,35].

Then, the first gene-silencing steps initiate. The recent discovery of Xist binding proteins, like SPEN, has given an additional understanding of how histone modifications, at the early stage of XCI establishment, take place[36].SPEN, which seems to be recruited by the A- Xist RNA region, is a protein factor that contains RNA-binding domains and belongs to a transcriptional repressor family[22,25,35]. This protein factor is critical for the earliest gene silencing[22]. However, SPEN does not work alone but through interaction with some cofactors like SMRT/NCOR2 and NCOR1 corepressors[25]. SMRT/NCOR2 and SPEN are together responsible for a correct histone deacetylase 3 HDAC3 activation and recruitment, which seem to be the master histone in that deacetylation process (Figure 4A)[22]. Thereby, there is a histone modification linked to active promoters like (H3K4me3, H4ac, H3K9ac, and H3k27ac) and enhancers (H3K27ac and H3K4me1) representing a loss of euchromatin markers[25,33,36]. As a result, euchromatic marks, which include histone H3 lysine 4 dimethylation and trimethylation (H3K4me2/me3) and histone H3 and histone H4 acetylation (H3/H4 Ac) are lost. Next, there

is a repressive mark accumulation like histone H3 lysine 9 dimethylation (H3K9me2) (Figure 6A)[33].

After these first events, it has been mentioned the role of two chromatin-associated protein complexes: Polycomb Repressive Complex 1 and 2 (PCR1 and PCR2), which are also recruited[33]. PRC1 is composed of Polycomb group Ring finger 3/5 (PCGF3/5) and recruited through the action of the heterogeneous nuclear ribonucleoprotein K (hnRNP K) (Figure 6B)[22,35]. The hnRNP K is a Xist interactor recruited by B- and C- Xist RNA repeats[35,36] PCR1 is responsible for a monoubiquitynation of lysine 119 of histone H2A (H2AK119) that, with the histone deacetylation previously mentioned, correspond to the first histone rearrangements[22].

Subsequently, PRC2, which is associated with trimethylations histone H3 Lys 27 (H3K27me3), accumulates in a PCGF3/5-PRC1- dependent manner, (Figure 6B)[22]. Both H2AK119ub and H3k27me3 are repressive histone marks that represent a correct establishment of XCI[33]. As the PRC2 recruitment occurs indirectly, it is mediated by another cofactor - JARID2, which is recruited by the B- and F- Xist RNA repeats[33,37].

Finally, the later chromatin changes take place. There is a histone variant macroH2A insertion into nucleosomes, followed by a CpG islands methylation, (Figure 6C)[22]. This epigenetic modification consists of the addition of a methyl group on the carbon 5 of cytosine (C-5) in CpG dinucleotides gene promoters. Those methyl groups are added by DNA methyltransferases (DNMTs) that maintain the process. After that, it occurs the formation of CpG islands, which are characterized by extensive methylation of CpG-rich areas in promoters. As a result, transcription factors do not bind to promoters causing an interruption in gene expression[38]. At the same time, it occurs demethylation of histone H3 Lys9 (H3k9me2) recruits a complex (constituted by a chromodomain Y-like protein (CDYL), a histone-lysine N-methyltransferase G9A, and the MAX gene-associated protein (MGA)). In its turn, this complex ensures the H3k9me2 propagation through the X chromosome, (Figure 6C)[22].

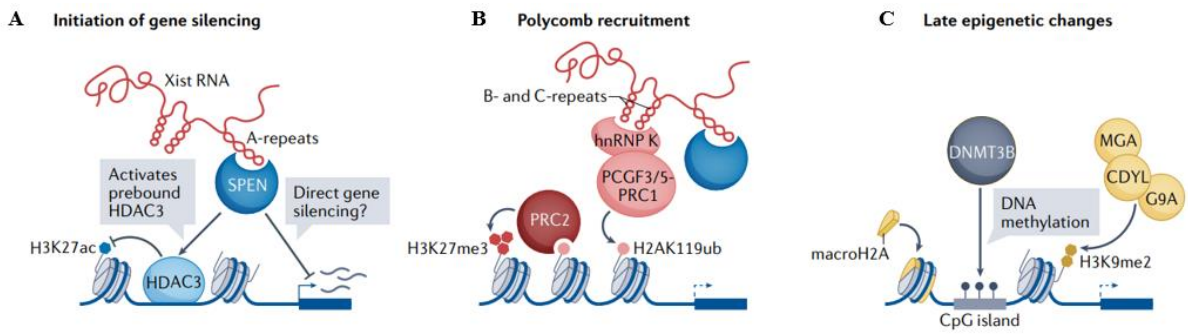

Figure 6 – Molecular mechanisms of X-chromosome Inactivation

A – Initiation X-chromosome inactivation. It occurs a gene silencing associated with SPEN recruitment, through the Xist RNA A-repeat. Before XCI there is already a Histone deacetylase 3 (HDAC3) present on the X chromosome and becomes activated through the recruitment of SPEN that starts the gene silencing by targeting acetylated histone H3 Lys27 (H3K27ac). Additionally, it has been proposed that SPEN also represses transcription through an independent HDAC3 mechanism. **B –** Polycomb repressive complex 1 (PRC1) and 2 (PRC2) recruitment. This step starts with the recruitment of the non-canonical PRC1, which includes Polycomb group RING finger 3 (PCGF3) or PCGF5 (PCGF3/5-PRC1), by heterogeneous nuclear ribonucleoprotein K (hnRNP K) bound to B- and C- Xist repeats. PCGF3/5-PRC1 monoubiquitylates histone H2A Lys 119 (H2AK119ub), allowing PCR2 recruitment, which trimethylates histone H3 Lys 27 (H3K27me3) and strengthens gene silencing. **C** – Late epigenetic changes. In this step, to maintain the XCI state, there is the histone variant macroH2A insertion in nucleosomes, methylation of cytosine at CpG island by DNA (cytosine-5)-methyltransferase 3B (DNMT3B), and histone H3 Lys9 dimethylation (H3K9me2). H3K9me2 recruits the chromatin Y-like protein (CDYL)-G9A-MAX gene-associated protein (MGA) complex, which might in turn facilitate H3K9me2 propagation on the inactive X chromosome. Adapted from[22].

Briefly, genes silencing is accompanied by some important modifications: X chromosome architecture reorganization, repressive complexes recruitment, histone modifications (active histone marks loss and inactive histone marks gain), histone variant macro H2A accumulation, and DNA methylation on CpG islands[17,25,33]. When the XC finally reaches its inactivated form, it evolves into a heterochromatic structure called Barr body[21,25]. The Barr body was first described by Murray Barr in 1949, through the evidence of a morphological difference between male and female neurons in cats, (Figure 7). He discovered the presence of a second body in the female nuclear structure that he defines as a nucleolar satellite located close to the nucleolus. He also postulated that this Barr body would derive from the sex chromosome chromatin[39].

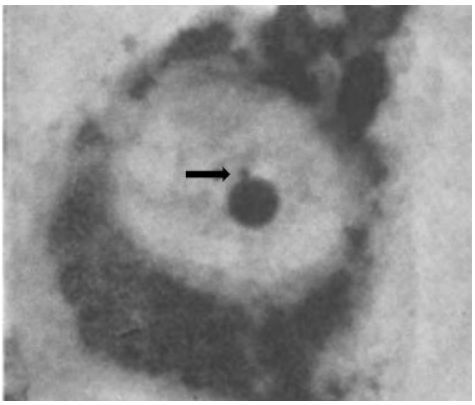

Figure 7 – Barr body discovery by Murray Barr

The female neuron of a cat where was found a heterochromatic structure derived from an X-chromosome described as Barr body (indicated by the arrow). Adapted from[39].

1.2.5- Escape genes and reactivation

Interestingly, not all genes are inactivated on the XC – a phenomenon often called incomplete XCI[40]. It is estimated that around 10 to 20% and 3 to 7% of X-linked genes are still being biallelically expressed in human and mice females, respectively[22,41]. This discrepancy is due to the existence of human escape regions, mostly located on the X short arm, that contain blocks of approximately 15 genes compared with blocks of 1 to 2 genes in mouse[19,32].

