Skewed X-chromosome inactivation and shorter telomeres associate with idiopathic premature ovarian insufficiency

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Objective: To analyze whether telomere length, X-chromosome inactivation (XCI), and androgen receptor (*AR*) GAG polymorphism are related to idiopathic premature ovarian insufficiency (POI).
Design: Case–control study.
Setting: University hospital.
Patient(s): A total of 121 women, including 46 nonsyndromic POI and 75 controls.
Intervention(s): None.
Main Outcome Measure(s): Age, weight, height, body mass index (BMI), systolic and diastolic arterial pressure, E_2, androstenedione, T, and C-reactive protein were assessed. Telomere length was estimated by quantitative real-time polymerase chain reaction, XCI was measured using the Human Androgen Receptor and X-linked retinitis pigmentosa 2 (*RP2*) methylation assays. *AR* and *FMR1* polymorphism was assessed by quantitative fluorescent polymerase chain reaction and sequencing.
Result(s): Premature ovarian insufficiency women had a higher mean age, weighed less, and exhibited lower C-reactive protein, E_2, and androstenedione levels. The *AR* polymorphism did not differ between the groups. Four patients had premutation (55–200 CGG repeats), and none displayed a full mutation in the *FMR1* gene. However, patients with POI showed shorter telomere length and higher frequency of skewed XCI. Extreme skewing ($\geq$90%) was observed in 15% of women with POI, and shorter telomeres correlated with XCI skewing in both groups.
Conclusion(s): Skewed XCI and shortened telomere length were associated with idiopathic POI, despite no alterations in the *AR* and *FMR1* genes. Additionally, there is a tendency for women with short telomeres to exhibit skewed XCI. (Fertil Steril® 2018;110:476–85. ©2018 by American Society for Reproductive Medicine.)
El resumen está disponible en Español al final del artículo.

Key Words: Anovulation, epigenetic mechanisms, DNA methylation, trinucleotide repeats, genomic instability

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Premature ovarian insufficiency (POI), also known as premature ovarian failure, is a common cause of female infertility, affecting approximately 1% of women of reproductive age. Premature ovarian insufficiency is defined as the premature cessation of ovarian function before 40 years of age. Primary or secondary amenorrhea, elevated serum gonadotropins, such as FSH levels >40 IU/L, and hypoestrogenism, also referred to as hypergonadotropic hypogonadism, are among the main characteristics of the disease (1, 2). In most cases the etiology of spontaneous POI is unknown, but there is evidence suggesting a genetic component in idiopathic ovarian insufficiency (2). X-chromosome-linked abnormalities have been reported in POI (3), including, for example, the expansion of trinucleotide repeats in genes such as fragile X mental retardation 1 (*FMR1*) (4).

The androgen receptor (*AR*) gene, located in the Xq12 region, contains a repetitive (CAG)n polymorphism that is associated with ovarian function and folliculogenesis (5). The expression of these repetitive sequences remains under epigenetic control, and the allele containing the expansion is often silenced through hypermethylation of the repeat and its regulatory regions (6, 7). In the inactive X chromosome, the CAG repeat in the exon 1 region of the *AR* gene is methylated and leads to the silencing of this gene. The methylation pattern of *AR* is the primary marker used to identify X-chromosome inactivation (XCI). Several epigenetic modifications are responsible for the inactivation of one of the two X chromosomes (X_i), resulting in the silencing of the majority of genes on X_i. Deoxyribonucleic acid methylation also plays an important role in the maintenance of this inactive state. X-chromosome inactivation is a random process, with an average 50:50 ratio of cells expressing either the maternal or paternal chromosome (8, 9). Nonrandom (or skewed) XCI is rare and related to X-linked disorders (10).

Mechanistically, the exhaustion of the ovarian reserve and subsequent reduction in female fertility may be due to abnormalities acquired over successive cell cycles, leading to oocyte senescence, which is a defining feature of POI (11). Decreased fertility in women with POI may be attributable to the depletion of the primordial follicular pool, resulting in a premature reproductive aging phenotype (3). The loss of the cellular proliferative capacity is a natural consequence of aging and is accompanied by genomic instability and telomere attrition. Telomere erosion is associated with reproductive senescence in women (12), which typically occurs after 40 years of age. This is mainly due to a reduction in the size and quality of the oocyte/follicle pool and the consequent loss of fertility (12–15). Indeed, the meiotic dysfunction that follows telomere loss reduces oocyte developmental competence and quality (14). Liu et al. (13) demonstrated that female germ cells do not undergo apoptosis after telomere shortening but are arrested in early meiosis and become aneuploid, suggesting that telomere shortening is associated with poor oocyte quality.

The intricate network of genomic interactions controlling the gonadal development provides the basis for understanding a complex disease such as POI (16). It is possible that several X-linked epigenetic markers, such as nonrandom X-inactivation or cryptic mosaicism (45,X/46,XX), *AR* (CAG)n expansion, or telomere dysfunction, may be related to oocyte senescence and loss of fertility in women with POI. In this context we sought to evaluate the association between telomere length, *AR* repetitive CAG polymorphism, skewed XCI, and the development of idiopathic ovarian insufficiency.

