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ORIGINAL RESEARCH & REVIEWS

Gender-Affirming Hormone Therapy Modifies the CpG Methylation Pattern of the ESR1 Gene Promoter After Six Months of Treatment in Transmen

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ABSTRACT

Background: Brain sexual differentiation is a process that results from the effects of sex steroids on the developing brain. Evidence shows that epigenetics plays a main role in the formation of enduring brain sex differences and that the estrogen receptor α (*ESR1*) is one of the implicated genes.

Aim: To analyze whether the methylation of region III (RIII) of the ESR1 promoter is involved in the biological basis of gender dysphoria.

Methods: We carried out a prospective study of the CpG methylation profile of RIII (−1,188 to −790 bp) of the ESR1 promoter using bisulfite genomic sequencing in a cisgender population (10 men and 10 women) and in a transgender population (10 trans men and 10 trans women), before and after 6 months of gender-affirming hormone treatment. Cisgender and transgender populations were matched by geographical origin, age, and sex. DNAs were treated with bisulfite, amplified, cloned, and sequenced. At least 10 clones per individual from independent polymerase chain reactions were sequenced. The analysis of 671 bisulfite sequences was carried out with the QUMA (QUantification tool for Methylation Analysis) program.

Outcomes: The main outcome of this study was RIII analysis using bisulfite genomic sequencing.

Results: We found sex differences in RIII methylation profiles in cisgender and transgender populations. Cismen showed a higher methylation degree than ciswomen at CpG sites 297, 306, 509, and at the total fragment ($P \leq .003$, $P \leq .026$, $P \leq .001$, $P \leq .006$). Transmen showed a lower methylation level than trans women at sites 306, 372, and at the total fragment ($P \leq .0001$, $P \leq .018$, $P \leq .0107$). Before the hormone treatment, transmen showed the lowest methylation level with respect to cisgender and transgender populations, whereas transwomen reached an intermediate methylation level between both the cisgender groups. After the hormone treatment, transmen showed a statistically significant methylation increase, whereas transwomen showed a non-significant methylation decrease. After the hormone treatment, the RIII methylation differences between transmen and transwomen disappeared, and both transgender groups reached an intermediate methylation level between both the cisgender groups.

Clinical Implications: Clinical implications in the hormonal treatment of trans people.

Strengths & Limitations: Increasing the number of regions analyzed in the ESR1 promoter and increasing the number of tissues analyzed would provide a better understanding of the variation in the methylation pattern.

Conclusions: Our data showed sex differences in RIII methylation patterns in cisgender and transgender populations before the hormone treatment. Furthermore, before the hormone treatment, transwomen and transmen showed a characteristic methylation profile, different from both the cisgender groups. But the

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hormonal treatment modified RIII methylation in trans populations, which are now more similar to their gender. Therefore, our results suggest that the methylation of RIII could be involved in gender dysphoria. **Fernández R, Ramírez K, Gómez-Gil E, et al. Gender-Affirming Hormone Therapy Modifies the CpG Methylation Pattern of the ESR1 Gene Promoter After Six Months of Treatment in Transmen. J Sex Med 2020;XX:XXX–XXX.**

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Key Words: Brain Masculinization; ERα; *ESR1*; Gender Incongruence; Methylation; RIII

INTRODUCTION

Gonadal steroids, testosterone and estradiol, are involved in the development of the sexual phenotype by binding to specific nuclear receptors (androgen receptor [AR] and estrogen receptors [ERs], α and β) that are transcription factors.[1] The receptor-ligand complex moves to the nucleus and triggers sex-specific gene expression. The presumptive route for brain masculinization is the direct induction of gene expression via activation of ERs α and β and the AR.[2–4]

Masculinization of the brain is largely induced by estradiol synthetized from testosterone by the aromatase enzyme CYP19, matching the karyotype, gonads, and genitals to the gender.[5] In contrast, the lack of early exposure to testosterone in women allows the development of a feminized brain, in concordance with their feminized phenotype (karyotype, gonads, and genitals).

In mammals, brain sex differences occur in some brain areas such as the preoptic area (POA), the bed nucleus of the stria terminalis, and also the hypothalamic nuclei.[6,7] In newborn male rodents, aromatized estradiol in the POA from testicular androgens induces major changes, which are generated in the first days after birth, affecting the volume and number of cells, and remain until adulthood.[8] Estradiol promotes neuritic growth, as well as changes in synapse and dendritic spine formation in several regions of the central nervous system. Synapses and afferences toward the arcuate nucleus decrease when the ovaries are removed and are restored by supplying estradiol again.[9] Estradiol increases the size of cell bodies and the volume of the hypothalamic ventromedial nucleus[10,11] as well as synapse density in this region.[10]

In addition, the membrane organization in these neurons is also sexually dimorphic, and the administration of high-dose prenatal estradiol to female rats irreversibly erases this sexual difference.[12]

But circulating steroid hormones also serve as precursors to the synthesis of neurosteroids.[13] The steroid precursors readily enter the brain, and new neurosteroids are synthesized that modulate neural excitability by rapid non-genomic actions.[14] Generally, neurosteroids modulate brain excitability primarily by interaction with neural membrane receptors and ion channels, principally GABA-A receptors.[15]

A mechanism of action of peripheral sex steroids is the modification of the activity of DNA methyltransferase enzymes by modifying DNA methylation in the C5 site of CpG dinucleotides, masculinizing behaviors and brain structures.[16] The neuronal DNA methylome is highly modifiable by exogenous steroids, with rapid changes in "de novo" methylation causing variations in the expression of genes associated with neural plasticity.[17] This mechanism can significantly affect the processes of cerebral sexual differentiation, so that the prescription of hormonal therapies for gender reassignment treatment can act as an epigenetic factor, modifying the methylome and consequently changing the expression of genes, such as those of AR and ER α and β.