The genes, which resist epigenetic silencing, and escape genes, are important to maintain sexual dimorphism and cell function[21,22,42]. On the other hand, genes that are silenced are considered subject genes[17]. The existence of escape genes was first hypothesized by Mary Lyon through the evidence of pairing genes, in humans, located in the pseudoautosomal region (PAR) between the X and Y chromosome[21]. Nowadays, it is known that all genes located in PAR1 escape XCI, while in the PAR2 region the two proximal genes are inactivated in both males and females[17]. Escape genes are classified into two groups: constitutive and facultative[22]. The constitutive escapee genes, which represent about 15% of escape genes, seem to be well conserved between humans and mice and are expressed regardless of the cell type, development type, or individual[22,42]. On the other hand, the facultative escape genes represent about 10% of escape genes and are variable between tissues and individuals and seem to be silenced and reactivated during time[21,22]. Additionally, constitutive escapee genes retained Y-chromosome homology during the sex chromosome differentiation from the autosomes[22].

For years, it was thought that the XC had only a unidirectional regulation in women[43]. However, it has been proved that, in females, the full XC regulation is obtained through a cycle of inactivation and reactivation[21,43]. In normal conditions, in humans, a reactivation of the Xi occurs on the germ cell to provide an assorted gene pool XC, where oocytes have two active XC[43,44]. On the other hand, in somatic cells, X reactivation can occur related to congenital or acquired diseases and aging[21,42].

1.3 – Skewed X-chromosome inactivation in Recurrent Pregnancy Loss

1.3.1 - Skewed X-chromosome inactivation

As described in the previous section, it is theoretically expected that women have around 50% of cells with the Xm active and 50% of cells with the Xp active (50:50 pattern)[41,45]. This expected random and cell-autonomous process follows a binomial distribution of those two cell populations, which is maintained in all descendants of a cell[45,46]. Interestingly, in the adult female the 50:50 distribution, first described by Mary Lyon, is not always observed[47].

When the XCI does not follow this expected pattern, women can have most or even all somatic cells with the same XC inactivated - Skewed X Chromosome Inactivation (Skewed XCI)[48–50]. There is no consensus from which percentage a skewed XCI should be considered, but it is usually defined as a cut off value higher than 75%[51]. Skewing is usually observed and related to some diseases. However, it is also expected in women of a healthy population because of different mechanisms[41,45,49]. Using the same methodology as in the present study (AR methylation assay) it was proposed a frequency varying from 1 to 23% in the female general population[52].

Skewed XCI can occur by chance or choice. It can occur by chance as a stochastic phenomenon due to the small cell population at the XCI onset[45,50,53]. Although the exact timing in humans has never been determined, the limited number of precursor cells giving rise to different germ layers may influence its representation in adult women[32]. As the XCI occurs early in embryonic development when there is a small progenitor cell pool, skewing can be a result of that small cell population or a later loss of some of those cells during life[30,32]. Additionally, it is expected that the probability for a woman to have an extreme skewing XCI (≥90%) by chance in blood, considering the simple binomial model, varies between 0,01% to 2,1% depending on the progenitor cell number during the XCI process[44].

Regarding choice, it is fundamental to distinguish a non-random or preferential inactivation (primary choice) from a subsequent cell selection (secondary choice). While the first refers to a preferential choice at the inactivation onset, the second refers to events occurring after the XCI establishment associated with unequal cell population distribution[45,53,54]. Considering a primary choice, some explanations have been developed over time. In mice, it was identified a locus - *X controller element* (Xce), that seem to have a role in primary choice[30,53,54]. However, in human females, this locus has never been associated with skewing[30,53]. It was also proposed that *Xist* itself can influence the primary choice, especially considering that some mutations can

prevent the inactivation of its respective chromosome[53]. In human females, a rare *Xist* mutation was seen in two families with skewed XCI, but these mutations did not give a complete understanding in terms of cause-effect[45]. The authors of this study conclude that the *Xist* mutation alone should not be the major cause of skewing in these women. They hypothesized that the major cause of this skewing was the co-inheritance of the *Xist* mutation with an X-linked disease locus. This postulation was sustained by the results obtained in one family where females with higher skewing patterns had both *Xist* and Snyder-Robinson mutations[55].

A secondary skewing may appear due to any epigenetic, genetic, or chromosomal abnormality that results in a proliferative cell advantage or disadvantage[30]. Secondary cell selection, during female adulthood, is considered the biggest contributor to skewing and is sometimes incorrectly confused with non-randomness[48,50]. Selection against X-linked disorders is the main cause of skewed XCI in healthy women who have no family history of any X-linked disorder[45]. It is consensual that structural abnormalities involving an XC (large deletions, duplications, and unbalanced X/autosome translocations) result in the inactivation of the involved XC in almost all cells[50,56]. However, in cases of balanced X/autosome rearrangements, the uninvolved XC is often inactivated to assure the euploidy and escape gene monosomies in the translocated autosome and disomies on the XC[30,50]. It is expected that, in both cases, it may occur a skewing caused by the selection of cells retaining the best genetic balance[56].

Extreme skewing patterns are usually observed in women who manifest X-linked genetic diseases and do not present the expected mosaicism (50:50 pattern). However, it is not possible to predict if this lack of mosaicism occurred during the XCI process itself or later. There are two hypotheses: there was a preferential inactivation of one XC (non-random) in favor or against the mutation or, after the random chose the two populations, cells were differentially selected due to their proliferative potentialities. It is possible that one cell population expanded more due to the presence of genes with growth advantages on the XC activated, while the other has the XC activated with genes that constitute a growth disadvantage[48].

Additionally, in cases of X-linked mutations, selection against the deleterious mutations leads to the normal chromosome being active in most cells[50]. This phenomenon is usually seen to prevent a possible undesirable phenotype outcome[42]. It is well known that some X-linked mutations are lethal in man (hemizygote). However, in women who are mosaic for some X-linked mutations possible effects of the mutation are attenuated or not even seen, since cells who express the wild-type allele are often enough to mask the effect of cells expressing the mutant allele or the cell lethality. Another possible explanation is the competition between cells carrying the mutation and normal cells resulting in a progressive elimination of the last ones[53].

However, in cases where X-linked mutations confer cell advantage growth X inactivation may be a problem if the chromosome is not inactivated[53].

The previously explained factors, associated with a natural process of cell differentiation, may result in a higher probability of skewing in older women. It is expected that for each female her skewing pattern rate is lower in the newborn phase than in adulthood. In the blood of healthy women, a pronounced increase in skewing, related to age, was seen for ages above 30. However, significant differences were only seen considering more than a decade as an age interval[57].

Among adults, extreme skewing (≥90%), when analyzed in blood, is significantly higher for women aged 60 or over than young women below 40 years old [50,58] Some explanations may be cell differentiation during adulthood or even a loss of methylation with age since the methods used for XCI are indirect methods based on DNA methylation[58]. Another well-explained fact is that the XCI pattern varies among tissues in a female and, as a result, the study of different tissues should be considered[42,45]. Differences in XCI patterns between tissue may be explained by a bottle-neck effect during differentiation[51]. It has been proved, in some studies with healthy women, that the concordance in terms of XCI pattern between tissues like blood, urine, and buccal mucosa decrease with age, and blood seem to be the tissue where skewing is most observed in older women[45,50]. The most accepted explanation is that blood is a highly mitotically active tissue, in which cells are constantly in division and have a short life span, where is expected that those cells suffer from a continuous selection during life. However, it has been well documented that when more than one tissue presents a skewed pattern, the preferentially inactivated XC is the same, which means that skewing follows a unique direction[50].

1.3.2 – Extreme skewed XCI in Recurrent Pregnancy Loss

Over time, several authors have been exploring the idea of an association between an extreme skewed XCI and Recurrent Pregnancy Loss (RPL)[59]. Consequently, it has been suggested that skewing is more frequent in women experiencing recurrent pregnancy losses than in healthy women of the general population[51]. However, while some studies showed a significantly increased frequency of extreme skewing in RPL women[60–63], others did not support this difference[46,52,63,64]. In some situations, such divergences can be a consequence of different chosen cutoffs, tissues, assay detection techniques, diagnosis criteria, and sample size

that all together may influence the outcome of statistical analysis[58,60]. Despite that, in such studies as ours, the XCI pattern is usually obtained using peripheral blood, which is the more easily accessible tissue, and age-matched controls are considered the best strategy since advanced age may influence results[51].