MATERIALS AND METHODS

Participants

The protocols were approved by the research ethics committee of the University Hospital (institutional review board) of Ribeirao Preto Medical School, University of São Paulo (protocol number 13305/2012) and ratified by the other participating institutions. All participants provided written, informed consent.

This was a prospective, case–control study recruiting consecutive patients from 2012 to 2016. Participants with POI (n = 46) were recruited at three academic medical centers in southeastern Brazil, including the Human Reproduction Division of the Department of Gynecology and Obstetrics of the Ribeirao Preto Medical School, University of São Paulo (FMRP-USP) (n = 23) and the Gynecology and Obstetrics Department of the University of Campinas (n = 13) and Federal University of Minas Gerais (n = 10).

Women aged 18–41 years, regardless of race, social status, or parity, were eligible. The idiopathic POI group (n = 46) included women with a history of amenorrhea and two serum FSH results >40 IU/L obtained before 40 years of age. At the time of the study the absolute majority of POI patients were receiving hormone therapy with oral or percutaneous E_2 and oral progestogen, and none was on androgen therapy. A 46,XX karyotype was required for women younger than 30 years. The control group (n = 73) was recruited at the University Hospital, FMRP-USP and included women with regular menstrual cycles of 24–38 days and a typical duration of 3–7 days, with no history of anovulation and FSH level <10 IU/L. The exclusion criteria were tobacco smoking, pregnancy or lactation, and a history of any other endocrine disorder. Syndromic POI cases (such as Turner's and Fragile X syndrome) and patients with autoimmune disorders, a history of radio- or chemotherapy, ovarian surgery, and chromosome abnormalities were excluded from the study.

Biochemical Measurements

Clinical and anthropometric characteristics were assessed during the study, including age, weight, height, body mass index (BMI), and systolic and diastolic blood pressure. The serum concentrations of E_2, luteinizing hormone (LH), follicle stimulating hormone (FSH), and C-reactive protein (CRP) were measured using chemiluminescence assays (IMMULITE 2000 Immunoassay System, Siemens Healthcare Diagnostics), whereas serum testosterone (T) and androstenedione (A) concentrations were measured using radioimmunoassays (Immulite 1000, Siemens Healthcare Diagnostics).

Genomic DNA Isolation

Human genomic DNA was extracted from peripheral blood leukocytes from all subjects. Briefly, the red cells were lysed, and the leucocytes were separated by centrifugation. Then the leukocytes were washed twice, and the pellet was resuspended in phosphate buffered saline. Genomic DNA was isolated using QIAamp DNA Microkits (Qiagen), according to the manufacturer's instructions. Deoxyribonucleic acid concentration and integrity were determined using a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific). All cases and an equal number of controls (n = 46) with good DNA yield were included in the sequencing and genotyping analyses.

FMR1 Analysis

The expansion of the CGG trinucleotide repeat sequences in the 5′ untranslated region of the *FMR1* (NM_002024.4) gene was assessed in the POI group using the *AmplideX FMR1* polymerase chain reaction (PCR) kit (Asuragen), according to the manufacturer's instructions. The electrophoretic profiles and areas under the curves for each allele were determined by capillary electrophoresis using an ABI Prism 3500 Series Genetic Analyzer (Applied Biosystems). This kit allows accurate detection of repeats for normal (<45 CGG), intermediate (45–54 CGG), premutated (55–200 CGG), and fully mutated (>200 CGG) alleles. The *FMR1* analysis was performed in the Chromosome Genomic Medicine facility (São Paulo, SP, Brazil).

X-Chromosome Inactivation Pattern

X-chromosome inactivation patterns was assessed according to the allele-specific DNA methylation of the *AR* exonic CAG repeat (Human Androgen Receptor [HUMARA] assay) and *RP2*-extragenic GAAA repeats, using a 5^{me}CpG-sensitive restriction endonuclease-based PCR assay (17, 18). Genomic DNA (500 ng) was digested with *Hpa*II (New England Biolabs) or mock-digested without restriction enzyme, according to the manufacturer's instructions, in a final volume of 10 μL.

Deoxyribonucleic acid genotyping was performed by capillary electrophoresis using quantitative fluorescence polymerase chain biplex reactions (QF-PCR) in approximately 50 ng of digested or mock-digested DNA. The following primers, as previously described (17, 18), were used: *AR forward* 5′-GTGCGCGAAGTGATCCAGAA-3′; *AR reverse* 5′-CCAGGACC AGGTAGCCTGTG-3′; *RP2 forward* 5′-TGACATAGCGAGACC CTGTG-3′; and *RP2* reverse 5′-GTGGTGGGTTCTCTAGCTGG-3′, in which the *forward* primer was fluorescently labeled. The amplification reaction was carried out using primer concentrations of 0.8 mM (*AR*) and 1.2 mM (*RP2*) with AmpliTaq Gold DNA Polymerase 1U (Thermo Fisher Scientific), according to Machado et al. (18). The thermal cycling conditions were 95°C for 11 minutes (1 cycle); 94°C for 1 minute, 59°C for 1 minute, and 72°C for 1 minute (30 cycles); and 60°C for 60 minute (1 cycle).