Transgender women, also known as transwomen or male-to-female transsexuals, are born with male genitalia and are assigned as male at birth, but they have a feminine gender. On the contrary, transgender men, also known as transmen or female-to-male transsexuals, are born with female genitalia and

Table 1. Methylation degree (%) at each CpG site and from the total fragment in the ESR1 promoter, in the cisgender and transgender populations, before and after 6 months (+6m) of gender-affirming hormone treatment

Methylation degree (%)								
ESR1 promoter CpG sites								
Groups	111	203	297	306	372	405	509	Total
Ciswomen	86.8	89.5	81.6	92.1	94.6	78.9	78.9	86.0
Cismen	92.0	92.0	97.7	100.0	88.5	77.0	97.7	92.1
Transwomen	96.2	87.7	93.8	99.2	86.2	68.5	86.2	88.2
Transwomen (+6m)	91.5	91.5	95.7	95.7	78.7	74.5	83.0	87.2
Transmen	97.9	89.4	95.7	82.6	70.2	53.2	89.4	82.6
Transmen (+6m)	86.8	97.4	94.7	89.5	98.7	72.4	80.3	88.5

Table 2. *P*-values of Fisher's exact test and the Mann-Whitney *U*-test for all groups and all ESR1 promoter CpG sites and total fragments

CpGs	Groups	Cismen	Transwomen	Transwomen (+6m)	Transmen	Transmen (+6m)
111	Ciswomen	0.509	0.048*	0.725	0.085	1.000
	Cismen	–	0.230	1.000	0.260	0.315
	Transwomen		–	0.248	0.687	0.023*
	Transwomen (+6m)			–	0.361	0.564
	Transmen				–	0.050*
	Transmen (+6m)					–
203	Ciswomen	0.734	0.791	1.000	1.000	0.176
	Cismen	–	0.374	1.000	0.753	0.177
	Transwomen		–	0.597	0.802	0.020*
	Transwomen (+6m)			–	1.000	0.201
	Transmen				–	0.105
	Transmen (+6m)					–
297	Ciswomen	0.003†	0.028*	0.071	0.071	0.040*
	Cismen	–	0.211	0.612	0.612	0.419
	Transwomen		–	0.733	0.733	1.000
	Transwomen (+6m)			–	1.000	1.000
	Transmen				–	1.000
	Transmen (+6m)					–
306	Ciswomen	0.026*	0.037*	0.652	0.330	0.749
	Cismen	–	1.000	0.121	0.0001‡	0.002†
	Transwomen		–	0.172	0.0001‡	0.002†
	Transwomen (+6m)			–	0.050*	0.315
	Transmen				–	0.407
	Transmen (+6m)					–
372	Cis women	0.347	0.251	0.058	0.005†	0.249
	Cismen	–	0.683	0.203	0.011*	0.011*
	Transwomen		–	0.248	0.018*	0.002†
	Transwomen (+6m)			–	0.478	0.0003‡
	Transmen				–	0.0001‡
	Transmen (+6m)					–
405	Ciswomen	0.823	0.231	0.798	0.022*	0.501
	Cismen	–	0.218	0.832	0.006*	0.588
	Transwomen		–	0.465	0.076	0.637
	Transwomen (+6m)			–	0.053	0.837
	Transmen				–	0.034*
	Transmen (+6m)					–
509	Ciswomen	0.001†	0.310	0.781	0.232	1.000
	Cismen	–	0.003‡	0.004†	0.051	0.0003‡
	Transwomen		–	0.633	0.626	0.325
	Transwomen (+6m)			–	0.552	0.814
	Transmen				–	0.217
	Transmen (+6m)					–
Total Fisher's exact test	Cis women	0.006*	0.339	0.716	0.2613	0.360
	Cismen	–	0.015*	0.020*	0.0001‡	0.044*
	Trans women		–	0.692	0.0107*	0.932
	Transwomen (+6m)			–	0.1030	0.590
	Transmen				–	0.019*
	Transmen (+6m)					–
Total Mann-Whitney *U*-test	Ciswomen	0.066	0.935	0.902	0.131	0.513
	Cismen	–	0.006*	0.095	0.0003‡	0.154
	Transwomen		–	0.881	0.021*	0.325

(continued)

Table 2. Continued

CpGs	Groups	Cismen	Transwomen	Transwomen (+6m)	Transmen	Transmen (+6m)
	Transwomen (+6m)			–	0.121	0.670
	Transmen				–	0.020*
	Transmen (+6m)					–

*Significant differences at level $P \leq .05$.
†Significant differences at level $P \leq .005$.
‡Significant differences at level $P \leq .0005$.

are assigned as females at birth, but they have a masculine gender. Transgender people generally experience gender dysphoria because the incongruence between their biological sex and their inner gender identity,[18,19] and for this reason, they often search for hormone therapy, with or without surgery, to align their physical and psychologic features with their inner gender. On the other hand, people without gender incongruence are classified as cisgender.[20]

The origin of gender incongruence seems to be multifactorial. It might be related to neurodevelopmental processes of the brain[19,21] probably as a consequence of a genetic vulnerability[22–32] that implicates variations in the nuclear receptor's sensitivity to their ligand. In our previous studies,[31] we first proposed a hypothetic genetic model based on ERα, ERβ, and AR allele combinations. We suggested that different allele combinations could modulate the sensitivity and therefore the efficiency of the ER to the ligand, and thus, different ERα, ERβ, and AR allele combinations may play a significant role in brain masculinization.

