

GENETIC CAUSES OF PRIMARY AMENORRHEA USING CHROMOSOMAL ANALYSIS AND PCR FOR SRY GENE

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ABSTRACT

BACKGROUND: Primary amenorrhea is defined as the absence of menstruation by age 14 without secondary sexual characteristics or by age 16, regardless of normal development. Chromosomal aberrations account for 14-60% of cases. This study aimed to evaluate chromosomal abnormalities in patients with primary amenorrhea referred to Elite Genetics Center, Khartoum-Sudan, for genetic analysis.

METHODS: Between 2017 and 2023, 248 patients with PA were investigated. Clinical data, hormonal profiles, and sonographic findings were recorded. Karyotyping was done initially, and PCR analysis for the SRY gene was used as a complementary test for patients with normal karyotypes. The clinical diagnosis was based on the history, clinical presentation, hormonal investigation, sonographic findings, and cytogenetic and molecular results.

RESULTS: The results showed that 68 (27.4%) had female karyotypes (46,XX), and 36 (14.5%) had male karyotypes. Numerical chromosomal abnormalities were seen in 97 (39.1%) cases, structural changes in 14 (5.6%), and mosaic abnormalities in 33 (13.3%). Turner syndrome was present in 137 (55.2%), Trisomy X (47, XXX) in 3 (1.2%) cases, and sex reversal (46, XY) in 7 (2.8%) cases. Clinical diagnosis included XY DSD in 21 (8.5%), Complete Androgen Insensitivity Syndrome in 8 (3.2%), Swyer's syndrome in 7 (2.8%), Ovotesticular DSD in 4 (1.6%), Mayer-Rokitansky-Küster-Hauser syndrome in 5 (2%), and Triple X in 3 (1.2%) cases.

CONCLUSION: The results highlight the need for cytogenetic analysis as an integral part of the PA diagnostic protocol. Complementary PCR for the SRY gene is essential in differentiating XY DSD cases.

KEYWORDS: Primary amenorrhea, Chromosomal abnormalities, Karyotyping, Cytogenetic study

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INTRODUCTION

Primary amenorrhea (PA) is a common gynecological problem in adolescent girls. Normal menstruation depends on the structural and functional integrity of the female genital tract, ovaries, pituitary gland, and hypothalamus.¹ Chromosomal abnormalities, hormonal disorders, endometritis, gonadal failure, and Mullerian dysgenesis can cause PA, defined as the failure of a female with normal secondary sexual characteristics to menstruate by age 16 or a female without secondary sexual characteristics by age 14.² PA is the sixth leading cause of female infertility, with an infertility rate of 15% worldwide.³ The prevalence of PA among women of childbearing age is about 2–5%.^{4,5}

Chromosomal abnormalities remain the primary cause of PA, occurring in 1 per 1000 live births and ranging between 10% to 25% of patients with PA.⁶ The SRY gene, located on the short arm of the Y chromosome at Yp11.31, encodes a 204 amino acid protein vital for initiating male sexual differentiation. SRY gene mutations, due to abnormal X/Y crossover, lead to sex reversal and PA.^{7,8} Therefore, PCR testing of the SRY gene is crucial in the genetic assessment of PA patients with normal karyotypes.

Different types of sex chromosome changes contribute to PA, making cytogenetic investigation essential for etiological diagnosis, risk assessment, and genetic counseling.^{9,10} This study, the largest of its kind, aimed to determine the frequency and types of chromosomal abnormalities associated with primary amenorrhea in Sudanese female patients.

METHOD AND MATERIALS

Patients for Cytogenetic Analyses A total of 248 female patients, all with primary amenorrhea, were referred to Elite Genetic Center for cytogenetic and molecular analysis between 2017-2023 and were enrolled in a retrospective cross-sectional study. The diagnosis of primary amenorrhea was confirmed, and only those aged 14 years and older with the absence of secondary sexual characteristics

or 16 years and older with secondary sexual characteristics were included.

Patients for SRY Gene Molecular Study As a complementary investigation, polymerase chain reaction (PCR) was performed in 108 patients with normal karyotypes (XX or XY).