The electrophoretic allele profile and areas under the curves for each allele were determined with an ABI Prism 3130 Genetic Analyzer (Thermo Fisher Scientific) and analyzed with GeneMapper software (Thermo Fisher Scientific). Fluorescent peak areas representing true alleles were normalized for the occurrence of stutter products according to Busque et al. (19). The XCI percentage was obtained from the frequency of the inactive and active X-chromosome (Xi/Xa), determined according to the methylation status at the RP2 GAAA onshore and the *AR* CAG repeat loci across women with varying extents of random and nonrandom XCI (skewed). Skewed XCI presents a frequency ≥75% for a Xa and Xi, and extreme skewing presents a frequency ≥90% for XCI (19).

Androgen Receptor (CAG)n Repeat Polymorphism

The number of (CAG)n triplets repeats in exon 1 of the *AR* gene expressed as base pairs (bp) was characterized by QF-PCR and genomic DNA sequencing. Three women who were homozygous for *AR* alleles in our previous XCI analysis were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) on an ABI Prism 3130 Genetic Analyzer (Thermo Fisher Scientific), according to the manufacturer's recommendations. Nucleotide sequences were analyzed using BioEdit Sequence Alignment software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The number of CAG repeats was calculated by combining the repeat length (determined by genomic DNA sequencing) and the electrophoretic allele size (determined using QF-PCR). Linear regression was performed between the three sequenced samples, as suggested by the *FMR1* protocol. The intercept point of the linear regression line corresponded to the correction factor (c_0), whereas the slope represented the mobility factor (m_0). The size of each peak was converted to repeat CAG length (CAGi) using the following equation: $CAG_i = (Peak_i - C_O)/m_O$, where $Peak_i$ is the size in base pairs of a given product peak; C_O is the size correction factor; and m_O is the mobility correction factor for each CGG repeat.

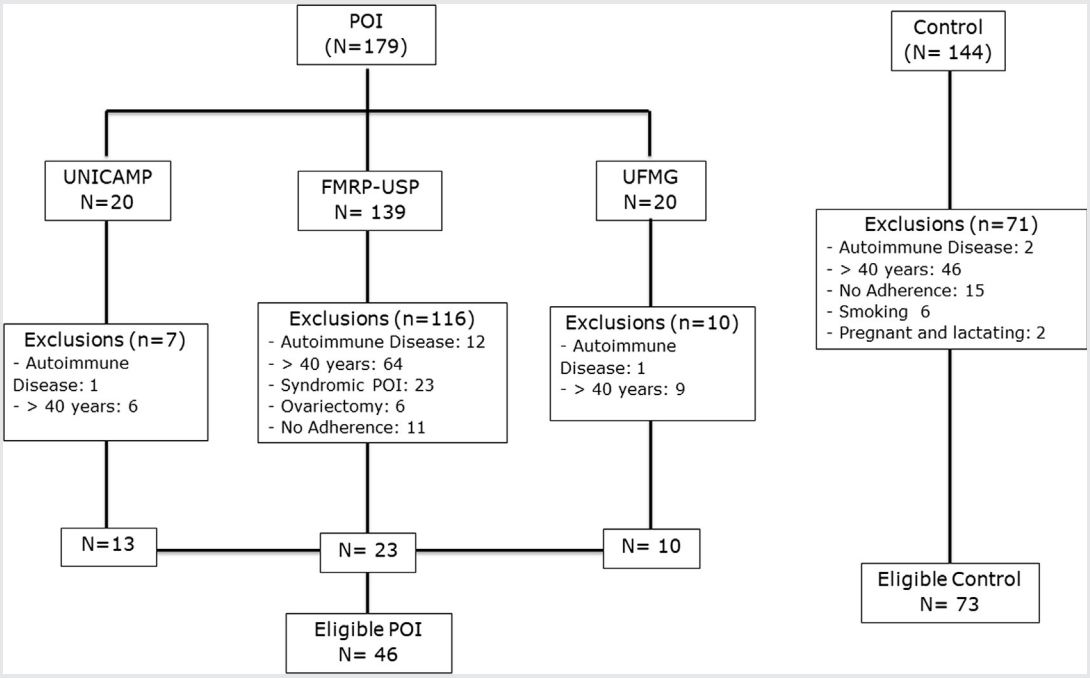
The CAG repeat number was represented in three different ways: the shorter allele (allele 1) alone, the longer allele (allele 2) alone, and the mean value of the two alleles (biallelic mean). The terms "shorter" and "longer" were relative for each individual and do not represent an absolute CAG repeat number.

Telomere Length Measurement

Telomere length was assessed by quantitative PCR as previously described (20–22). This method is highly sensitive and specific when compared with Southern blot and is able to distinguish short telomeres (23). We used the following primer sequences: T-Fw 5′-CGGTTTGTTTGGGTTTGGGT TTGGGTTTGGGTTTGGGTT-3′ and T-Rv 5′-GGCTTGCCTTA CCCTTACCCTTACCCTTACCCTTACCCT-3′; S-Fw 5′-CAGCA AGTGGGAAGGTGTAATCC-3′ and S-Rv 5′-CCCATTCTATCA TCAACGGGTACAA-3′.