It was initially proposed that women who are carriers of X-linked male-lethal mutations would present an extreme skewed XCI, due to a secondary choice through a cell selection to eliminate cells where the mutated chromosome is activated[60]. Consequently, male embryos of such carriers who inherit the mutation are not viable and aborted[58,60]. Then, it would be expected that such mutations would cause male lethality up to 20 weeks and explain recurrent abortions in carriers[51]. Although well accepted, in the scientific community, this explanation has been questioned[44]. First, if these mutations were the major cause of extreme skewing in RPL women, it would be expected a high number of aborted males in these RPL women[51]. However, as in some studies the comparison of RPL women, with and without extreme skewing, did not show any difference in the sex ratio of abortions[51,65]. Furthermore, it is not always possible to access the karyotype of abortions[62]. Moreover, female carriers of X-linked male-lethal mutations may have a significant chance to have successful pregnancies. Their female embryos will be viable, even if they have a 50% probability of being carriers and may have a skewed pattern as their mothers. On the other hand, males have a 50% probability to survive depending on the X inherited[51]. Moreover, this does not explain the incidence of chromosomal abnormalities usually observed in aborted karyotypes of RPL women with skewed XCI[58,65].

Alternatively, Robinson et al. proposed that an association between extreme skewed and RPL may be explained through a reduction in the embryo's progenitor cell size at the XCI onset. According to the authors, and as explained in the previous section, any event causing a reduction in the number of progenitor cells in the embryo should result in an extreme skewed pattern[51]. Later, these events may impact women's reproductive life. Then, it is necessary to understand that what happens in a female embryo may have repercussions on her offspring.

Recurrent pregnancy losses, and skewed patterns, due to a reduction in embryonic precursor cells could be explained in two different ways: 1) the existence of trisomic cells in oocytes in women whose own embryonic development was associated with mosaic trisomy and 2) a reduction of primordial ovary follicles[51,65].

It is known that chromosomal abnormalities, especially mosaic trisomy, are common in embryos during early development and, in most cases, high levels of trisomy are lethal. However, cell selection favoring diploid cells over trisomic may result in successful pregnancies[51,58]. Embryos may face a trisomy rescue that consists of the reversion of the initial

trisomic conceptus and results in a diploid embryo being trisomic cells confined to the placenta[2,58]. Interestingly, many female fetuses/newborns with detected trisomy mosaicism of meiotic origin showed a 100% skewed XCI pattern in most diploid fetal tissues because of that trisomy rescue[51,58]. As the trisomy rescue may occur after the primordial germinative line is sequestered from the remaining embryo, and differentiated in an independent pathway, it is expected that it would incorporate trisomic cells[58]. Briefly, these embryos may present a trisomy confined to the placenta (confined placental mosaicism) and trisomic cells in their germinative line (gonadal mosaicism)[58,65]. That was supported by a report of an aborted female fetus with a germ cell aneuploidy and placental trisomy of 16 chromosome with no evidence of trisomy in the remaining fetus[51].

Later, when these females reach their reproductive age, they may be at a higher risk of spontaneous abortions due to gonadal mosaicism[51]. They would have a higher risk of trisomic pregnancy losses due to the fertilization of disomic oocytes[58]. Considering that complete skewing was observed in diploid cells from trisomy rescue embryos, gonadal mosaicism can explain skewed XCI in women with trisomic pregnancies[58]. This explanation is supported by the fact that gonadal mosaicism in one parent is the cause of 2% of the 21 trisomy cases and the most accepted explanation for young women with recurrent pregnancies of 21 trisomies.

Another supportive finding is that, according to some studies, the presence of the 21 trisomies in fetuses is frequent in women presenting extreme skewing.[58]

Additionally, aneuploid germ cells may also lead to atresia in primary oocytes and follicles, to a higher degree than diploid cells, and result in an ovarian reserve reduction[51,58,65]. In mice studies, it was observed that the absence of an ovary increases the risk of aneuploid pregnancies and reduces fertility age. Moreover, women with premature ovarian insufficiency are more like to experience pregnancy losses than women in the general population. The relation between a trisomy risk in the fetus and follicular atresia in the mother suggests that extreme skewed patterns may reflect a reduction in the number of follicles during birth, or a growth restriction when the mother herself was an embryo, that affected the oocyte pool[65].

Briefly, a reduction in the total number of primary follicles in the ovary, caused by any mentioned events, will result in higher rates of trisomic oocytes and losses of trisomic pregnancies[51]. Then, it is expected that RPL women who experienced trisomic pregnancies and have extreme skewed XCI, may have a reduced follicle pool. Beever et al. sustained that hypothesis by finding a higher incidence of extreme skewed patterns in RPL women with trisomic losses than in RPL women with no story of trisomic abortions[65]. Following all these

assumptions, the relation between extreme skewed XCI and trisomy risk has been investigated in RPL women[2,44,46].

Additionally, Dorothy Warburton et al. suggested that, as oocyte maintenance in the ovary depends on the presence of two normal X chromosomes, extreme skewed XCI in the blood may be an indicator of X chromosome mutations or chromosome abnormalities that can also lead to a decrease in oocyte pool[44].

Although extreme skewed patterns may not justify RPL in women, they may show a strong genetic influence[51]. Consequently, the study of XCI patterns in women may be an important marker to identify increased risks of aneuploid oocytes, which lead to trisomic pregnancies that can result in recurrent losses [58]. Additionally, a higher frequency of skewing in women experiencing trisomic pregnancies shows that a reduction in embryonic precursor cells, and ovarian reserve, may originate an increased susceptibility for pregnancies with chromosomic abnormalities[65]. Extreme skewed cannot be considered a diagnostic tool but may be highlighted as a trisomy and RPL indicator for future pregnancy outcomes[51,65].

1.4 - Telomere Length

In mammals, chromosome extremities end in hexameric (TTAGGG)n non-coding conserved tandem repeats, responsible for genomic integrity maintenance, known as telomeres[66–68]. Telomeres are composed of a proximal double-stranded and a distal single-stranded DNA region, which are separated, from the remaining chromosome, through a subtelomeric and interstitial region[68], as shown in Figure 8.

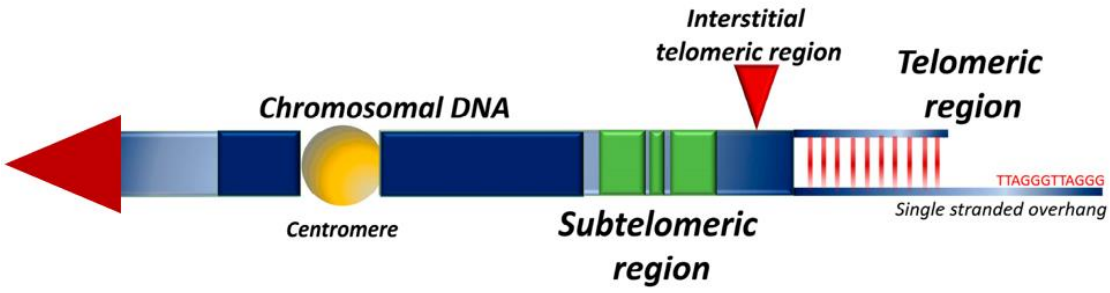

Figure 8 – Representation of telomeric and subtelomeric regions on a chromosome

Schematic representation of chromosomal constituents: centromere (yellow), chromosomal DNA (dark blue), subtelomeric (green), interstitial (red arrow), and telomeric region (represented as a double-stranded followed by a single-stranded DNA (TTAGGG)n). Adapted from [68].

The single-stranded, which is longer, consists of a 50-300 nucleotide guanine-rich (G-rich) tail, while the opposite stranded is cytosine-rich (C-rich), and is responsible for telomere configuration[68–70]. The G-rich tail has de ability to fold back into the double-stranded DNA forming a T-loop structure. Then, the telomere end folds back on itself and the 3' G strand overhand invades the double-stranded DNA forming a D-loop structure[68,69], as shown in Figure 9. Additionally, the 3' end of the G-tail forms G-quadruplexes structures that form hydrogen bonds thought to protect and maintain telomeres integrity[68,69].