DNA methylation in gender incongruence has not been studied in depth, although research shows that it is involved in the development and plasticity of mammalian brains.[33] Evidence has suggested that changes in the methylation of the ESR1 promoter could participate in the conformation of cerebral dimorphism in specific brain areas[34] because DNA methylation adds stability to transcription repression when it is located at the promoter sites of mammalian genes.[35] DNA methylation can alter chromatin structures and prevents the access of transcription factors to gene promoters—[36] regulating gene expression. On the other hand, there is evidence showing that postnatal experiences have a lasting effect on adult ERα expression.[37,38]

The influence of gender-affirming hormone treatment on the global methylation level of *ESR1*, *ESR2*, and *AR* genes was studied by Aranda et al.[39] They conducted a prospective observational study in 12 transwomen and 6 transmen, comparing the degree of global methylation before and after hormonal treatment, finding an increase in the overall methylation of the *ESR1* in transmen, after 6 and 12 months of hormonal treatment. But they did not contrast cisgender and transgender populations, nor did they examine the CpG methylation profiles using bisulfite genomic sequencing, obtaining overall methylation levels. Other studies have shown that promoters are capable of being activated or inhibited in particular physiological situations or by the action of exogenous compounds with estrogenic activity.[40,41]

In this article, we study the CpG methylation pattern of region III (RIII) of the ESR1 promoter.[42] RIII corresponds to a tissue-dependent and differentially methylated region (T-DMR) as described by Maekawa et al.[43] It is distant from the transcription start site (−1,188 to −790 bp), and its methylation is strongly associated with downregulation of ESR1 expression.

The analysis of RIII methylation was performed in a population of transmen and transwomen, before and after 6 months of gender-affirming hormone treatment, and they were compared with a cisgender population with the same geographical origin, sex and age, with the following objectives: (i) to find out if there are methylation patterns associated with cisgender and transgender variants, which will provide us with information about the possible function of methylation in the process of sexual differentiation of the brain, which would support our previous hypothesis that estrogen and the *ESR1* participate in brain sexual differentiation in our species and (ii) to establish whether the hormonal treatment of gender affirmation produces any modification on the methylation pattern of the *ESR1* in blood.

METHODS AND MATERIALS

We carried out a prospective analysis of the CpG methylation profile of RIII (−1,188 to −790 bp) of the ESR1 promoter,[42] using bisulfite genomic sequencing, in a cisgender and in a transgender Spanish population, before and after 6 months of gender-affirming hormone treatment. To carry out the investigation, the steps followed are as follows.

Study Design and Subjects

The population analyzed was 10 transgender men (ie, natal female individuals transitioning to male individulas), 10 transgender women (ie, natal male individuals transitioning to female individuals), 10 cisgender men, and 10 cisgender women. The transgender population was recruited, diagnosed, and treated with gender-affirming hormone treatment at the Gender Unit of the Clínic Hospital of Barcelona (Spain) from July 2015 to November 2018. The inclusion criteria for transgender groups were the presence of gender incongruence as per International Classification of Diseases, 11th Revision,[44] identification with the other gender (male or female), the early presence of onset of gender non-conformity (before puberty); to be erotically attracted to persons with the same anatomical sex, and to have no prior

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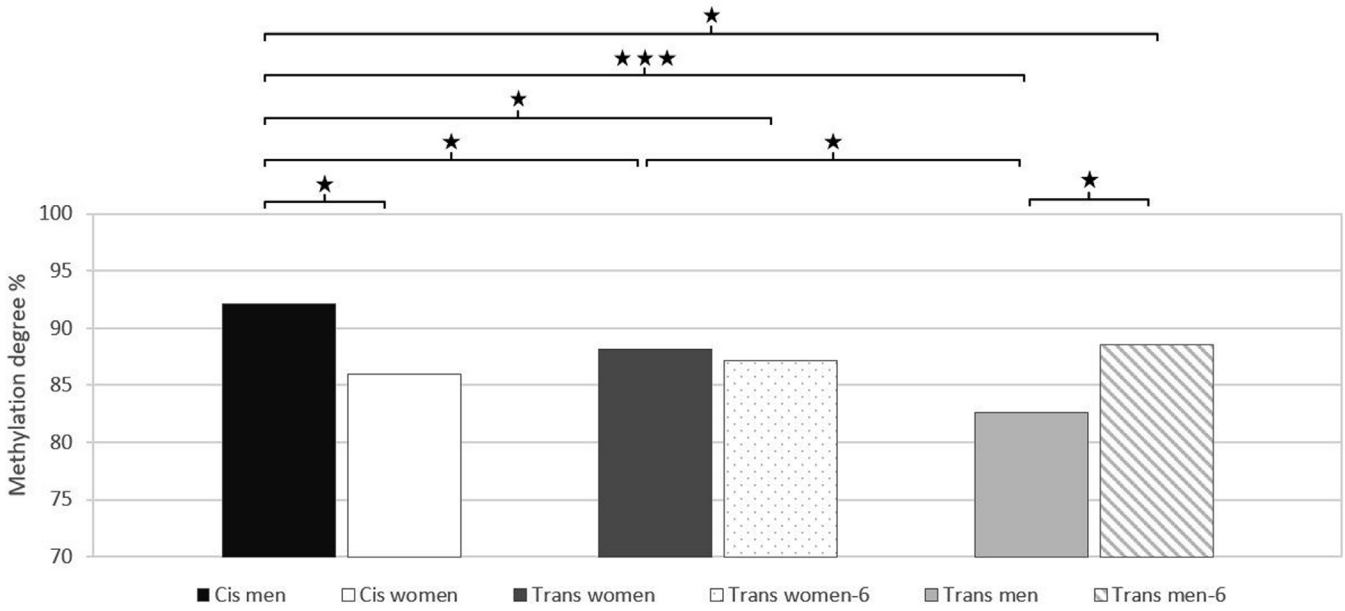