Sampling Around 5ml of venous blood was collected from each patient under complete aseptic conditions. Instantly, 3 ml were placed in a sodium heparin vacutainer for cytogenetic analyses, and 2ml were placed in an EDTA tube for later genomic DNA extraction and SRY gene molecular analyses when necessary.

Methods Cytogenetic Analyses All samples were processed for cytogenetic analyses as described earlier.⁹ In brief, cultures were set by adding 10-15 drops of blood to 10 ml of McCoy's 5A Modified Media (Sigma®), supplemented with L-glutamine, penicillin (100 IU/mL), streptomycin (200 µg/mL), 25% fetal bovine serum (Sigma®), and 3.4 mL phytohemagglutinin (10 µg/mL) (Sigma®). The specimens were incubated in a 5% CO₂ incubator at 37 °C for 72 hours.

The harvesting started after 72 hours by adding 100 µg/ml Colcemid (10 µl/ml) (Sigma®) for 30 minutes to arrest the cells (lymphocytes) in the metaphase stage. The cell suspension was exposed to hypotonic shock in 0.05 M KCl and then fixed three times in methanol: acetic acid (3:1). The cell suspension was centrifuged at 1300 rpm for 10 min before removing the supernatant and resuspending in approximately 0.5-1 ml of fresh fixative (3:1 methanol: acetic acid). Around 1-4 drops of cell suspension were placed on a clean, dry glass slide. The quality and spreading of chromosomes were assisted under a phase contrast microscope after 2-4 slides were prepared for each patient.

The slide-DNA-aging was obtained overnight by keeping the slide in the oven at 60 °C. The slides were immersed in a buffer solution (2XSSC) and kept in a water bath at 60 °C for 2-3 hours. After that, the buffer solution was poured off, and the slides were washed repeatedly with running tap water and air-dried for 1 hour before staining.

G-banding was obtained using Wright's stain, and for each case, 5 to 25 metaphases were analyzed using the CytoVision system, Applied Imaging®. The clonality criteria and karyotypic descriptions were done according to the recommendations of the International System for Chromosomal Nomenclature (ISCN 2020)¹⁰.

Molecular analysis for SRY gene

DNA was extracted from the patient's blood samples collected in EDTA containers using Wizard Genomic DNA Purification Kit Promega®. The DNA samples were kept at -20 °C until PCR analysis was performed. PCR amplification was performed using primers for the SRY gene (Table 1).

Table 1 shows the DNA primer sequence for the SRY gene and the temperature used for the DNA

Primer	DNA sequence(5' to 3')	Temperature (°C)
Forward	TACAGGCCATGCACAGAGAG	60
Reverse	TAAGTGGCCTAGCTGGTGCT	60

In brief, the SRY gene was PCR amplified for the DNA of the patients, as well as from the fertile XX female and fertile XY male controls. The PCR mixture of each sample consisted of 1.0 µL of genomic DNA, 0.2 µL (1.0 U) of Taq polymerase, 3.0 µL of each primer, 1.0 µL dNTPs, 1.5 µL MgCl₂, 12.8 µL H₂O, and 2.5 µL PCR buffer in a final volume of 25 µL. The PCR conditions were 5 min at 95 °C for preheating, 35 cycles of 94°C for 20 seconds (denature), 61°C for 45 seconds (annealing), and 72 °C for 1½ min (extension), and 72 °C for 5 min. Reaction products were electrophoresed on 1.5% agarose-TBE gels containing 0.5 µg/ml Ethidium bromide (for staining) and documented with gel electrophoresis. Hyper ladder 100 bp has been used as a marker.

The statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 20. Data were expressed as frequencies and percentages (%).

Ethical Considerations The ethical approval for the present study was obtained from the Ethical

Committee at Al Neelain Stem Cell Research Center at Al Neelain University. The data were anonymized, and written consent from all patients or their parents was obtained before enrollment.