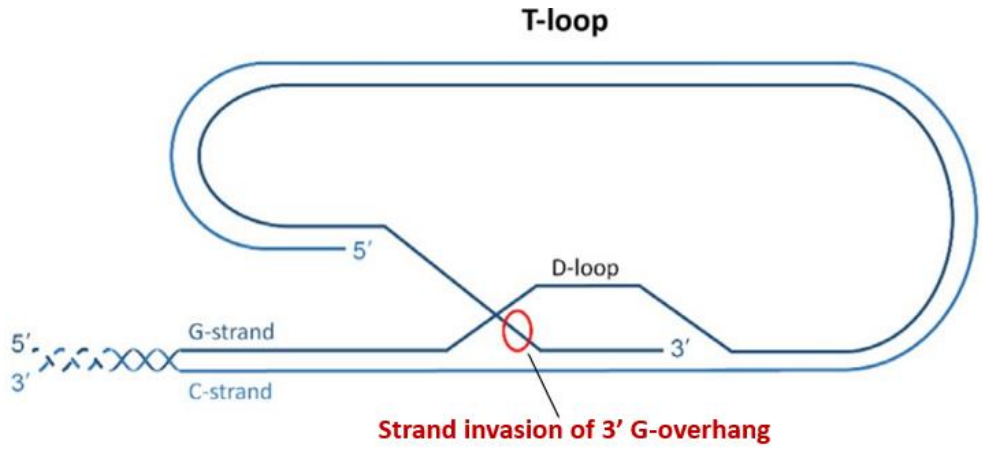

Figure 9 – Telomere configuration

Schematic representation of T-loop and D-loop formation gives rise to telomere organization in the chromosome. Adapted from[70].

Telomeres are enriched by heterochromatic histones, such as H3K9me3, responsible for their structural maintenance[68]. In turn, subtelomeres are regions of genes intermixed with repeat elements that are transcriptionally active[68]. Telomeres are associated with different proteins from which the shelterin complex, also known as telosome, is highlighted[68,69]. The shelterin complex, which has a central role in telomere maintenance, is composed of six proteins subunits: TRF1 (Telomeric-repeat-binding factor 1), TRF2 (Telomeric-repeat-binding factor

2), POT1 (Protection of telomeres 1), TIN2 (TRF1-interacting nuclear protein 2), TPP1 (TIN2-interacting protein) and RAP1 (Repressor and activator protein 1)[67,69]. While TRF1, TRF2, and POT1 bind to DNA, T1N2, TPP1, and RAP1 work as adaptors. Altogether, these proteins work as a dynamic complex protecting telomere through the repression of DNA-damage response signals, homologous and non-homologous recombination, and end joining[67–69,71].

For a full acknowledgment of telomeres, it is crucial to focus on their biological and cellular role and length dynamics. The «end replication problem» is a phenomenon that consists of incomplete replication of chromosomal ends resulting in progressive telomere shortening during each cell division and increasing with age[68,71,72]. This has a protective measure since the loss of a telomeric region is not as prejudicial as the loss of a protein DNA sequence[66,71].

Briefly, the well-known semi-conservative DNA replication process consists of the use of both DNA strands as templates to form new complementary strands[68]. The process starts with the synthesis of a new strand in the 5' to 3' direction, through the action of a DNA polymerase and a single RNA primer, toward the so-called replication fork[68]. Then, this DNA polymerase is replaced by a secondary DNA polymerase which permits the elongation and formation of the leading strand. During this process, multiple primers elongation form Okazaki fragments in the opposite sense leading to the lagging strand[68]. As a normal result of replication, primer degradation origins internal gaps which are filled by an additional DNA polymerase and ligated to form the strand. However, the gap left by the terminal primer is not filled, resulting in a DNA loss at the 5' end of the lagging strand[68]. Consequently, the loss of nucleotides at the end of chromosomes leads to a decrease in the telomeric G-rich strand [68]. Considering that every human telomere has an estimated length of 8 to 15kb, and a loss of 50 to 250 bp per mitosis, it is expected that telomeres may resist for a while that telomere erosion[68].

Nevertheless, over time, this leads to a progressive telomere shortening, due to the DNA polymerase's incapacity to fully replicate telomeres, and results in cellular senescence (irreversible cell cycle arrest)[67,68]. Additionally, Leonard Hayflick observed that healthy human cells stop dividing after a few dozen cycles – Hayflick limit[67,68]. Once chromosomes acquire a critically short length, the shelterin complex dissociates and becomes unable to operate properly. Consequently, the resulting DNA damage makes the cell leave the G1 stage to enter in a G0 stage[66,72]. Furthermore, the accumulation of senescent cells leads to a reduction in mitotically active cells limiting the potential growth and repair of tissue, and accumulation of proteases, inflammatory and growth factors that may affect neighboring cells[66]. Ultimately, it has been proposed that such accumulation may result in tissue and organismal aging[66].

Despite that, in mitotically active cells, such as stem cells, telomere shortening is attenuated through the actuation of the ribonucleic protein - telomerase[68,69]. Telomerase is composed of a catalytic subunit and an RNA component, that acts as a template for *de novo* (TTAGGG) repeats extension on the G-rich strand, telomerase reverse transcriptase (TERT) and telomerase RNA component (TERC), respectively, and other coadjutant factors[67–69]. As an evolutive process, in most adult cells and somatic tissues, telomerase does not have an active role[67,68,71]. Therefore, *TERT* expression is often downregulated in somatic cells in which telomerase activity is stunted[68,69]. Moreover, as its expression and, consequently, its activity decrease with age an inverse relationship between age and telomere length has been frequently reported[71].

Telomere length homeostasis involving telomerase is not completely understood. However, two models have been proposed[68]. The first defends preferential inaccessibility of telomerase in longer telomeres to favor shorter telomeres elongation[68,69]. The second model supports a switch between telomerase-accessible and non-accessible states, with a more frequent telomerase-accessible state switch in shorter telomeres[68].

Telomerase has a highlighted role in cancer since is upregulated in about 85-90% of tumors resulting in telomere elongation and cell proliferation[68–70]. In the remaining 10-15% an alternative lengthening of telomeres (ALT), which is telomerase-independent and based on homologous recombination, takes place[68,71]. When ALT occurs cancer cells usually present different telomeres lengths that can be ultra-long (>50kb) or short (<5kb)[68]. Interestingly, telomerase reactivation is also common in tumors and multiple causative factors have been revealed and are mostly associated with genetic and epigenetic factors[68]. *TERT* (5p15.33) and *TERC* (3q26.3) gene amplification, *TERT* inter-chromosomal translocations, and *TERT* promoter mutations have been associated with some clinical cancer outcomes due to their upregulation expression[68]. Additionally, the hypermethylation of THOR (*TERT* hypermethylated oncological region), which is enriched in CpG sites, can prevent *TERT* repression leading to its upregulation and telomerase activation[68].

In a healthy population telomere length may also vary due to genetics and/or environmental factors[66,68]. A couple of loci and *TERC* and *TERT* single nucleotide polymorphisms have been associated with telomere length. However, more studies need to be addressed to understand the relationship between such findings[68]. Nevertheless, the major factors influencing telomere length are non-genetic and result in heterogenous attrition rates, (rate of gradual telomeres loss), between individuals[68]. Several epidemiological studies showed that lifestyle and environmental factors such as exposure to carcinogens and oxidative and physiological stress may influence

telomere length[68]. It is known that psychological stress results in high levels of cortisol release suppressing telomerase activation[71].

Meanwhile, oxidative stress caused, by an imbalance between antioxidants and reactive oxygen species (ROS), is the major cause of telomere shortening because of the presence of telomeric guanine-enriched structures that are targets of oxidative damage[68,73]. Alternatively, it has also been suggested that ROS may affect *TERT* expression and telomerase function[71]. Additionally, oxidative stress can be induced by ultraviolet and ionizing radiation exposure. Lifestyle also plays a role in oxidative damage since cigarette smoking, alcohol consumption, obesity, and little physical activity have been associated with shorter telomeres[66,68].

Telomere length is usually related to lifespan and longevity in different species[67,71]. Additionally, the higher the average lifespan the slower telomeres shortening occurs[67,72]. In humans, shorter telomeres are observed in people 60 years or older and associated with higher mortality[67]. Interestingly, it was postulated that telomeric attrition is faster in men than women due to higher levels of estrogen that reduce ROS production, [66,71]. On the other hand, testosterone, which does not have antioxidant properties, increases oxidative stress and telomere attrition[71].

Several research studies have been conducted to identify an association between shorter telomeres and some disorders. Nowadays, it is known that shorter telomeres are more frequent in individuals with cardiovascular conditions, like hypertension and atherosclerosis, dementia, diabetes mellitus, and premature aging disorders[6,14,66]. More recently, as will be explained in the next section, it has been hypothesized that shorter telomeres may be a cause underlying RPL.

1.5 – Telomere Length in Recurrent Pregnancy Loss

The association between telomere shortening and reproductive disorders has never been completely explored[73]. Although controversial in the scientific community, telomere length has been associated with couples who experienced RPL, but little is known about telomere erosion in such situations due to limited studies in literature[9,14,73].