Figure 1. Global RIII methylation degree in the cisgender and transgender populations before and after gender-affirming hormone treatment. Cismen showed a higher RIII methylation degree than ciswomen, and it was the highest methylation degree. Transwomen initially showed an intermediate RIII methylation level between both cisgender populations, and after 6 months of treatment, they showed a slight demethylation and still showed an intermediate RIII methylation degree between both cisgender populations. Transmen showed the lowest RIII methylation level before the hormone treatment, but after 6 months of hormone treatment, they showed an important RIII methylation increase reaching an intermediate level between ciswomen and cismen. The Bonferroni correction estimated the significant differences at level $P \leq .007$. * Significant differences at level $P \leq .05$. *** Significant differences at level $P \leq .005$. RIII = region III.

history of hormonal treatment and no disorders of sexual development. Sexual orientation was established by asking which partner (a man, a woman, both, or neither) they would prefer or feel attraction to if they were completely free to choose and their body did not interfere.

The exclusion criteria were head trauma, neurologic and hormonal disorder, major medical condition (we excluded those with a history of cardiovascular disease, diabetes, or positive HIV), and a history of alcohol and/or drug abuse. No one had concomitant treatment. To dismiss the presence of psychiatric disorders and substance abuse of all participants, the Mini-International Neuropsychiatric Interview[45] was administered.

The cisgender population was selected from the biobank generated from the Pizarra country census (Pizarra, Málaga, Spain) matching by geographical origin, sex, and age. The main characteristics of the Pizarra census were described in a previous study[46]: individuals were excluded if they had been hospitalized for any reason in the 4 weeks before the evaluation, if they were pregnant, or had a severe medical or psychiatric disorder (these exclusion criteria were also applied to the transgender population). Furthermore, Pizarra participants were asked if they had acute or chronic medical conditions (history of cardiovascular disease, diabetes, or positive HIV), a history of alcohol and/or drug abuse, and if they identified themselves as a woman or a man.

Written informed consent was obtained from the transgender group after a full explanation of the procedures. The Pizarra participants signed informed consent for the donation of the samples to the biobank of the Regional Universitario Hospital of Málaga (previously Carlos Haya Hospital) for medical research studies. The study was approved by the Ethics Committee of the Clinic Hospital (Barcelona) and the Regional Universitario Hospital (Málaga).

Transgender and cisgender groups were matched by age and sex. The age range of the transgender group at the beginning of the investigation was 18−38 years (mean 24.5) and 18−40 years (mean 24.8) for the cisgender group.

Gender-Affirming Hormonal Treatment in the Transgender Population

The hormonal treatment in the transmen population consisted of intramuscular administration of testosterone ciclopentilpropionato (cipionato) (250 mg every 21−28 days), whereas the transwomen population received estradiol valerate (2−4 mg/day), associated with acetate of cyproterone (50 mg/day).

Bisulfite Genomic Sequencing

Genomic DNAs from the transgender population were extracted from 5-ml tetrasodium ethylenediaminetetraacetate dihydrate blood samples using the DNeasy Blood & Tissue Kit from Qiagen (Madrid, Spain) following the manufacturer's protocol. The DNAs were extracted twice before starting gender-affirming hormone treatment and after 6 months of treatment. Subsequently, 200−500 ng DNAs from