Each sample was assayed in triplicate with Rotor-Gene SYBR Green PCR Master Mix (Qiagen). Telomere length was determined by calculating the telomere to single copy gene ratio (T/S ratio) using ΔCt [Ct(telomere)/Ct(single gene)]. The T/S ratio of each sample (x) was normalized relative to the

FIGURE 1



Flow chart of the study. FMRP-USP = Ribeirao Preto Medical School, University of São Paulo; POI = premature ovarian insufficiency; UFMG = Federal University of Minas Gerais; UNICAMP = University of Campinas.

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mean T/S ratio of the reference sample [2 − (Δctx − Δctr) = 2 − ΔΔct], which was used to construct standard curves, both as a reference and a validation sample.

Statistical Analysis

The sample size for the study was calculated from data previously published by Chatterjee et al. (24) that identified a difference of 3.5 bp in *AR* GAG repeat length between women with POI and controls. This suggested that it was necessary to include 40 participants per group to detect such a difference with 90% statistical power and a level of significance of 0.05. We fulfilled this number in both groups and further expanded the control group to increase the precision of other quantitative estimates. Quantitative variables were summarized as the mean and SD, whereas qualitative variables were described as absolute and relative frequencies. Nonparametric Mann-Whitney tests were used to compare quantitative variables between the POI and control groups. Chi-square test was used to investigate the association between POI and XCI. The general linear mixed model was used to compare the telomere length between groups (POI and control) and to determine the effects of possible confounding variables, such as age, BMI, or levels of E_2, CRP, and A. The final model was $y = \alpha + \beta 1X1 + \beta 2X2 + \beta 3X3 + \beta 4X4 + \beta 5X5 + \beta 6X6 + \varepsilon$, in which y is the estimated response, α is the intercept, $\beta 1$, $\beta 2$, $\beta 3$, $\beta 4$, and $\beta 5$ correspond to the coefficient of the regression model associated with the variables X1 (group), X2 (age), X3 (BMI), X4 (E_2), X5 (CRP), and X6 (A), and ε is the error associated with the estimate.

A logistic regression model was used to determine the effects of possible confounding variables on the XCI pattern, such as group (POI and control), age, BMI, E_2, CRP, and A. Spearman's correlation coefficients were used to correlate the quantitative variables (age, BMI, E_2, A, and CRP) with telomere length in the POI and control groups. All calculations were performed using SAS 9.0 software (SAS Institute), with a level of significance of 5% ($P<.05$).

RESULTS

Clinical Characteristics and Biochemical Measurements

The study design is shown in Figure 1. The study included 46 patients with POI and 73 healthy subjects. The mean FSH serum levels were 46.50 ± 41.45 IU/L in the POI group and 4.08 ± 2.74 IU/L in the control group. The mean LH serum concentrations were 23.38 ± 21.82 IU/L in the women with POI and 3.75 ± 2.56 IU/L in the controls.

The physical and hormonal features of the study participants are shown in Table 1. The mean age was 32.1 years in the POI group and 28.8 years in the control group, whereas the mean body weight was 64.5 kg in the POI group and 73.0 kg in the control group. No differences in height or BMI were observed. Biochemically, the serum concentrations of the inflammatory biomarker CRP ($P<.0001$), A ($P=.0001$), and E_2 ($P=.0006$) serum concentrations were lower in women with POI than in controls. Other variables were not significantly different between groups.

TABLE 1

Quantitative variables measured in the POI and control groups

Variable	POI (n = 46), mean (SD)	Control (n = 73), mean (SD)	*P* value
Age (y)	32.10 (6.81)	28.76 (4.98)	.0010[a]
Weight (kg)	64.54 (12.73)	72.99 (18.59)	.0328[a]
Height (m)	1.59 (0.07)	1.61 (0.06)	.1326
BMI (kg/m^2)	25.41 (4.64)	27.11 (7.58)	.2769
SBP (mm Hg)	116.06 (12.96)	114.16 (8.42)	.4773
DBP (mm Hg)	74.97 (9.36)	76.41 (8.74)	.4430
E_2 (pg/mL)	88.56 (133.35)	112.48 (84.11)	.0006[a]
CRP (mg/L)	0.93 (2.2)	3.88 (3.91)	<.0001[a]
T (dg/dL)	58.89 (45.13)	61.95 (27.99)	.0969
A (dg/dL)	75.0 (61.85)	112.57 (53.85)	.0001[a]

Note: DBP = diastolic blood pressure; SBP = systolic blood pressure.
[a] *P*< .05.

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FMR1 Allele Investigation

Polymorphic alterations in the *FMR1* gene were assessed only in the POI group to evaluate fragile X syndrome-related mutations. Among 46 patients, 4 showed premutation (55–200 CGG repeats) in the *FMR1* gene. A fully mutated allele (>200 CGG repeats) was not observed in the POI group; all other participants (n = 42) showed normal trinucleotide expansion (<45 CGG repeats).

