

# Y-Chromosome Microdeletion Screening in Senegalese Infertile Men

Adjie Dieynaba Diallo<sup>1,2,\*</sup>, Arame Ndiaye<sup>1,\*</sup>, Fatou Diop Gueye<sup>1,2</sup>, Ndiaga Diop<sup>1,2,3</sup>,  
Rokhaya Ndiaye<sup>1,4</sup>, Amath Thiam<sup>5</sup>, Abdoulaye Segal Diallo<sup>1,3</sup>, Mame Venus Gueye<sup>1,2,3</sup>,  
Mama Sy Diallo<sup>1,3</sup>, Oumar Faye<sup>1,3</sup>

<sup>1</sup>Clinical Cytology, Cytogenetics, Reproductive Biology and Human Development Laboratory, Aristide Le Dantec Hospital, Dakar, Senegal

<sup>2</sup>Doctoral School of Life, Health and Environmental Sciences, ED-SEV, Biology and Human Pathologies, Faculty of Medicine, Pharmacy and Odontology, Cheikh Anta Diop University, Dakar, Senegal

<sup>3</sup>Histology-Embryology-Cytogenetics Laboratory, Department of Biology and Functional Explorations, Faculty of Medicine, Pharmacy and Odontology, Cheikh Anta Diop University, Dakar, Senegal

<sup>4</sup>Immunogenetic Laboratory Faculty of Medicine, Pharmacy and Odontology, Cheikh Anta Diop University, Dakar, Senegal

<sup>5</sup>Urology Department, Ouakam Military Hospital, Dakar, Senegal

## Email address:

adjidieynaba.diallo@ucad.edu.sn (Adjie Dieynaba Diallo), emarasn@gmail.com (Arame Ndiaye)

\*Corresponding author

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**Abstract:** Background: Significant progress has been made in recent years towards understanding the pathogenesis of spermatogenic arrest and infertility. Genetic factors contribute to 10-15% of male infertility. After chromosomal abnormalities, Azoospermia factor (AZF) microdeletions in the Yq region are the second most prevalent spermatogenic disorder among infertile men. Our study aimed to assess rates of chromosomal abnormalities and AZF microdeletions in Senegalese men diagnosed with azoospermia and oligozoospermia. Methods: Twenty-three men with azoospermia and oligozoospermia were chosen for molecular studies. Blood samples were analyzed for karyotyping and identification of Y chromosome microdeletions. Multiplex polymerase chain reaction was used to identify the complete deletion of AZF using six Sequence-Tagged Sites (STSs) (sY84 and sY86 in the AZFa region, sY127 and sY134 in the AZFb region, and sY254 and sY255 in the AZFc region). Results: During karyotyping analysis, it was observed that no chromosomal abnormalities were present except for four patients who had Klinefelter syndrome (XXY or XY/XXY; XX/XXY mosaics). Furthermore, AZF microdeletions were detected, with the most common being in the AZFc region, followed by AZFa and AZFb. Ten patients (62.5%, 10/16) exhibited deletion of AZFc markers sY254 and sY255. Of these, two patients (20%, 2/10) had sY254 deletion, one patient (10%, 1/10) had sY255 deletion, and seven patients (70%, 7/10) had sY254 + sY255 deletion which was significantly related to azoospermic phenotype (80%, 8/10). Additionally, four patients (25%, 4/16) had deletion of AZFa at marker sY86 and this was linked to both azoospermic and oligozoospermic. Finally, two patients (12.5%, 2/16) exhibited deletion AZFb in marker sY127, which was associated solely with azoospermia. However, microdeletions of the Y chromosome were detected in four azoospermic patients with abnormal karyotype. Conclusions: Our study indicates the presence of abnormal chromosome and Y chromosome microdeletions in the infertile Senegalese men, suggesting that screening for these should be part of their diagnostic.

**Keywords:** Male Infertility, Karyotype Analysis, AZF (Azoospermia Factor) Deletions

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## 1. Introduction

A question often asked by people around a couple after

they have been together for a few years is: "So when are you going to have a baby?". This often-harmless question can sometimes lead to a difficult situation for the couple if they are facing infertility.