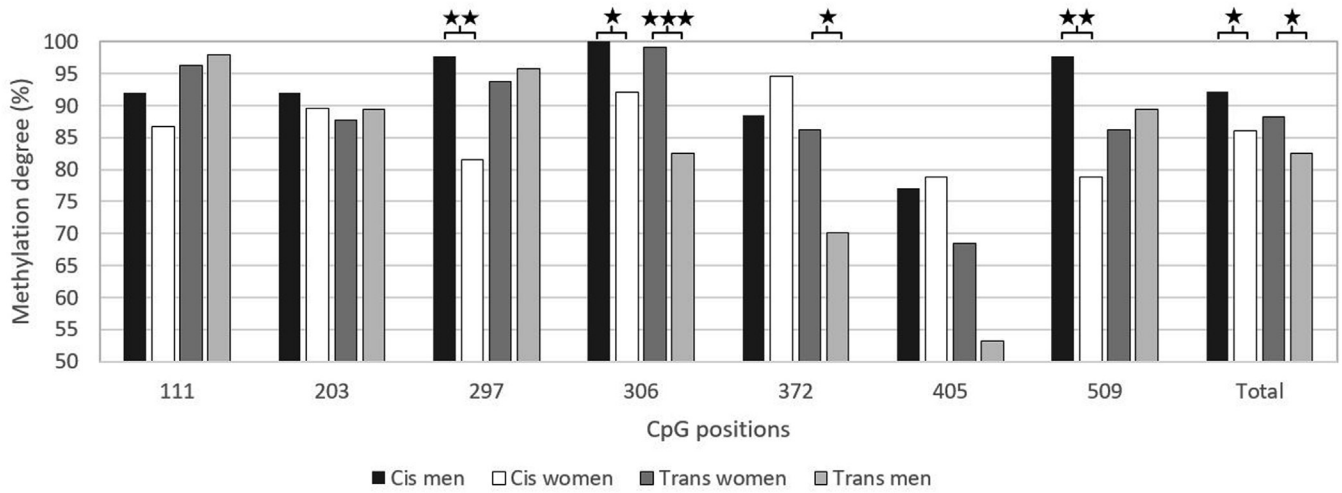

Figure 2. Comparative RIII methylation analysis between cismen, ciswomen, transwomen, and transmen. The analyzed RIII fragment has 7 CpGs in sites 111, 203, 297, 306, 372, 405, and 509. When the methylation degree was compared between ciswomen and cismen, statistically significant differences were found at the CpG sites 297, 306, and 509. Furthermore, the methylation degree of the total fragment was statistically significant between the 2 groups. The methylation degree was higher in cismen than in ciswomen in all statistically significant CpGs and in the total fragment. When comparing transwomen and transmen, statistically significant differences were found at sites 306 and 372 and at the total fragment. In both statistically significant sites (306 and 372), and also at the total fragment, the methylation degree was higher in transwomen than in transmen. The Bonferroni correction estimated the significant differences at level $P \leq .007$. * Significant differences at level $P \leq .05$. ** Significant differences at level $P \leq .005$. *** Significant differences at level $P \leq .0005$. RIII = region III.

transgender and cisgender populations were treated with bisulfite using the MethylCode Bisulfite Conversion Kit (Invitrogen, Madrid, Spain) following the manufacturer's protocol. In this reaction, unmethylated cytosine is transformed into uracil, and 5-methylcytsine remains non-reactive, allowing the analysis of the methylated DNA profiles with high resolution when bisulphite conversion is followed by sequencing.[47]

RIII Amplification

We amplified by polymerase chain reaction (PCR) a 568-bp fragment of the ESR1 promoter, described as RIII by Asada et al[42] using 4 μl of the methylated DNA.The PCR conditions were 95°C for 10 min and 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 10 min. Primers were described by Asada et al.[42] The resulting products were examined in a 1.8% agarose gel electrophoresis

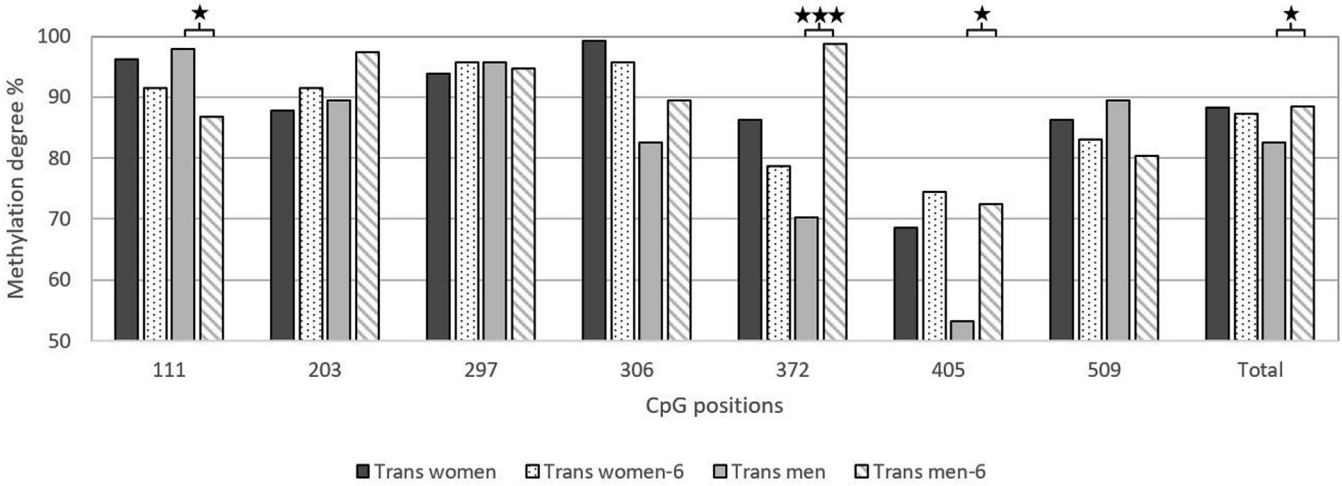

Figure 3. Comparative RIII methylation analysis in transgender populations before, vs after, the hormonal treatment. No statistically significant differences were found at any CpG site, nor in the total fragment when comparing the methylation profiles in the transwomen group before, vs after, the hormone treatment. And statistically significant differences were found at sites 111, 372, 405, and also at the total fragment when we compared methylation levels in the transmen population before, vs after, 6 months of hormone treatment. After the hormonal treatment, the methylation degree at the 111 CpG site decreased, whereas at sites 372, 405 and at the total fragment, there was a significant increase. The Bonferroni correction estimated the significant differences at level $P \leq .007$. * Significant differences at level $P \leq .05$. *** Significant differences at level $P \leq .0005$. RIII = region III.