AR (CAG)n Polymorphism and X-Chromosome Inactivation Pattern

The average biallelic means (the mean of the length of the two *AR* alleles) were similar in patients (n = 46; 21.0 ± 2.3 bp) and controls (n = 46; 21.6 ± 2.1 bp; *P*=.2398) (Supplemental Fig. 1). The short repeat length ranged from 14.4 to 24.6 bp in women with POI, whereas it ranged from 13.8 to 24.4 bp in controls. The longer allele ranged from 18.1 to 27.8 bp in the POI group and from 18.8 to 29.2 bp in the control women. The length of neither the short (*P*=.7057) nor long (*P*=.1219) alleles differed significantly between the two groups.

The *AR* and *RP2* genes showed heterozygosity rates of 84.8% and 83.7%, respectively, and their combined heterozygosity rate was 95.7%. The *AR* and *RP2* markers showed the same electrophoretic profiles, before and after the digestion with *Hpa*II (Fig. 2A), as well as the same XCI pattern (Fig. 2B). Nonrandom (or skewed) XCI was identified with a higher frequency in the POI group (34.1%) compared with the control group (15.2%, *P*=.0373; Fig. 2C). In the control group, random XCI was more frequent, and 84.8% of individuals had this XCI inactivation pattern. Conversely, only 65.9% of the women in the POI group demonstrated random XCI (Fig. 2C). Interestingly, extreme skewing, in which more than 90% of cells have the same inactivated X chromosome, was observed in 15.2% of patients (7 of 46) with POI; and in 46.7% (7 of 15) of those with POI and skewed XCI. We used a logistic regression to investigate the effects of age, BMI, and levels of E_2, CRP, and A on the XCI pattern, and we observed that none of these variables seemed to interfere with the random or skewed XCI (*P*>.05). Additionally, the degree of *FMR1* premutation did not seem to be associated with a skewed XCI in the POI group: three of the four women with premutations in their *FMR1* gene had random XCI, and only one had skewed XCI.

Telomere Length

The telomere length, expressed as the T/S ratio, was lower (0.93 ± 0.23) in patients with POI when compared with controls (1.07 ± 0.27; *P*=.0006). We also investigated the effects of confounding variables on telomere length. The adjusted regression model for age (*P*=.6236), BMI (*P*=.4834), and levels of E_2 (*P*=.9871), CRP (*P*=.6183), and A (*P*=.7187) showed that these variables did not influence telomere length. Only the group variable (whether an individual had POI or not) was found to affect telomere length (*P*=.0068) (Fig. 3A).

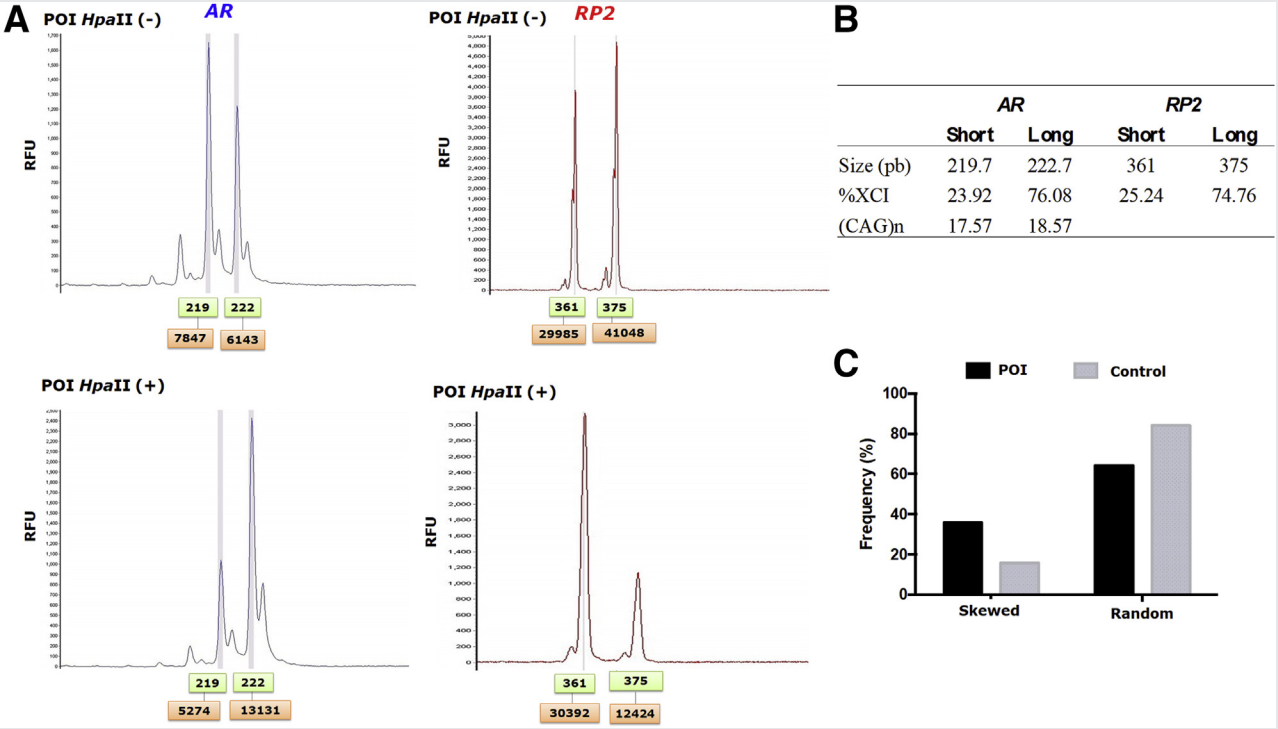
Despite differences in the age of the analyzed individuals, this variable was not correlated with telomere shortening in the POI group (r^2 = −0.2083; *P*=.1856), controls (r^2 = 0.0794; *P*=.5135), or overall (r^2 = 0.0999; *P*=.2943) (Fig. 3B). When the data were grouped by age into two groups, either ≤28 years or >28 years, irrespective of being POI or control, no correlation was observed between younger and older women (*P*=.6821) (Fig. 3C). However, we did find that telomere length and XCI were related biological events; therefore, women with reduced telomere length were more likely to have skewed XCI (*P*=.0492).