In Africa, infertility is experienced as a tragedy, a subject that is still taboo today, as having children is considered the primary reason for marriage [1]. In popular consciousness, the responsibility for infertility is often attributed to women, as a man's fertility remains inseparable from his virility. Conversely, Africa appears to have the highest prevalence of infertility, reaching 15-20% in some regions [2]. In Senegal, men are involved in 38.30% of cases [3].

Male infertility is the result of an alteration in the processes of spermatogenesis and/or sperm transit in the seminal tract. This infertility can have various causes: toxic, environmental, traumatic, or genetic.

Chromosomal abnormalities are currently being investigated as a possible cause of both azoospermia and oligospermia [4] with frequencies of 10-35% and 1.1-13.5%, respectively [5-9].

Since it has been shown that azoospermic patients have deletions or other cytogenetic abnormalities affecting the long arm of the Y chromosome, the Yq11 region, located above the heterochromatic portion, has been assigned an important role in maintaining spermatogenesis. Subsequently, microdeletions, in particular of the azoospermia factor (AZF), in this region of the Y chromosome and male infertility have been described [10].

The AZF has three distinct regions, including AZFa, which is located on proximal Yq11 (Yq11.21), and AZFb and AZFc, which are located on distal Yq11 (Yq11.23) [11], according to the current model [12]. The AZFa-c region encodes gene families for 27 unique proteins, some of which are specific to the Y chromosome, while others have homologs on the X chromosome or the autosomes [13]. AZF-encoded proteins are essential for spermatogenesis processes such as germ cell cycle regulation and meiosis [14].

Considering that Y chromosome microdeletion is the second most common cause of male infertility after chromosomal abnormalities. The aim of this study was to combine cytogenetic and Y chromosome microdeletion screening in a group of infertile Senegalese men seen at the Aristide Dantec University Hospital, to provide valuable insights into the genetic factors contributing to male infertility in Senegal.

## 2. Materials and Methods

### 2.1. Ethical Approval

This medical research study was performed according to the tenets of the Declaration of Helsinki. Ethical approval was obtained from the Research Ethics Committee of the Cheikh Anta Diop University (CER/UCAD / AD / MsN / 014 /2020), Dakar, Senegal. Written informed consent was provided from all participants.

### 2.2. Study Population and Inclusion/Exclusion Criteria

With the aim of investigating the underlying causes of infertility, patients were referred to the Clinical Cytology, Cytogenetics, and Reproductive Biology Laboratory at

Aristide Dantec Hospital in Dakar, Senegal. A total of 23 patients with a homogeneous phenotype of azoospermia type (N = 16) and severe oligozoospermia (sperm concentration below <5 million/ml, N = 7) were recruited and included in this study.

In addition, patients with moderate oligospermia (sperm count > 10 to <39 million/ejaculate) and normospermia (sperm count 39 million/ejaculate) were excluded from the study.

The male control was a man with a normal, normospermic phenotype that met the current World Health Organization reference ranges [15].

After signing the informed consent, all participants were interviewed about their family and medical history. Blood samples were collected from both patients and controls using EDTA (ethylenediaminetetraacetic acid) tubes.

### 2.3. Sperm Analysis

Semen samples were collected by ejaculation after a 3-day abstinence period. Semen analysis was performed according to the recommendations and standards of the World Health Organization (WHO) [15].

### 2.4. Barr's Chromatin

Barr's chromatin test was performed for all patients using the GUARD staining technique [16]. The percentage is obtained by the ratio between the nuclei with sexual chromatin in a total of 200 nuclei observed. If this rate is between 0 and 5%, the chromatin sex is male; between 15 and 30%, the sex is female; and if this rate is between 6 and 14%, the sex is intermediate.

### 2.5. Karyotype GTG

For this purpose, a cell culture (72 h at 37°C with 5% CO<sub>2</sub>) was performed on 0.5 ml of whole venous blood collected in a heparin tube according to a protocol described previously [17]. The cell bases thus obtained were then spread on a microscope slide and denatured by the enzymatic action of trypsin to reveal the G bands. Finally, the observations were made on a Leica CW4000 Cytogenetics Imaging Station to assemble the karyotype of the different patients and to detect possible chromosomal abnormalities. The karyotypes were described according to the current International Human Cytogenetic Nomenclature (ISCN) [18].

### 2.6. Detection of AZF Microdeletions

Genomic DNA was isolated from peripheral blood leukocytes using the Quick-DNA<sup>TM</sup> Miniprep kit (D3024) according to the manufacturer's instructions. The extracted DNA was stored at -20°C for future use.