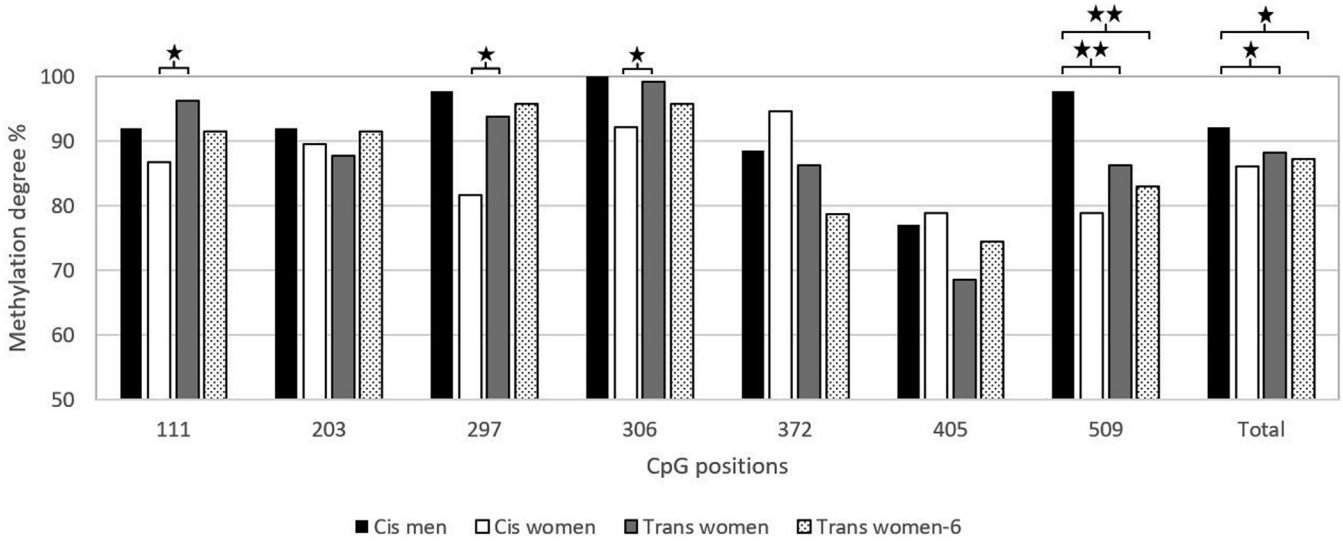

Figure 4. Representation of the CpG methylation degree in RIII, in cisgender populations vs transwomen, before and after the hormonal treatment. Before treatment, transwomen vs ciswomen showed a statistically higher methylation degree at sites 111, 297, and 306. With respect to cismen, transwomen showed a statistically lower methylation degree at site 509 and also at the total fragment. After 6 months of hormone treatment, the methylation analysis between transwomen and ciswomen showed no statistically significant differences at any site, whereas with respect to cismen, it showed a statistically lower methylation degree at 509 site and at the total fragment. Overall, the transwomen population showed a slight methylation decrease after the hormonal treatment, but it remained at an intermediate methylation level between both cisgender groups. The Bonferroni correction estimated the significant differences at level $P \leq .007$. * Significant differences at level $P \leq .05$. ** Significant differences at level $P \leq .005$. RIII = region III.

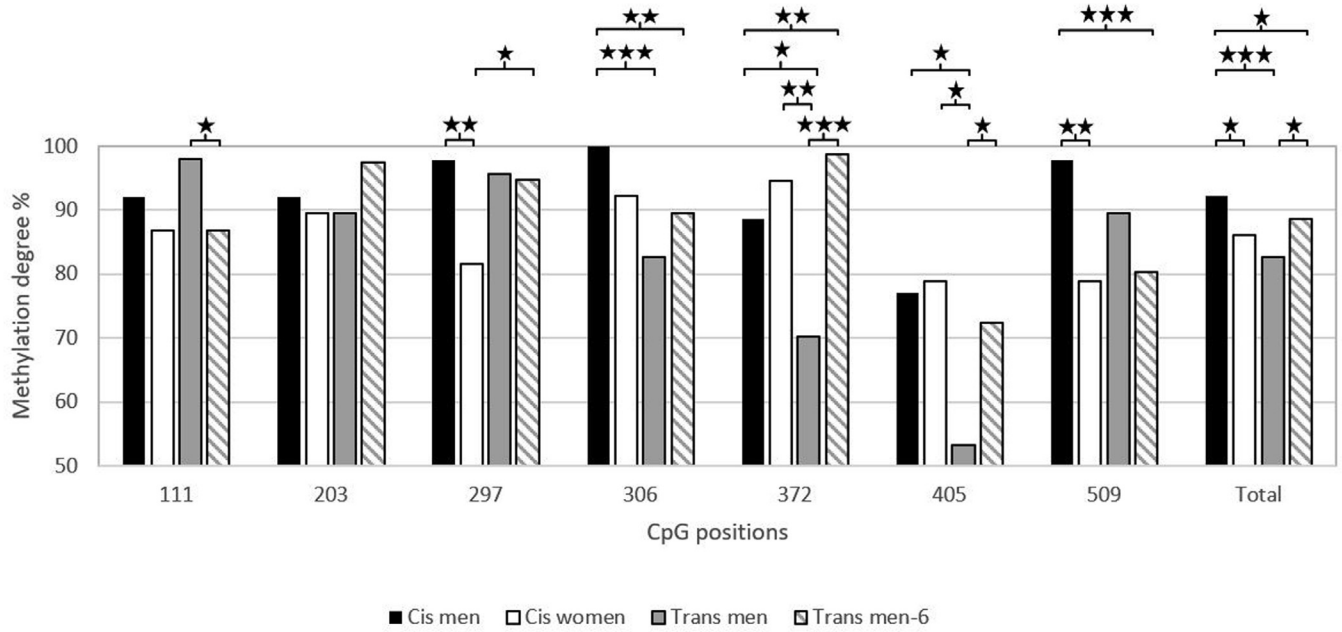

Figure 5. Representation of the CpG methylation pattern in RIII, in cisgender populations vs transmen, before and after the hormonal treatment. Before the hormone treatment, and with respect to cismen, the transmen showed a statistically lower methylation degree at CpG sites 306, 372, and 405 and also at the total RIII fragment. With respect to ciswomen, the transmen population also showed a lower methylation degree statistically significant at CpG sites 372 and 405. After the hormone treatment, the transmen population showed, with respect to cismen, a significantly lower methylation degree at CpG sites 306, 509, and also at the total fragment. In site 372, the methylation degree was higher in transmen than in cismen. With respect to ciswomen, there were statistically significant differences only at site 297. The Bonferroni correction estimated the significant differences at level $P \leq .007$. * Significant differences at level $P \leq .05$. ** Significant differences at level $P \leq .005$. *** Significant differences at level $P \leq .0005$. RIII = region III.

and purified using a QIAquick Gel Extraction Kit (Qiagen, Madrid, Spain).