It was reported that in both feminine and masculine germ cells, telomeres have the function to help in meiotic recombination and homologous chromosome pairing[73,74]. Moreover,

telomeres are necessary for a correct chromosomal alignment that guarantees the formation of synapsis and chiasmata for crossingover[75]. Following that, it has been proposed that shortened telomeres may result in replicative senescence, impair embryonic and fetal viability and result in a higher incidence of aneuploidies due to chromosome missegregation[5,73].

In studies performed in telomerase-deficient mice, it was observed that telomeres length may have a role in fertilization since shortened telomeres, in both sperm and oocyte, resulted in abnormal cleavage and embryonic development[73]. Evidence also showed that shorter telomeres result in premature aging, impaired oocyte quality, aneuploid arrested, and fragmented embryos when compared with *wild-type* mice[5,73,76]. Following that, as aneuploidies are commonly found in aborted products of RPL, and shorter telomeres may lead to aneuploidies, it has been hypothesized that short telomeres may be a potential cause underlying iRPL[14,73].

Moreover, as mentioned in the previous section, telomere shortening is associated with aging, as a reflection of higher levels of oxidative stress and is considered a potential cause of age-dependent reproductive complications such as pregnancy morbidity and mortality as well as impaired offspring outcomes[5,73]. David L. Keefe et al. proposed that telomere shortening may be involved with reproductive aging in women[75]. In experimental mice studies, in which telomere shortening was genetically or pharmacological induced, shorten telomeres produced oocytes with reduced chiasmata and synapsis leading to abnormal chromosomal alignment and consequently embryonic development arrest[75]. Then, they hypothesized that shortening telomeres in oocytes, due to a chronic ROS exposition during life, may cause reproductive aging in women and, consequently, miscarriages[77]. Moreover, the fertilization of null telomerase eggs, with either null telomerase sperm or wild-type eggs, showed that telomerase is dispensable in early cleavage and embryonic development[77]. Additionally, the authors defended that such shortening may affect egg quality and promote aneuploidy in mammals as above described[77]. Ultimately, shorted telomeres in eggs may predispose to genomic instability, cell cycle arrest, and apoptosis of embryos[77]. This is explained by the fact that in mature oocytes telomerase activity is low and increases only at the blastocyst stage and the interval between the birth of oocytes and ovulation, which in some cases can occur up to 45 years, would increase the exposure to ROS and increase telomere shortening[77].

Although telomeres are restored across generations, telomerase remains inactive in oocytes and embryos until the blastocyst stage[77]. Additionally, the longer the interval between oocyte birth and ovulation the higher the exposure of ROS and, consequently, telomere shortening[77]. In a recent preliminary study, Michaeli et al. compared telomere leukocyte length of women with extended fertility and normal fertility and observed that longer telomeres were prevalent

in the first group and proposed that telomere length may reflect higher oocyte quality and be considered in future studies for pregnancy outcome predictions[6]. Following the same line of thought, some authors support that the loss of ovarian function and menopause in women under 40 years old is associated with shorter telomeres and that late reproduction is associated with women's health and longevity[6,14].

As telomere length is usually used as a biological aging marker it is thought that may be considered an indirect risk factor for aged RPL women[14]. In women, aging is accompanied by increased meiotic and fertility dysfunction (reproductive aging)[75]. As oocytes do not divide but their precursors' oogonia did, it was postulated that the last ovulated oocytes in older women, which later exited during fetal life, may result in shorter telomeres into adulthood[76]. Another hypothesis is that a late exit from the primordial line followed by a prolongated exposure to ROS in the adult ovary may result in shorter oocytes in older women[76]. While most aneuploid oocytes and embryos undergo apoptosis before implantation, resulting in infertility cases, others escape and result in later miscarriages or fetuses with congenital problems[75]. It has been proposed that oocytes may be the locus of reproductive aging that result in meiotic spindle disruption, genomic instability, and cell cycle arrest in the associated embryo.

Additionally, it has also been reported that unsuccessful in vitro fertilization (IVF) cycles and aneuploidies, associated with fertility treatments, were caused by shortened telomere length[76].

As telomeres get shorter during time and oocytes do not increase their telomeric length in early embryogenesis, it was predicted that reduced telomeres may lead to a bad prognosis for the correspondent oocyte and consequent pregnancy[78]. Consequently, this may result in recurrent pregnancy losses or an increased risk of malformations in survival fetuses[73]. However, studies in germ cells are still needed to support these ideas[14,73].

2 – Objectives

This study has the main goal to investigate potential genetic and epigenetic causes underlying Recurrent Pregnancy Losses (RPLs). The research project evaluated the possible existence of a relation between extreme skewed XCI patterns and/or shortened telomeres in RPL women. Considering that, RPL and healthy women were studied, and compared, to analyze if there are:

- An association between extreme skewed XCI patterns and RPL women.
- A relationship between maternal increased age and extreme skewed XCI patterns.
- An association between shortened telomeres and RPL women.
- A relationship between maternal increased age and shortened telomeres.

Ultimately, as around 50% of RPL cases remain idiopathic and represent a stressful condition for couples, this study aims to improve the knowledge of RPL causes and provide new insights into genetic counseling for future pregnancies.

3 – Materials and methods

3.1- Samples collection and characterization

Two groups of women were included in this study: a group of 23 women who experienced recurrent pregnancy losses, considering the ESHRE definition, named the RPL group, and an age-matched control group of 27 healthy women with proven fertility, named the CTRL group. The RPL group was composed of women aged between 23 to 42 years old (average age of 33,7 years old) and the number of abortions varied from 3 to 11(average of abortions per woman of 4,2). In the RPL group, additional information about the maternal and/or fetal karyotype was obtained for a few women, (for all 46,XX or 46,XY for fetus), (see Table 1 in Attachments for more detailed information).

DNA samples were previously extracted from peripheral blood for both RPL and CTRL in the Genetic Unit, Department of Pathology of Medicine of Porto. For all included women the confidentiality of individual data was guaranteed. The present research project was approved by the Ethics Committee of Centro Hospitalar Universitário de São João, Porto (Research Projected - 32/22).

3.2- DNA extraction

DNA extraction was done through an automatic extraction (*PREPITO* Protocol), based on a magnetic particle separation (*chemagic Prepito® Instrument*, Ref 2022-0030, PerkinElmer). Briefly, the separation consists of the use of magnetic rods and automated dispensing buffers into standard plastic devices, resulting in a quick and purified isolation product (DNA). (https://www.perkinelmer.com/lab-solutions/resources/docs/PRD_Prepito_RUO_CT2-10-1701.pdf for more details). After its quantification using the *NanoDrop*™ 2000, DNAs were stored at -20ºC until their use.

3.3- X-chromosome Inactivation Pattern Analysis

The X-chromosome Inactivation Pattern was obtained through the human androgen receptor (HUMARA) assay, a PCR-based methodology. HUMARA assay allows the distinction

between the two XC in a female cell population accessing the percentage of parental XC active cells[79]. As described in the introduction, one of the mechanisms of gene silencing is CpG methylation islands in the gene's promoter. One of the two XC is inactivated in female cells through hypermethylation, and this assay relies on an indirect methodology with methylation sensitivity. The human androgen receptor (*HUMARA*) gene, which is located at Xq11.2, contains a highly polymorphic and commonly heterozygous (CAG)n region on the 5' of the coding gene region that is used for methylation activity comparison of the X chromosome[79]. The protocol consists of four fundamental steps: **1)** Methylation-sensitive restriction endonuclease, *HpaII*, digestion; **2)** Polymerase Chain Reaction (PCR); **3)** Fragments analysis **4)** XCI pattern calculation.

3.3.1 - Methylation-sensitive restriction endonuclease, *HpaII*, digestion

This first step permits the *HpaII* to cleave the recognition DNA site (C^CGG) in the case when the adjacent CpG island is undermethylated, consequently, the digestion only occurs on the active *HUMARA* gene of the active X chromosome[79]. DNA concentrations of 4 ng/µl were considered for the following prepared mix: 11µl of H2O B. Braun, 2 µl Buffer Tango, 2 µl *HpaII* (mix volume = 15 µl) which was distributed and added 5 µl of each DNA sample (4 ng/µl), within a total volume of 20µl per sample. Next, samples were incubated at 37ºC in a thermal cycler for, at least, 12 hours. Additionally, DNA male samples were used as digestion controls as they do not have their X inactive, and consequently methylated, and may be fully digested by *HpaII*.