Following the guidelines of the European Academy of Andrology (EAA) and the European Molecular Genetics Quality Network (EMQN), we utilized 50 ng of gDNA for testing conventional AZF microdeletions via multiplex PCR, validated by single STS-PCR. Each gDNA sample underwent 2 multiplex PCR reactions, as detailed above.

Six STS markers were used to detect microdeletions in the AZF region of the Y chromosome, in accordance with laboratory recommendations from the European Academy of Andrology (EAA) and the European Molecular Genetics Quality Network (EMQN) [19]. Additionally, an internal control was utilized through the SRY (sex-determining region of Y) and ATL1 (ATL1 locus). The ATL1 sequence in the FMR1 gene located on the long arm of the X chromosome (reference [20]) was chosen to serve as internal controls of PCR for detecting Y and X/Y chromosomes. A blank sample without matrix was included in the analysis to verify reagent contamination. The selected sequence-marked sites (STSs) are provided below.

**Table 1.** Gene and STS primers using for a PCR analysis.

Genes/STS	Primers sequences	Size (bp)
	5' → 3'	
ATL1	F: CCCTGATGAAGAACTTGATCTC R: GAAATTACACACATAGGTGGCACT	300
SRY	F: CATGAACGCATTCATCGTGTGGTC R: CTGCGGGAAGCAAACCTGCAATTCTT	254
sY84 (AZFa)	F: AGAAGGGTCTGAAAGCAGGT R: GCCTACCTGGAGGAGGCTTC	326
sY86 (AZFa)	F: GTGACACACAGACTATGCTTC R: ACACACAGAGGGACAACCT	320
sY127 (AZFb)	F: GGCTCACAACGAAAAGAAA R: CTGCAGGCAGTAATAAGGGA	274
sY134 (AZFb)	F: GTCTGCCTCACCATAAAACG R: CCGTGTGCTGGAGACTAATC	301
sY254 (AZFc)	F: GGGTGTTACCAGAAAGGCAAA R: GACCGTATCTACCAAAGCTGC	400
sY255 (AZFc)	F: GTTACAGGATTCGGCGTGAT R: CTCGTCATGTGCAGCCAC	126

PCR was performed on 2 µL (50 ng/µL) of each patient's genomic DNA in a 23-µL reaction volume. The reaction mixture comprised of 2X Master Mix with Standard Buffer, 0.5 µL of each primer (10 mM), 12.5 µL of Taq DNA polymerase (OneTaq® Quick-Load®), and 9.5 µL milliQ water. The ProFlex PCR system was used to conduct the reactions. The thermal cycling program included an initial denaturation of 5 min at 95°C, followed by 35 cycles of 30s at 95°C (melting), 30s at 60°C (AZFa and AZFb), 58°C (AZFc) (annealing), and 4 min at 72°C (extension). Reaction products were stored at 4°C until they were loaded into agarose gels for analysis.

Next, 5 µl of the amplicons were separated on 2,5% agarose gels by electrophoresis in TAE 50X buffer diluted 1/10 with 5 µl of safeview dye at room temperature using a voltage gradient of 8 volts/cm for 30–60 min. The amplicon size was estimated using a 100 bp Quick-Load® Purple DNA Ladder size marker. Each multiplex PCR included positive controls (fertile man and woman) and negative controls.

## 3. Result

### 3.1. Clinical Characteristics of Cohort

A total of 23 patients were recruited for the study. The Ouolof and Halpulaar ethnic groups had the highest

representation, accounting for 30.43% and 28.09%, respectively. Consanguinity was present in 11 out of 23 patients, representing 47.83%. The mean age at the time of medical diagnosis was 41.01% for patients in the 40 - 50 years age group. Moreover, 60.87% (14/23) of patients had a normal body mass index. Hormonal data were only available for 13 patients, constituting 56.52% of the study population.

### 3.2. Genetic Investigation

Regarding the Barr chromatin, 78.26% of patients had a male sex chromatin, while 17% had results compatible with a female or intermediate chromatin sex.