RESULTS

Of the 248 primary amenorrhea cases studied, the karyotype analysis showed that 68 (27.4%) of the cases revealed female karyotype complement (46,XX) consistent with their female sex, whereas 36 (14.5%) showed male karyotypes (46,XY). Of all cases, 144 (58.1%) showed abnormal karyotypes. Of these, numerical chromosomal abnormalities were seen in 97 (39.1%) cases, whereas structural chromosomal changes were seen in 14 (5.6%) cases (Table 2).

Table 2 shows the results of cytogenetic analyses of 248 patients referred to Elite Genetics Center with Primary Amenorrhea.

Cytogenetic category	Karyotype	Number (%)
Normal	46,XX	68 (27.4%)
	46,XY	36 (14.5%)
Trisomy chromosome X	47,XXX	3 (1.2%)
Monosomy X	45,X 94 (37.9%)	
Structural abnormalities	46,X,del(X)(p10)	8 (3.2%)
	46,X,i(X)(q10)	6 (2.4%)
Mosaic without numerical aberration	46,XX/46,XY	4 (1.6%)
Mosaic with numerical aberration	46,XX/45,X	17 (6.9%)
Mosaic with Structural abnormalities	46,XX/46,X,del(X)(p10)	4 (1.6%)
	46,XX/46,X,i(X)(q10)	4 (1.6%)
	\45,X/46,X,i(X)(q10)	1 (0.4%)
	46,XX/46,X,del(X)(p10)/46,X,i(X)(q10)	2 (0.8%)
Mosaic with numerical and Structural abnormalities	46,XX/45,X/46,X,i(X)(q10)	1 (0.4%)

Apart from cases with normal karyotype, changes involving chromosome X, the most common chromosome in this study, were seen in most cases. Of the 248 patients with PA, Turner syndrome was the most common cause of PA seen in 137 (55.2%), of which monosomy of chromosome X was seen in 94 (37.9%) (Figure 1), mosaic karyotype (46,XX/45,X) seen in 17(6.9%), del(Xp) seen in 8 (3.2%) (Figure2), i(Xq) seen in 6 (2.4%) of the cases (Figure 3), mosaic karyotype 46,XX/46,X,del(X)(p10) and 45,X/46,X,i(X)(q10) were seen in 4(1.6%) cases each, 46,XX/46,X,del(X)(p10)/ 46,X,i(X)(q10) seen in 2 (0.8%), both 45,X/46,X,i(X)(q10) and 46,XX/45,X/46,X,i(X)(q10) karyotypes were seen in 1 (0.4%) cases each. Trisomy of the X chromosome (47, XXX) was in 3(1.2%) cases, and sex reversal females (46, XY) were in 7(2.8%) cases (Figure 4). Trisomy of the X chromosome (47, XXX) was in 3(1.2%) cases, and sex reversal females (46, XY) were in 7(2.8%) cases (Figure 4).

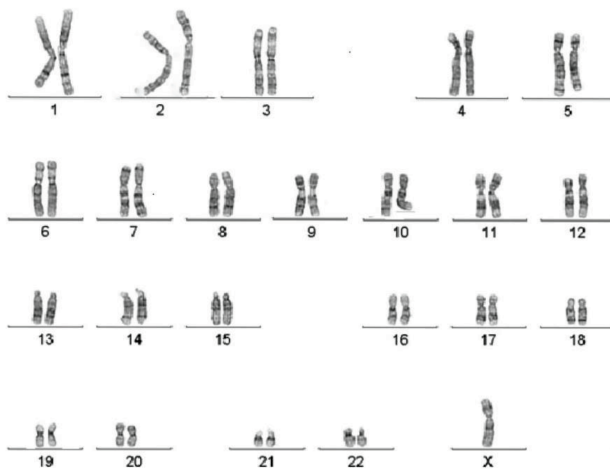


Figure 1. Karyotypic monosomy of the X chromosome (45,X) is consistent with the Turner syndrome diagnosis. The karyogram showed a loss of the second copy of chromosome X.