3.3.2 - Polymerase Chain Reaction (PCR)

After the digestion, digested and non-digested DNA from the same samples were amplified by PCR. At this stage, only undigested regions, which represent methylated regions, amplify effectively[79]. The PCR mix included: 5 µl of H2O B. Braun, 0.5 µl of Forward HUMARA primer (10 µM), 0.5 µl of Reverse HUMARA primer (10 µM) (see Table 2 in Attachments for primers sequences), 10 µl of Type-it enzyme, 3 µl of Q. solution, within a total volume of 19 µl per sample, which was distributed and 1 µl of each DNA sample was added. Samples were

put on a thermal cycler with the following program: (95ºC for 5 minutes), (94ºC for 45 seconds, 62.5ºC for 1 minute, 72ºC for 1 minute) x 30 cycles, (60ºC for 30 minutes), and (4ºC to ∞).

3.3.3 - Fragments Analysis

The capillary electrophoresis was possible with the following prepared mix: 13,7 µl of Formamide and 0,3 µl of Liz600, within a total volume of 14 µl per sample which were distributed through the plate wells, and 1 µl of PCR product added. Then, samples were denatured at 95ºC for 3 minutes in a thermal cycler. Finally, samples were analyzed in the 3500 Genetic Analyzer (Applied Biosystems) within the *Fragment Protocol* program.

3.3.4 - XCI pattern calculation

Through the comparison between the peak areas of the digested and undigested *HUMARA* alleles, it was possible to determine the XCI pattern in the cell population (peripheral blood) of each sample[79]. The XCI pattern is here presented in percentages varying between 50% to 100% and calculated according to the following formula[32] :

$$\frac{\dfrac{\text{Xc1 (digested)}}{\text{Xc1 (undigested)}}}{\dfrac{\text{Xc1 (digested)}}{\text{Xc1 (undigested)}} + \dfrac{\text{Xc2 (digested)}}{\text{Xc2 (undigested)}}} \times 100$$

Xc1 digested – Allele 1 area of the digested X chromosome
Xc1 undigested – Allele 1 area of the undigested X chromosome
Xc2 digested – Allele 2 area of the digested X chromosome
Xc2 undigested – Allele 2 area of the undigested X chromosome

3.4- Telomere Length Analysis

The average telomere length was measured using the *Absolute Human Telomere Length Quantification qPCR Assay Kit* (AHTLQ) by ScienCell™ Research Laboratories. The kit consists of two primer sets one of which targets and amplifies telomere sequences (TLM primer set) and another which amplifies a 100 bp-long region on human chromosome 17 (SCR primer set) used as data normalization. Additionally, it contains a Reference Human genomic DNA sample for telomere length calculation of the target samples (known telomere length of 1.23 ± 0.09 Mb). (https://www.sciencellonline.com/absolute-human-telomere-length-quantification-qpcr-assay-kit.html#product_tabs_description_tabbed for more details).

Two steps were considered using this kit: **1)** qPCR reaction and **2)** Quantification Method.

3.4.1 - Quantitative Polymerase Chain Reaction (qPCR)

Two qPCR reactions were prepared for the reference genomic DNA sample and each DNA sample: one for the TLM mix and another for the SCR mix. The TLM mix was prepared with 2 µl of TLM primer, 10 µl of GoldNstart TaqGreen, and 7 µl of Nuclease-free H2O. Then, a total of 19 µl were distributed by the wells, and 1 µl of the Reference and 1 µl of each DNA sample (2ng/µl) were added to each corresponding well. In its turns, the SCR mix was prepared with 2 µl of SCR primer, 10 µl of GoldNstart TaqGreen, and 7 µl of Nuclease-free H2O. Then, a total of 19 µl were distributed on each well's plate, and 1 µl of the Reference and 1 µl of each DNA sample (2ng/µl) were added to each corresponding well. Additionally, the qPCR program selected included an initial denaturation at 95ºC for 10 minutes (1 cycle) and denaturation at 95ºC for 20 seconds, annealing at 52ºC for 20 seconds, and extension at 72ºC for 45 seconds (32 cycles). The experiment was performed using duplicates to obtain more reliable results.

3.4.2 - Quantification Method

The average telomere length was obtained for each sample through a Comparative ΔΔCq (Quantification Cycle Value) Method, using the following formula:

1. For telomere (TEL), ΔCq (TEL) is the quantification cycle number difference of TEL between the target and the reference genomic DNA samples.

   ΔCq (TEL) = Cq (TEL, target sample) - Cq (TEL, reference sample)

2. For single copy reference (SCR), ΔCq (SCR) is the quantification cycle number difference of SCR between the target and the reference genomic DNA samples.

   ΔCq (SCR) = Cq (SCR, target sample) - Cq (SCR, reference sample)

3. ΔΔCq = ΔCq (TEL) - ΔCq (SCR)
4. Relative telomere length of the target sample to the reference sample (fold) = $2^{-\Delta\Delta Cq}$
5. The total telomere length of the target sample

   = Reference sample telomere length x $2^{-\Delta\Delta Cq}$

Finally, considering that there are 92 chromosome ends in one diploid cell, the telomere length given by the previous formula was divided by 92 and presented in kb per chromosome end, as described and suggested in the protocol.

3.5- Statistical Analysis

Statistical analysis was performed using the IBM SPSS Statistics Software version 27. Data related to the XCI pattern was analyzed through a Fisher's Exact test to compare the proportion of extreme skewed XCI between RPL and CTRL groups and Pearson's correlation test was performed to analyze a possible relationship between increased maternal age and an increase in skewed XCI patterns in both groups. For telomere length data, the normality distribution was first analyzed through the Kolmogorov-Smirnov test, the means between RPL and CTRL groups were compared through Student's t-test and, finally, a Pearson's correlation test was performed to analyze a possible relationship between increased maternal age and shortened telomeres in both groups. During the data analysis, results were considered statistically significant at $p<0,05$.

4 - Results

4.1 - XCI pattern

4.1.1- Extreme skewed XCI in RPL and CTRL group

Although there is no consensus about which cut-off must be considered as an extreme skewed XCI pattern influencing RPL women[58,60], in this study the comparison between groups was performed using two different cut-offs ≥85 % and ≥ 90%, which are the most considered in the scientific community. One of the 23 women in the RPL group and five of the 27 women in the CTRL group were not included in the analyses because were found to be homozygous for the *HUMARA* gene and then, non-informative, as it is not possible a distinction between peaks and, consequently, perform XCI pattern calculation.

Considering a cut-off of ≥85 % for extreme skewed XCI, in the RPL group 17 of 22 (77,3%) women presented an XCI pattern varying between 50 to 85%, and 5 of 22 (22,7%) women presented an extreme skewed XCI pattern varying between 85 to 100%. In its turn, in the CTRL group, 20 of 22 (90,9%) women have an XCI pattern varying between 50 to 85%, and 2 of 22 (9,1%) women present an extreme skewed XCI pattern varying between 85 to 100%. Although, no statistically significant differences, between groups (p=0,41), were observed when comparing the frequency of extreme skewed patterns, (Table 1).

Table 1 - Frequency of XCI pattern in RPL and CTRL group (cut-off ≥ 85 %)

		XCI pattern (%)	
		[50,85[	[85,100]
Group of study	RPL	17/22 (77,3%)	5/22 (22,7%) *
	CTRL	20/22 (90,9%)	2/22 (9,1%)

**p=0,41*, Fisher's Exact test when comparing RPL and CTRL groups

Considering an extreme skewed XCI as a cut-off of ≥90 %, in the RPL group 20 of 22 (90,9%) women presented an XCI pattern varying between 50 to 90% and 2 of 22 (9,1%) women presented an extreme skewed XCI pattern varying between 90 to 100%, exactly as in

the CTRL group. Then, no significant differences, between groups (p=1,00), were observed when comparing the frequency of extreme skewed patterns, (Table 2).

Table 2 - Frequency of XCI pattern in RPL and CTRL group (cut-off ≥ 90 %)

		XCI pattern (%)		
		[50,90[	[90,100]	
Group of study	RPL	20/22 (90,9%)	2/22 (9,1%)	*
	CTRL	20/22 (90,9%)	2/22 (9,1%)	

*$p=1,00$, Fisher's Exact test when comparing RPL and CTRL group

4.1.2 - Effect of maternal age on extreme XCI pattern

- RPL Group

When a possible correlation between increased maternal age and an increase in extreme skewed patterns was statistically analyzed, in the RPL group, a negative correlation (correlation coefficient=-0,52; p=0,01) was observed between maternal age and XCI pattern, (Figure 10).