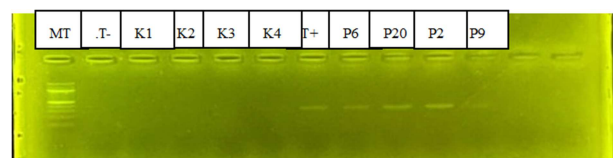
Cytogenetic analysis of at least 20 metaphases revealed that 18 out of 23 patients had a seemingly normal karyotype (46, XY), while 1 had poor quality after observation and 4 of 23 patients (10.5%) had abnormalities in their karyotype (47, XXY) (see Figure 1).



A: morphology of the klinefelter patient 2 (47, XXY/46, XY mosaïc): (1m73), sparse body, micropenis, small testes



B: morphology of the klinefelter patient 2 (47, XXY) large stature (1m95), small testes, Discreet gynecomastia, gynoid aspects of the hip, sparse body hair and hypogonadism



C: Samples of multiplex PC

R analysis:

Lane T- illustrates the negative control representing a normal female. Lanes K1, K2, K3, and K4 present patients with a 47, XXY karyotype, and exhibited a microdeletion in the AZFc region (SY254).

Lanes P6, P7, P8, and P9, which feature with a 46, XY karyotype and demonstrate patients without any deletions in the AZFc region (SY254).

Lane T+ displays the positive control (normal male).

**Figure 1.** Two patients with Klinefelter syndrome (KS) and had Y chromosome microdeletions.

The frequency of chromosomal abnormalities in patients with azoospermia is 17.39% (n= 4/23). Chromosomal abnormalities were not detected in the groups with oligozoospermia.

Out of four patients with chromosomal abnormalities, one had a homogenous form of 47, XXY, two had mosaic forms 47, XXY/46, XY, and one had mosaic forms 47, XYY/46, XX.

**Table 2.** Correlation between barr chromatin and karyotype.

Phenotype	N	Barr chromatin		Karyotype		
		Normal frequency (%)	Middle frequency (%)	Female frequency (%)	Normal frequency (%)	Abnormal frequency (%)
Azoospermia	16	68.75 (11/16)	1/16	4/16	68.75 (11/16)	25 (4/16)
Oligozoospermia	7	100 (7/7)	-	-	100 (7/7)	-
	23	78.26 (18/23)	4.35 (1/23)	17.39 (4/23)	78.26 (18/23)	17.39 (4/23)

Molecular analysis was conducted to determine the presence of Y chromosome microdeletions in 23 patients. The results revealed that the AZFc region had the highest frequency of microdeletions (62.5%, 10/16), followed by

AZFa (25%, 4/16) and AZFb (12.5%, 2/16). Furthermore, AZF microdeletions was significantly linked with azoospermic phenotype (87.5%, 14/16) (refer to Table 3). None of our positive controls exhibited any deletions.

**Table 3.** Pattern of sequence-tagged site (STS) deletions in the 23 infertile men.

ID	Phenotype	AZFa		AZFb		AZFc	
		sY84	sY86	sY127	sY134	sY254	sY255
1	Azoospermic	+	-	+	+	-	-
2	Azoospermic	+	+	+	+	+	+
3	Azoospermic	+	+	-	+	+	+
4	Azoospermic	+	+	+	+	-	-
5	Azoospermic	+	+	+	+	-	+
6	severe oligozoospermic	+	+	+	+	+	+
7	Azoospermic	+	+	+	+	+	+
8	Azoospermic	+	+	+	+	+	+
9	severe oligozoospermic	+	+	+	+	+	+
10	severe oligozoospermic	+	+	+	+	-	-
11	Azoospermic	+	+	+	+	-	-
12	severe oligozoospermic	+	-	+	+	+	+
13	Azoospermic	+	+	+	+	+	-
14	severe oligozoospermic	+	+	+	+	+	+
15	Azoospermic	+	+	+	+	+	+
16	severe oligozoospermic	+	+	+	+	-	+
17	Azoospermic	+	-	+	+	+	+
18	severe oligozoospermic	+	-	+	+	+	+
19	Azoospermic	+	+	+	+	+	+
20	Azoospermic	+	+	+	+	+	+
21	Azoospermic	+	+	+	+	-	-
22	Azoospermic	+	+	+	+	-	-
23	Azoospermic	+	+	+	+	-	-

Among these patients with Y-chromosome microdeletions, four had abnormal karyotypes the Klinefelter syndrome (47; XXY) (figure 1) and twelve had a normal Karyotype (46; XY).