DISCUSSION

In this study, POI was associated with shorter telomeres and nonrandom (skewed) XCI, despite the lack of changes in the length of *AR* and *FMR1* polymorphisms. Women with POI demonstrate hormonal changes that characterize the disease, such as elevated FSH and LH concentrations and reduced estrogen levels (hypoestrogenism) (1, 3). The POI group had a lower mean weight and reduced CRP and A levels but no significant changes in BMI. Elevated CRP levels have been previously associated with elevated weight and BMI (25), although weight loss in obese postmenopausal women decreases plasma CRP levels (26). Additionally, A is associated with adiposity indices because adipose tissues stimulate androgen production irrespective of hyperandrogenic disorders (27). This suggests that there is a putative link among body weight, hormone levels, and POI that requires further investigation.

Follicular alterations are a primary cause of syndromic POI, and variation in the expansion of the CAG repeats in *AR* has been shown to associate with the disease owing to their role in folliculogenesis (5, 24). However, we did not observe any differences in the lengths of the short or long alleles, or in the biallelic means (POI, 21.03 ± 2.31 bp; controls, 21.64 ± 2.05 bp) between *AR* alleles in the POI and control groups. Indeed, the variation found in our study for this polymorphic region is within the range expected range for a normal population (8–31 bp) (17). The *AR* CAG repeat-based assay (HUMARA) is often the primary marker used for determining XCI in X-linked disease. However, combining the examination of *AR* (Xq12) and *RP2* (Xp11-23) methylation assays allows XCI to be determined in

FIGURE 2



	AR		RP2	
	Short	Long	Short	Long
Size (pb)	219.7	222.7	361	375
%XCI	23.92	76.08	25.24	74.76
(CAG)n	17.57	18.57		

X-chromosome inactivation pattern. **(A)** *AR* and *RP2* allele profile at capillary electrophoresis, digested (*Hpa*II +) and undigested (*Hpa*II −). **(B)** Representation of the values observed in the electropherogram, with short and long alleles size of both *AR* and *RP2* markers, XCI percentage, and CAG repeat number of each *AR* allele. **(C)** Frequency of skewed and random XCI in women with POI and control women (P=.0373).

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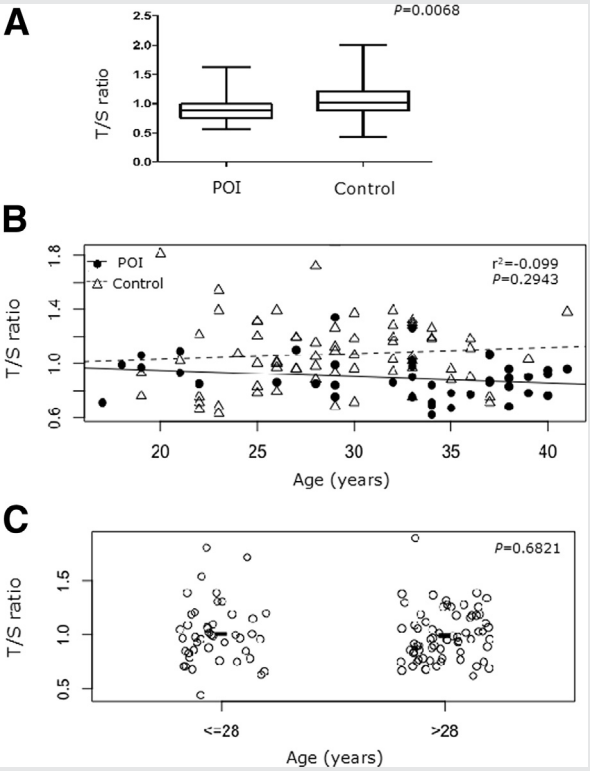
95.65% of a studied population, revealing a greater heterozygosity rate (18).

Normally, XCI is a random process, with an equal number of cells expressing the maternal (X_m) and paternal (X_p) chromosomes at a 50:50 ratio. Moreover, the female population consists of a mosaic of XCI. However, skewed XCI (>70%) can arise owing to alterations on the X chromosome. This leads to several diseases, including the fragile X syndrome (9, 28). Skewed XCI has been previously related to POI (28, 29). We have also found that this phenomenon is more frequent in women with POI, including patients with extreme XCI (>90%). Supporting our findings, some studies have suggested that women with POI show an increased frequency of skewed XCI as a protective measure that selects against the expression of genes from an altered X-chromosome (28). For example, Laisk et al. (30) have found that the number of CAG repeats in *AR* is associated with POI. However, an increase in skewed XCI was not observed in their study, and random XCI was frequent in both the POI and control groups.