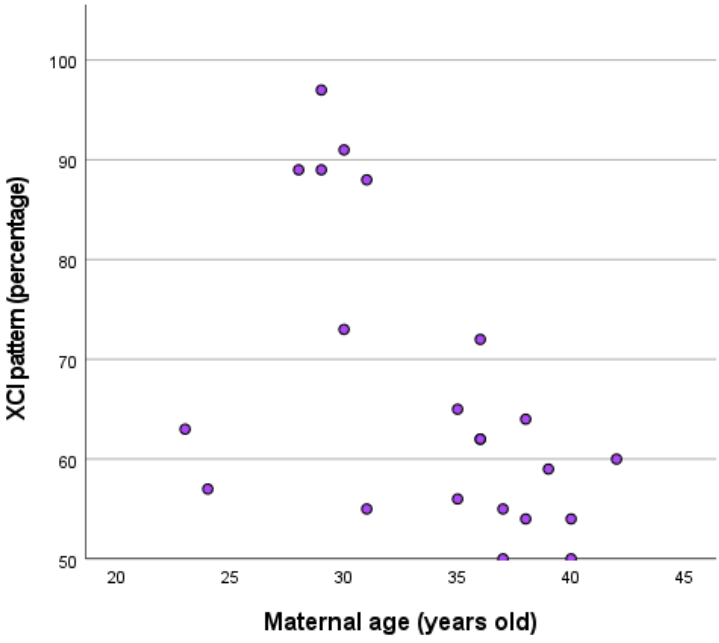

Figure 10 - Association between XCI pattern (percentage) and maternal age (years old) in the RPL group

- CTRL group

When a possible correlation between increased maternal age and an increase in extreme skewed patterns was statistically analyzed, in the CTRL group, no statistically significant correlation (coefficient correlation=0,04; p=0,86) was observed between maternal age and XCI pattern, (Figure 11).

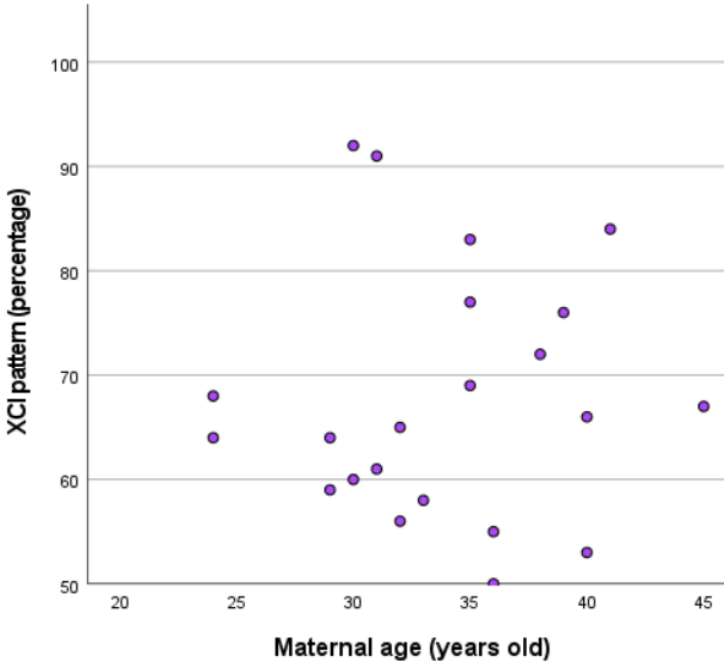

Figure 11 - Association between XCI pattern (percentage) and maternal age (years old) in the CTRL group

4.2 - Telomere Length

4.2.1 - Telomere Length in RPL and CTRL women

When comparing the mean telomere length in the RPL group (7,24 ± 2,57 kb per chromosome end) and the mean telomere length in the CTRL group (7,37 ± 1,94 kb per chromosome end), no statistically significant differences (p=0,85) were observed, (Figure 12).

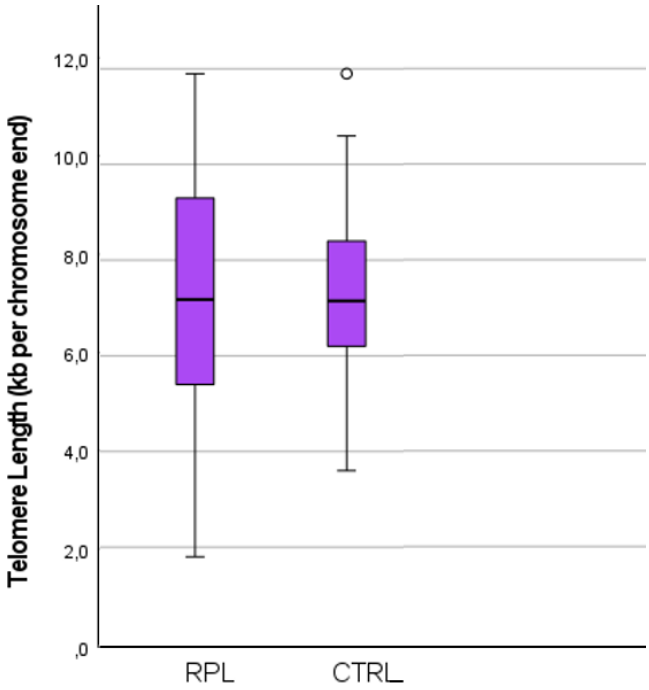

Figure 12 - Comparison of Telomere Length (kb per chromosome end) in the RPL and CTRL group

4.2.2 - Effect of maternal age on Telomere Length

- RPL group

When a possible correlation between increased maternal age and a decrease in telomere length was statistically analyzed, in the RPL group, no statistically significant correlation (correlation coefficient=0,09; p=0,69) was observed between maternal age and telomere length, (Figure 13).

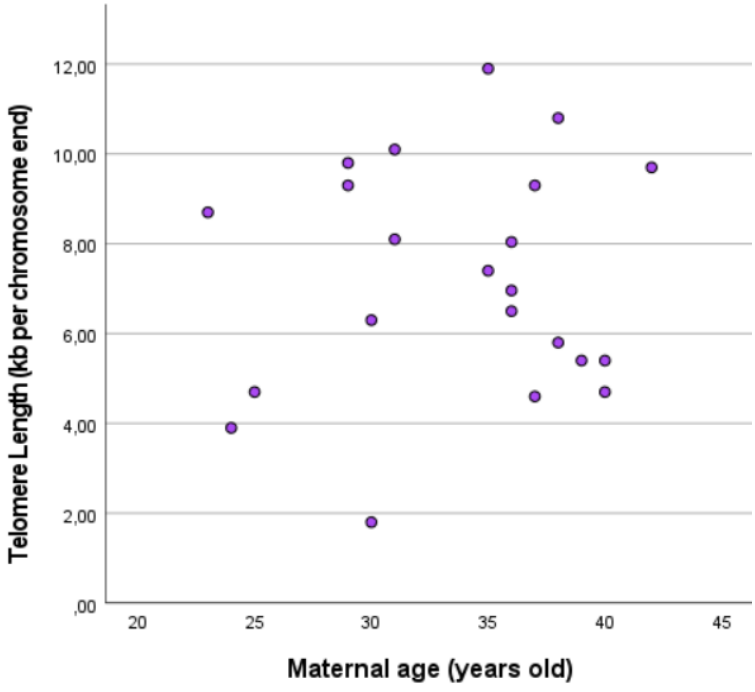

Figure 13 - Association between Telomere Length (kb per chromosome end) and Maternal age (years old) in the RPL group

- CTRL group

When a possible correlation between increased maternal age and a decrease in telomere length was statistically analyzed, in the CTRL group, no statistically significant correlation (correlation coefficient=0,28; p=0,21) was observed between maternal age and telomere length, (Figure 14).