**Table 4.** Genetic and hormonal profile of patients with Klinefelter syndrome and Y-chromosomal microdeletions.

IDs	Age (years)	Biochemical Characteristics			Semen analysis	Karyotype	STS deleted	Y-microdeletion
		FSH	LH	T				
21	31	24.34	18.42	0.35	Azoospermia	47, XXY	sY254, sY255	AZFc
22	48	10.71	NA	16.51	Azoospermia	47, XXY/46, XY	sY254, sY255	AZFc
23	40	23.62	24.45	0.68	Azoospermia	47, XXY/46, XY	sY254, sY255	AZFc
4	43	58.28	NA	14.78	Azoospermia	47, XXY/46, XX	sY254, sY255	AZFc

Reference values: FSH 0.95-11.95 mIU/ml; LH 0.57-12.07 mIU/ml; T 1.42-9.23 ng/ml; NA Not available

## 4. Discussion

In this study, elevated levels of FSH and LH were observed in certain patients in comparison to normal levels. These findings are consistent with a study conducted by

Ramesh Babu *et al.* [1]. The presence of FSH plays a crucial role in initiating spermatogenesis and facilitating sperm maturation. Elevated FSH concentrations have demonstrated their reliability as indicators of damage to the germinal epithelium as well as conditions like azoospermia and oligospermia [21].

Furthermore, normal testosterone levels combined with elevated levels of LH and FSH can serve as an indicator for identifying primary testicular failure [22].

According to guidelines from American Urological Association and the European Academy of Andrology guidelines, men with a sperm count below  $5 \times 10^6$  spermatozoa/mL and non-obstructive azoospermia should undergo karyotype analysis (AUA/ASRM 2006, [23]). Other recommendations suggest performing karyotype analysis for males with a sperm count lower than  $10 \times 10^6$  spermatozoa/mL and for couples who have been unable to achieve pregnancy after one year of unprotected sexual intercourse [24], or for those with a family history of recurrent spontaneous abortions, malformations, and intellectual disability [25].

Chromosomal abnormalities are linked to male infertility and the frequency of cytogenetic aberrations has been estimated to be between 2.1% and 28.4% among infertile men [26]. A prior analysis of pooled data from 11 surveys, which comprised of 9,766 infertile males exhibiting sperm count fluctuations, discovered a 5.8% incidence of chromosomal abnormalities. Sex chromosomes anomalies had a frequency of 4.2%, whereas autosomal abnormalities were less pervasive with a prevalence of only 1.5% [27]. Chromosomal abnormalities related to sex chromosomes are often the cause of infertility issues. Sex chromosome aneuploidies corresponding to Klinefelter syndrome were observed in four azoospermic patients in this study.

A study by Ferlin et al. reported that Klinefelter syndrome (KS) frequency among infertile men was 10% and 5% in azoospermic and severe oligospermic men respectively [28]. Klinefelter's syndrome is a sex chromosomal aneuploidy that our patients exhibit during chromosome analyses. Our study identified two patients with a mosaic karyotype of 46, XY [75]/47, XXY [25], one patient with a 46, XX [90]/47, XXY [10] mosaic karyotype, and another with a 47, XXY homogenous karyotype. It is worth noting that most Klinefelter's syndromes are characterized by homogeneous 47, XXY cells in the blood. However, 10% of cases exhibit a mosaic pattern of 46, XY cells along with 47, XXY cells [29].

In Klinefelter's syndrome, the function of the human Y chromosome is inhibited by the extra X chromosome, resulting in Sertoli cell degeneration, Leydig cell hyperplasia, and hyalinization of the seminiferous tubules [30]. The patients presented the traditional phenotypes attributed to Klinefelter's syndrome (Figure 1 A and B), characterized by tall stature, small testes, gynecomastia, gynoid aspects of the hips, sparse body hair, and hypogonadism. But, in most cases, Klinefelter syndrome presents with mild symptoms and can be difficult to differentiate from the general population.

After Klinefelter syndrome, the most common genetic cause of human male infertility is Y chromosomal microdeletions (AZF regions) [31]. AZF regions, namely AZFa, AZFb, and AZFc, are specific genetic regions implicated in human spermatogenesis.