X-chromosome inactivation is also an important epigenetic marker used for the detection of cryptic mosaicism that is related to chromosomal alterations (5, 24). The extreme skewing observed in our POI group is a condition frequently found in X-linked disorders and 45,X/46,XX mosaics with syndromic POI, such as Turner's syndrome (30). Therefore, XCI patterns may represent an important molecular marker for both clinical management and prognostic counseling. This may assist in the preservation of fertility in affected patients. Previously the expansion of trinucleotide repeats in *FMR1* was found to associate with POI (4). *FMR1* is the main marker of fragile X syndrome and is located at a "POI segment" on the X-chromosome (Xq27.3). The number of trinucleotide repeats (CGG)n in the gene can range from 6 to 53 copies in a normal population (31). The expansion in the number of CGG repeats in *FMR1* to more than 200 (termed full mutation) has been related to mental retardation, ovarian dysfunction, and syndromic POI phenotypes (32, 33). Barad et al. (34) observed higher percentages of skewed XCI among infertile women aged 30–40 years with less than 26 or more than 34 CGG repeats in *FMR1* compared with those with 26–34 CGG repeats, but this relationship was not present in younger oocyte donors (34). In the present study, skewed XCI was not related to *FMR1* genotype since only one patient had both skewed XCI and *FMR1* premutation.

In addition to skewed XCI being more frequent in women with POI, these patients also showed reduced telomere length compared with controls. Although our women with POI were older than the controls, the mean age difference between

FIGURE 3



Telomere length (T/S ratio) in women with POI and control women. **(A)** Telomere length between POI and control women adjusted by age, BMI, E_2, CRP, and A. **(B)** Relationship between T/S ratio and age in the POI and control groups. **(C)** Telomere length grouped by age (≤28 years or >28 years), irrespective of being in the POI or control group.

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groups was 3.3 years. This small difference did not affect telomere biology in our study groups, because telomere shortening is a relatively slow process (~52 bp/y) (35). Nevertheless, we performed the general linear mixed model adjusted by age and other confounding variables (BMI, A, E_2, and CRP) to ensure the robustness and accuracy of the results. We observed that these variables did not affect telomere biology, because the difference between groups persisted after the adjustment. Moreover, there was no correlation between age and telomere content in the study population, even when we stratified ages into younger (≤28 years) and older (>28 years) women, possibly because the age range of the participants was relatively narrow. Therefore, other genetic and environmental factors might be affecting telomere biology in women with POI.

Accelerated telomere shortening may contribute to follicular depletion or atresia in women with POI, leading to earlier reproductive aging, premature menopause before the age of 40 years, and a loss in fertility (12,36–38). Supporting our results, Butts et al. (39) observed a shortening of the telomeres and reduced telomerase activity in granulosa cells from patients with infertility and a history of FSH elevation at premenopausal levels. Recently, shortened telomeres and a reduced telomerase activity in leukocytes and granulosa cells have been associated with biochemical POI. In this case, patients with biochemical POI were classified with FSH levels ranging from 10 to 40 IU/L and regular menstruation, also representing an intermediate stage of POI in which the primordial follicle pool has not been fully depleted. Despite the nature of the POI groups and the cells used to assess telomere content in these previous studies, both are compatible with our results and lead to the hypothesis that telomere length plays an important role in the pathophysiology of the disease (40).

Hanna et al. (41) reported an increased telomere length in leukocytes with an established POI phenotype (FSH >40 IU/L). The authors suggested that exposure to elevated E_2 level during the menstrual cycle, before menopause, and a lower rate of cell division or autoimmunity might explain these results (41). However, they did not measure the E_2 level. In contrast, we observed shorter telomeres, which were accompanied by reduced A and E_2 levels in women with POI. Although the hormonal levels did not correlate with telomere content in this study, steroid hormones may influence telomere maintenance by stimulating telomerase activity (42, 43). Moreover, aging ovaries produce less androgens (44), which could contribute to reduced telomerase activation and therefore to poor telomere maintenance and shorter telomeres in the POI group. Therefore, further studies are needed to verify the relationship between hormonal exposure and telomere length in women with diminished ovarian reserve.

Finally, nonrandom or skewed XCI, especially extreme skewing (>90%), is a rare phenomenon in a normal population. However, it is frequently observed in women with X-chromosome alterations and has been shown to be involved in many disorders (19). The relationship between genetic biological markers, such as telomere length and XCI pattern, has not been investigated across the general female population. Our study revealed that reduced telomere length and skewed XCI are related events, because women with skewed XCI had shorter telomeres, independently of the group. This represents evidence for a relationship between XCI and telomere maintenance. Telomere maintenance and XCI are complex biological processes that share DNA methylation as a primary regulatory mechanism. For example, XCI often coincides with multiple epigenetic changes, especially a gain in DNA methylation (8, 9), whereas telomere maintenance and telomerase activity both positively correlate with subtelomeric DNA methylation (45–47). This suggests a putative link between the two mechanisms that involves DNA methylation, potentially impacting the study of both processes. A more detailed examination in the future will reveal whether this is indeed the case.