Cloning and Sequencing

Amplified PCR products (3 μl) were cloned with a T&A Cloning Vector plasmid (Yeastern Biotech Co, Ltd, Madrid, Spain), following the commercial indications. The transformation was subsequently performed in competent *Escherichia coli* cells (Yeastern Biotech Co, Ltd, Madrid, Spain) and plated with solid Lysogeny broth agar medium at 37°C overnight. The white colonies were selected, and the size of the insert was examined by PCR. Subsequently, the clones with the correct size (748 bp = 568 bp fragment + 180 pb vector) were grown in 1.5 ml Lysogeny broth agar medium at 37°C overnight, and the fragments were isolated using the miniprep technique following the manufacturer's protocol (QIAprep Spin Miniprep Kit, Qiagen, Madrid, Spain).

To define the methylation profiles, at least 10 clones per individual, from independent PCRs, were sequenced using a 3130xl Genetic Analyzer (Applied Biosystems, Madrid, Spain).

Statistical Analysis

To align, visualize, and quantify the methylated CpGs from 671 bisulfite sequences, we used the QUMA (QUantification tool for Methylation Analysis) software http://quma.cdb.riken.jp.[48] Statistically significant differences between CpG sites were evaluated using the Fisher's exact test because it is suitable for binary data in unpaired samples. The 2 × 2 table is used to compare methylation effects in CpG sites in 2 groups.[49] Significance between groups was evaluated by the Mann-Whitney U-test because it can be used for the comparison of a non-normally distributed parameter in 2 unpaired samples (http://quma.cdb.riken.jp/).[49]

The conditions of exclusion of low-quality DNA sequences suggested by QUMA software were 5 bases as the maximum limit of CpGs without conversion, 95% as the minimum limit of CpGs with conversion, 10 bases as the maximum limit of alignment defects, and 90% as the minimum limit of percentage of identity. We highlighted results from the analysis that reached statistical significance based on $P < .05$ and also with a more stringent Bonferroni-corrected P threshold ($P < .05/7 = .007$). The CpGs passing the more stringent Bonferroni-corrected P were considered our primary findings.

RESULTS

RIII has 7 CpGs in sites 111, 203, 297, 306, 372, 405, and 509. The methylation degrees at the 7 CpG sites and also from the total fragment in cisgender and transgender populations, before and after gender-affirming hormone treatment, are shown in Table 1. The P values of Fisher's exact tests and Mann-Whitney U-tests are shown in Table 2.

a) Comparative methylation analysis in ciswomen vs cismen: The methylation degree was statistically significantly higher in cismen than in ciswomen and passed the Bonferroni correction at the CpG sites 297 ($P \leq .003$) and 509 ($P \leq .001$) and also at the total fragment ($P \leq .006$) (Tables 1 and 2, Figures 1 and 2).
b) Comparative methylation analysis in transwomen vs transmen before gender-affirming hormone treatment: The methylation degree was statistically significantly higher in transwomen than in transmen and passed the Bonferroni correction at the CpG site 306 ($P \leq .0001$) but did not pass the Bonferroni correction at the total fragment ($P \leq .0107$) (Tables 1 and 2, Figures 1 and 2).
c) Comparative methylation analysis in transwomen before, vs after, the hormonal treatment: No statistically significant differences were found at any CpG site, nor at the total fragment, in the methylation pattern comparing transwomen before vs after the hormone treatment (Tables 1 and 2; Figures 1 and 3).
d) Comparative methylation analysis in transmen before, vs after, the hormonal treatment: After the hormone treatment, the transmen population showed a statistically significant increase that passed the Bonferroni correction at the CpG site 372 ($P \leq .0001$) (Tables 1 and 2; Figures 1 and 3).
e) Comparative methylation analysis between the transwomen and cisgender population, before and after the hormone treatment: Before treatment, transwomen vs ciswomen showed a statistically ($P < .05$) higher methylation degree at sites 111 ($P \leq .048$), 297 ($P \leq .028$), and 306 ($P \leq .037$). With respect to cismen, transwomen showed a statistically lower methylation degree at site 509 ($P \leq .003$) that passed the Bonferroni correction (Tables 1 and 2, Figures 1 and 4). After 6 months of gender-affirming hormone treatment, the methylation analysis between transwomen and cismen showed a statistically lower methylation degree at 509 CpG site ($P \leq .004$) (Tables 1 and 2; Figure 4). Overall, the transwomen population showed a slight methylation decrease after the hormonal treatment, which still remains at an intermediate methylation level between ciswomen and cismen populations.
f) Comparative methylation analysis between the transmen and cisgender population, before and after the hormone treatment: Before the hormonal treatment, the transmen population showed statistically significant differences with respect to both cisgender populations. The methylation degree was higher in cismen than in transmen and passed the Bonferroni correction at CpG sites 306 ($P \leq .0001$) and 405 ($P \leq .006$) and at the total fragment ($P \leq .0001$) (Tables 1 and 2; Figures 1 and 5). With respect to ciswomen, the methylation degree was statistically higher in ciswomen than in transmen at CpG sites 372 ($P \leq .005$) (Tables 1 and 2; Figures 1 and 5). After the hormone treatment, the comparative analysis between transmen and cismen showed statistically significant differences at CpG sites 306 ($P \leq .002$) and 509 ($P \leq .0003$)

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(Tables 1 and 2, Figures 1 and 5). The methylation degree was lower in transmen at both CpG sites, 306 and 509.