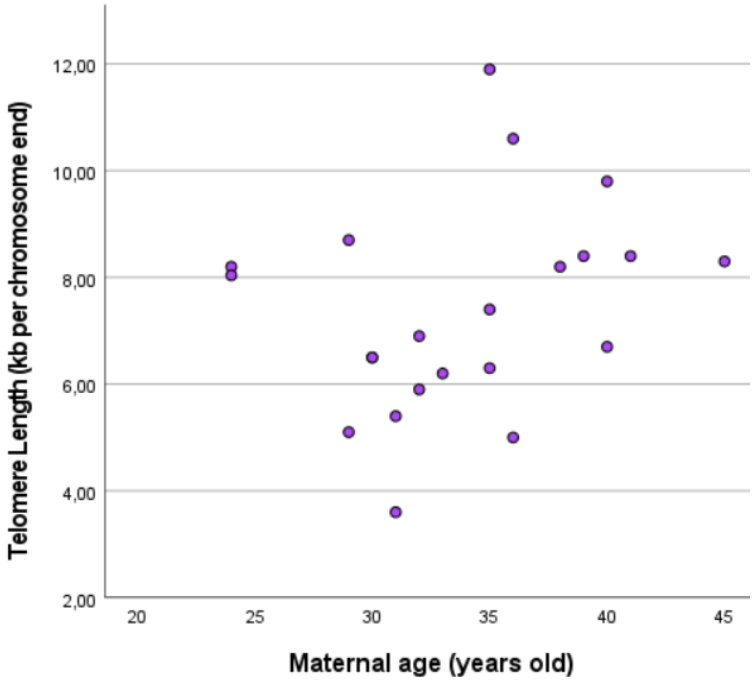

Figure 14 - Association between Telomere Length (kb per chromosome end) and Maternal age (years old) in the CTRL group

5 - Discussion

As previously mentioned, the hypotheses explored in this study are not consensual among scientists. While some results, using *HUMARA* assay, age-matched controls, and peripheral blood as the preferential tissue analyzed, support a strong association between an extreme skewed XCI and RPL women, others did not. In our study, for both cut-offs of ≥85% and ≥90% of extreme skewed XCI patterns, no differences were observed between groups, which suggests no association between extreme skewed XCI and RPL women. However, the percentage of extreme skewing found in the CTRL group, for both cut-offs, is according to the literature, since it has been estimated that 1 to 23% of the female general population present a skewed pattern caused by chance or choice[52].

Both associations of X-linked male lethal mutations, leading to a higher lethality of male embryos, and a higher risk of trisomy with extreme skewed XCI patterns[51,65] in RPL women, could not be analyzed in this study because this was a retrospective study and clinical information about all pregnancy losses were incomplete in RPL group, namely information of fetal karyotype which would indicate the fetal sex and possible existence of chromosomal abnormalities. According to the Hospital protocol, a fetal karyotype study should be performed only after two spontaneous abortions which makes impossible to have information about fetal chromosome complement for all the pregnancies (see Table 1 in Attachments).

A correlation between maternal increased age and extreme skewed XCI patterns has also been proposed in the literature as was addressed in detail above in this study. Therefore, we tested this possible correlation for both groups, and a negative and no correlation was found in the RPL and CTRL groups, respectively. These results might be caused, in both cases, by the reduced sample size that may affect the statistical outcome. Moreover, as a relatively young population of females was analyzed in both groups and considering that higher differences associated with age were reported in older women, the age effect must be masked and not as obviously seen as though[50,58].

In addition, there are some authors who question the HUMARA assay since they do not consider that the methylation of the *AR* locus may always reflect a correct interpretation of the X chromosome inactivation state[80]. However, despite that, it continues to be considered the most cost-effective, reliable, and routinely performed assay[58]. Nevertheless, according to some authors, other methodologies may be tested and compared with HUMARA assay results to ensure a better assumption of the XCI pattern in women[81]. In future approaches, quantitative techniques described by Sabina et al. as significantly reliable and accurate, such as

Quantification of SNPs by pyrosequencing, may also be tested and compared with HUMARA assay [80].

The inclusion of new loci must also be weighted. As an example, access to the methylation status of the *FMR1* gene has been mentioned as a good mirror of the XCI pattern obtain through the *AR* gene methylation status and may overcome unsolved cases due to homozygous *AR* gene[81].

Additionally, it must be noted that our analysis was performed in blood peripheral samples which may not faithfully translate what happens in the germline, in some cases, especially considering that the XCI varies between tissues[50]. Despite that, it is considered the most accessible and reliable studied tissue and representative of such phenomenon in individuals.

Considering that no telomere length differences were found between RPL and CTRL groups, this study does not suggest an association between shortened telomeres and RPL women. However, some aspects must be discussed, especially because the telomere length results presented are an average of the telomere length population tissue of each sample and may not reflect the erosion rate of each single chromosome which may vary according to different previously mentioned factors in the introduction[71]. Then, possible differences between women and groups may be smoothed.

This approach has some well-defined advantages, especially considering that is easy to conduct, needs a small amount of DNA for analysis, and is widely used in comparison population studies[82]. A comparison between the qPCR assay and other methodologies, for example *Terminal Restriction Fragment analysis*, and the use of the *Single Telomere Length Analysis technique* (STELA), which allows the telomeres measurement of individual chromosomes[82], might also be considered in future analysis.

It is also important to note that in this study only the telomere length of RPL women was considered and shortened telomere length in men of RPL couples has also been documented and associated with RPL cases[9].

A possible relation between shortened telomeres and replicative senescence, causing impaired embryonic and fetal viability and resulting in a higher incidence of aneuploidies due to chromosome missegregation, underlying the RPL condition in women could not be analyzed due to lack of clinical information. Moreover, little has been explored in the scientific community about telomere shortening and reproductive disorders[73].

The hypothesis of a decrease in telomere length with increased maternal age was also tested but no correlation was observed in both the RPL and CTRL groups. As predicted for the XCI pattern, this may be caused by a reduced sample size which may affect the statistical outcome

and relieve a possible relation between such variables or comparison between groups of women with a reduced age interval difference.

In addition, it must also be highlighted that the results obtained were using peripheral blood samples which may not reflect what happens in the germline as previously mentioned for XCI pattern. Thus, the possibility of carrying out this study in other tissues such as ovaries samples, considering that RPL is a reproductive disorder, would also be interesting to explore.

Briefly, according to our results, it is not possible to suggest that an extreme XCI pattern and/or shortened telomeres are responsible for or may predispose to RPL condition in the present studied RPL women group. Nonetheless, this work represents one more valid contribution, to uncover the cause underlying RPL. In future studies, a more enriched data assessment, including more cases (RPL and CTRL groups), different tissues, and new strategies may provide more knowledge and new evidence.

6 - Conclusion

Although this study's results do not support the hypothesis of either, an association between extreme skewed XCI or shortened telomeres with RPL women, considering the above-explained evidence, such propositions should not be discarded as potential causes underlying Recurrent Pregnancy Loss.

Moreover, considering the high number of iRPL cases, which was several times mentioned as a stressful condition, for both couples and physicians, further investigations need to be addressed and other variables need to be considered for new potential risk factors and/or causes identification.

Considering its clinical relevance, the necessity of continuous scientific community investment in the matter is unquestionable.

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Attachments

Table 1 - RPL women and respective aborted products known information

Sample ID	Maternal age	Pregnancy Losses number	Maternal karyotype	Fetal karyotype
1	23	5	unknown	46,XX (F); 45,XO(F); 3 unknown
2	24	4	unknown	46,XX (F); 3 unknown
3	28	3	unknown	46,XY (M); 46,XX (F); 1 unknown
4	29	6	unknown	46, XY (F); 5 unknown
5	29	3	unknown	46,XY (M); 46,XX (F); 46,XX (F)
6	30	3	46,XX	46,XX (F); 2 unknown
7	30	7	unknown	46,XY (M); 46,XX (F); 46,XX (F); 4 unknown
8	31	4	46,XX	46,XX (F); 3 unknown
9	31	3	46,XX	46,XX (F); 2 unknown
10	32	3	unknown	46,XX (F); 2 unknown
11	35	3	unknown	47,XY+13 (M); 2 unknown
12	35	3	46,XX	46,XX (F); 47,XY+21 (M); 1 unknown
13	36	4	unknown	46,XX (F); 3 unknown
14	36	3	unknown	46,XX (F); 48,XX,+18+21 (F); 69,XXY(M)
15	36	3	46,XX	46,XX (F) 46,XX(F); 1 unknown
16	37	11	46,XX	46,XY (M); 10 unknown
17	37	3	unknown	46,XY (M); 2 unknown
18	38	4	46,XX	4 unknown
19	38	3	46,XX	46,XY (M); 2 unknown
20	39	9	unknown	46,XY (M); 8 unknown
21	40	3	unknown	46,XX (F); 2 unknown
22	40	3	46,XX	46,XX (F); 2 unknown
23	42	4	46,XX	46,XX (F); 3 unknown

Table 2 - HUMARA primer sequences

Forward	5´ TCC AGA ATC TGT TCC AGA GCG TGC 3´
Reverse	5´ GCT GTG AAG GTT GCT GTT CCT CAT 3´