One of the limitations of the present study is that we did not assess the ovarian volume of the patients. This may have affected other measurements. To minimize this risk, patients in the POI group were selected carefully, and clinical diagnoses and assessments of FSH and LH levels confirmed their anovulatory condition, with two serum results obtained before 40 years of age. Additionally, to exclude syndromic

POI before 30 years of age, we assessed both karyotype and *FMR1* mutations.

Shortened telomere length and nonrandom (skewed) XCI were both found to associate with idiopathic POI, despite no alterations in *AR* and *FMR1* X-linked genes. The inflammatory biomarker CRP did not seem to represent the main cause of telomere disruption in women with POI; therefore, other factors likely contribute to telomere erosion and ovarian aging. Our study has revealed that telomere length and XCI patterns are (epi)genetic markers for POI. This phenomenon may be related to changes in DNA methylation that lead to oocyte senescence or apoptosis. Further investigation may lead to better diagnosis and treatments to predict the ovarian resources and preserve fertility before a complete loss of ovarian function in patients with POI.

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La inactivación sesgada del cromosoma X y los telómeros más cortos se asocian con insuficiencia ovárica prematura idiopática

Objetivo: Analizar si la longitud de los telómeros, la inactivación del cromosoma X (XCI) y el polimorfismo de los receptores de andrógenos (AR) GAG están relacionados con la insuficiencia ovárica prematura idiopática (PDI).

Diseño: Estudio de casos y controles.

Localización: Hospital Universitario. Departamento de Ginecología y Obstetricia de la Facultad de Medicina de Ribeirao Preto, Universidad de Sao Paulo, Universidad de Campinas y Universidad Federal de Minas Gerais.

Paciente (s): Un total de 121 mujeres, incluyendo 46 POI no sindrómicas y 75 controles.

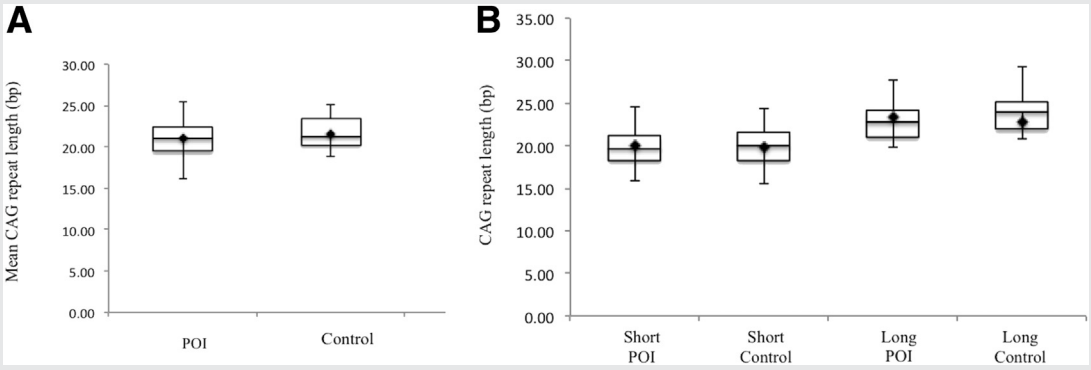
Intervención (es): Ninguna.

Principales medidas de resultado: Se evaluaron edad, peso, talla, índice de masa corporal (IMC), presión arterial sistólica y diastólica, E2, androstenediona, T, y la proteína C-reactiva. La longitud de los telómeros se estimó mediante la reacción en cadena de la polimerasa cuantitativa en tiempo real, y la XCI fue medida utilizando ensayos de metilación del Receptor de Andrógeno Humano y la retinitis pigmentosa ligada a X 2 (RP2). El polimorfismo AR y FMR1 se evaluó mediante la reacción en cadena de la polimerasa fluorescente cuantitativa y secuenciación.

Resultado (s): Las mujeres con insuficiencia ovárica prematura tenían una edad media mayor, pesaban menos y presentaban una proteína C-reactiva, E2 y androstenediona más bajos.

El polimorfismo AR no fue diferente entre los grupos. Cuatro pacientes tuvieron premutación (55-200 repeticiones CGG), y ninguna mostró una mutación completa en el gen FMR1. Sin embargo, los pacientes con POI mostraron una longitud de telómero más corta y una mayor frecuencia de inactivación sesgada del cromosoma X. Se observó un sesgo extremo (R90%) en el 15% de las mujeres con POI, y los telómeros más cortos se correlacionaron con inactivación sesgada del cromosoma X en ambos grupos. Conclusión (s): La inactivación sesgada del cromosoma X y el acortamiento de la longitud de los telómeros se asociaron con PDI idiopática, a pesar de que no hubo alteraciones en la AR y genes FMR1. Además, existe una tendencia a que las mujeres con telómeros cortos presenten inactivación sesgada del cromosoma X.

SUPPLEMENTAL FIGURE 1



Androgen receptor (*AR*) CAG repeats length in women with POI and control women. **(A)** Mean values of two alleles (biallelic mean) ($P>.05$); **(B)** short and long allele repeat length ($P>.05$).

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