DISCUSSION

Our data support the existence of a sexually dimorphic pattern of RIII methylation in blood and in cisgender and transgender populations before hormonal treatment. RIII was defined as a T-DMR by Maekawa et al[43] demonstrating that ESR1 expression is regulated by DNA methylation at this T-DMR, rather than by DNA methylation of regions RI or RII, at the promoter region.

RIII was more densely methylated in cismen than in ciswomen and in transwomen more than in transmen. This RIII methylation pattern in blood is observed in all CpG sites with statistical significance that passed the Bonferroni correction, and also in global RIII methylation.

Before the hormone treatment, the transgender population showed a characteristic methylation profile in blood that is different from cismen and ciswomen. Transwomen showed an intermediate methylation level between both the cisgender groups, higher than ciswomen but lower than cismen. However, transmen showed the lowest RIII methylation degree of the study (lower than transgender groups, before or after the hormone treatment, and also lower than both the cisgender groups).

But after 6 months of hormone treatment, transmen and transwomen reached an intermediate methylation level between ciswomen and cismen. Transmen showed a global RIII methylation increase that was very strong at 372 and 405 CpG sites but inverse at site 111.

Consequently, the data obtained indicate that estradiol treatment did not produce significant changes in RIII methylation in transwomen, unlike testosterone that induced a significant increase in RIII methylation in transmen. These different results imply the existence of different physiological mechanisms of exogenous estradiol and testosterone.

Our data are consistent with previous methylation studies of the POA of the rat.[50,51] This area has sexual dimorphism because men show higher levels of methylation and lower levels of ESR1 expression from day 2 postnatal to adulthood. This implies a sexually different expression of the *ESR1*, which arises in early postnatal development and remains until adulthood, influencing the hormonal sensitivity of the ERα receptor. Therefore, an understanding of the effects of the exogenous steroid on RIII methylation could be a relevant aspect in understanding sexual differentiation.

In addition, Schwarz et al[52] found that in rats, males show higher methylation levels at the ERα promoter than females; female adult rats showed almost 30% less methylation than males. Moreover, Auger et al[53] correlated the highest methylation degree in males with a reduction in the ERα protein. In humans, the demethylation of the ESR1 promoter also correlates with its enhanced expression.[54] Thereby, according to methylation and expression studies, in humans and other species, there is a sexually different methylation pattern of the *ESR1*.

According to our data, cismen showed the highest RIII methylation level, and based on expression studies,[43] it should correspond to the lowest expression of the *ESR1* gene. In the case of transmen, before the hormone treatment, they showed the lowest RIII methylation level that should be related to the highest *ESR1* gene expression. Nevertheless, transwomen showed an intermediate RIII methylation degree in blood that is between cismen and ciswomen and that should correspond to an intermediate *ESR1* gene expression, higher than cismen but lower than ciswomen. Therefore, before hormone treatment, the transgender population showed a RIII methylation degree that did not match with either of the cisgender groups, and after 6 months of treatment, both transgender groups reached an intermediate RIII methylation level between ciswomen and cismen, more similar to their gender but that was still characteristic of those groups.

To our knowledge, there is only 1 study about ESR1 methylation in transgender individuals undergoing hormone treatment. Aranda et al[39] compared a transsexual population before and after hormonal treatment. In this respect, our data are consistent with this work because they also found an increase in the methylation degree of the *ESR1* gene in transmen and found no statistical significance in transwomen.

On the other hand, these data are also consistent with our previous genetic data on the participation of the *ESR1* gene in the genetic basis of gender dysphoria.[30,31] Here, we propose that variations in RIII methylation are related to the process of masculinization of the brain and, therefore, to the etiology of gender incongruence.

Limitations of the Study

This work presents some limitations. In the first place, given the limited number of individuals analyzed, the inclusion of a larger sample would be necessary to achieve greater statistical power.

Undoubtedly, a longer follow-up study (at 12 and 18 months of treatment) would provide important additional information, although Colizzi et al[55] advised that metabolic changes are more noticeable during the first 12 months of hormonal treatment.

In addition, increasing the number of regions analyzed in the ESR1 promoter and increasing the number of tissues analyzed would provide a better understanding of the variation in the methylation pattern.

Moreover, the cisgender population was asked whether they identified themselves as men or women, but the question about other no binary identities was not asked. Nevertheless, the probability of a non-binary identification in our sample is extremely low.

Another limitation of our work is the lack of a study of the *ESR1* gene expression that proves that changes at RIII methylation cause a decrease in the *ESR1* protein.

The strength of our study is that it is the first to analyze the methylation pattern of RIII in the ESR1 promoter in a transgender population using bisulfite genomic sequencing.

CONCLUSIONS

The present study showed (i) that in both cisgender and transgender populations, men and women had different RIII methylation patterns in blood; (ii) that before the hormonal treatment, both transgender groups showed a characteristic RIII methylation profile in blood, which did not match any of the cisgender groups; (iii) that in transmen, the treatment with exogenous testosterone acted as an epigenetic factor that significantly modified the RIII methylation pattern in blood, although it still did not coincide with any of the cisgender population patterns. In this context, the increase in RIII methylation with the consequent decrease in ESR1 expression in transmen supports that the ERα receptor must have an important role in the mechanisms of masculinization; (iv) that in transwomen, the estrogen treatment did not produce significant changes in the RIII methylation pattern; and (v) as a consequence, the different effects of hormonal treatment on the RIII methylation are of clinical interest in the follow-up of transgender men.

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