The criteria for performing Yq microdeletion analysis typically rely on sperm count and encompass conditions like

azoospermia and severe oligozoospermia [19]. Existing literature indicates that the majority of clinically significant deletions are detected in individuals with azoospermia or severe oligozoospermia, characterized by a sperm count of less than  $2 \times 10^6$  spermatozoa/mL. In rare cases, such deletions may be identified in patients with a sperm concentration ranging from 2 to  $5 \times 10^6$  spermatozoa/mL [32]. The occurrence of deletions is extremely low (<1%) in patients with a sperm concentration ranging from 5 to  $20 \times 10^6$  spermatozoa/mL [33, 34]. Indeed, screening for Y microdeletions has both diagnostic and prognostic value and can impact therapeutic options. Thus, it should be conducted routinely. The AZFc region accounts for the majority of Y microdeletions (80%), followed by AZFb (1%-5%), AZFa (0.5%-4%), and AZFbc (1%-3%) [35]. In this study, DNA analysis of the AZFa, AZFb and AZFc regions revealed Y chromosome microdeletions in 34.78% of infertile patients. It is important to note that the majority of deletions were identified in the AZFc region, with a higher prevalence in the azoospermia group (87.5%, 14 out of 16 cases) compared to the oligozoospermia group. These findings support prior research indicating that deletions in AZFc are the most common and are linked to various clinical phenotypes [36, 37].

Microdeletions were found in 62.5% (5 out of 8) of patients with normal karyotypes, including 3 cases of azoospermia and 2 cases of oligozoospermia. Generally, residual spermatogenesis is present in AZFc microdeletions. In such cases, sperm retrieval by TESE (Testicular Sperm Extraction) has a 50% chance of retrieving sperm, and ICSI (Intracytoplasmic Sperm Injection) can be used to conceive children [36]. However, it's important to note that the defective Y chromosome can be inherited by the male offspring, with the risk of having a son with impaired spermatogenesis and a high probability of infertility, similar to his father [38].

Nonetheless, it's worth noting that certain men with a Y-chromosome microdeletion exhibit sperm production that diminishes over time. It is advisable to conduct Y-chromosome microdeletion testing for male family members of AZFc cases or those displaying partial AZFb or AZFa deletions. Conversely, such testing is not advisable for individuals with complete AZFa, AZFb, AZFbc, or AZFabc deletions, as these deletions typically preclude sperm production [36].

Abnormal karyotypes were discovered in 37.5% (3 out of 8) of cases with Y-chromosome microdeletions (refer to Table 3), and all of these patients were diagnosed with Klinefelter syndrome 47, XXY. This study differed from Liu et al. report, indicating that there might not be a significant link between the cause of azoospermia and Y chromosome microdeletion in Klinefelter syndrome patients. This suggests that AZF microdeletion detection might not be necessary for these patients [39]. Similarly, in Ambasudhan's study which combined cytogenetic and Y chromosome microdeletion screening, no Y chromosome deletions were detected in Klinefelter syndrome patients [37]. However, the results are

in line with Mitra *et al.* research, which identified Y chromosome microdeletions that spanned the azoospermia factor (AZF) and AZFb loci in four azoospermic Klinefelter syndrome patients [40]. Performing a karyotype analysis is recommended for males diagnosed with Y-chromosome microdeletions.

In this study, we observed patients who presented no chromosomal abnormalities or Y chromosome microdeletions. Their infertility can result from multiple factors, such as genetic variation in the sexual cascade gene, epigenetic modifications, or exposure to endocrine disruptors.

## 5. Conclusion

In male infertility management, it is commonly accepted that causation is multifactorial. Our study highlights the value of cytogenetic (karyotype) and molecular (Y chromosome microdeletion) studies as a complement to semen analysis in the management of patients with severe disorders of spermatogenesis.

Although our study population was small, chromosomal abnormalities and chromosomal microdeletions were observed with a frequency of 17.4% (4/23 patients) and 69.56% (16/23), respectively. In this regard, we believe that karyotyping and Y-chromosome microdeletion analysis should be performed for a better diagnosis in men with infertility.

Additionally, a study is currently underway examining nucleotide variations in the NR5A1 gene, a nuclear receptor that controls the transcription of genes related to sexual development and reproduction, in the same patients.

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## Conflicts of Interest

The authors declare no conflicts of interest.

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