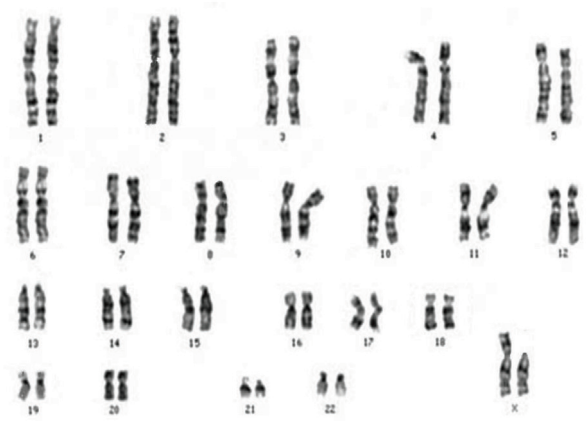


Figure 2. Shows loss of the p arm of the X chromosome. 46,X,del(X)(p10) karyotype consistent with the diagnosis of Turner's syndrome.

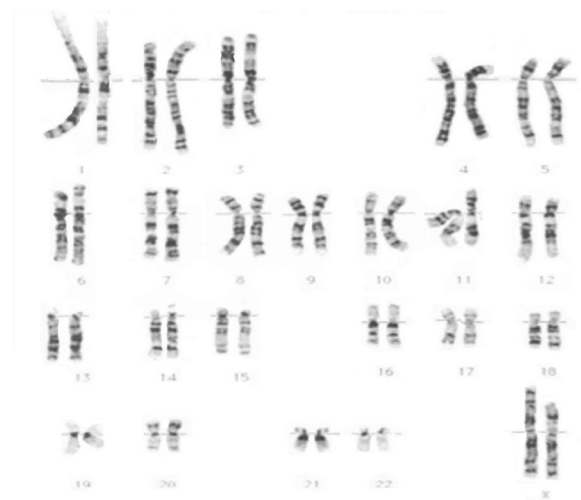


Figure 3. Shows an iso q arm of chromosome X. 46,X,i(X)(q10) karyotype consistent with the Turner syndrome diagnosis.

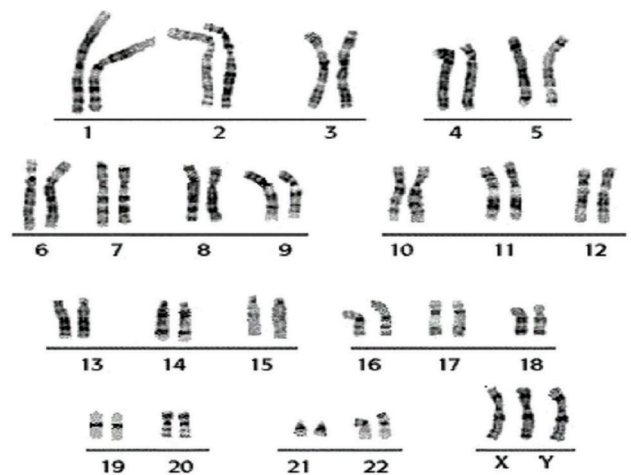


Figure 4. Shows extra copy of chromosome X. 47,XXX karyotype with trisomy X consistent with the diagnosis of Triple X syndrome.

As a complementary test, PCR for the SRY gene was performed in 108 patients with normal karyotypes (46,XX, 46,XY). Out of 108 cases, the SRY gene was detected in 33 cases (30.6%). Among them, 29 cases had a 46,XY karyotype, and 4 had a mosaic karyotype of 46,XX/46,XY. The remaining 75 (69.4%) cases showed negative SRY genes.

According to the clinical examination, imaging findings, hormonal, cytogenetic, and PCR for SRY

gene results, 137 (55.2%) of the patients revealed a clinical diagnosis of Turner's syndrome, 21 (8.6%) XY-DSD, 8 (3.2%) Complete Androgen Insensitivity Syndrome (CAIS), 7 (2.8%) Swyer's syndrome, 4 (1.6%) Ovotesticular DSD, 5 (2%) Mayer-Rokitansky-Küster-Hauser (MRKH) syndrome, and 3 (1.2%) complete premature ovarian failure (Triple X syndrome) (Table 3).

Table 3 shows the clinical findings, genetic work-up, and provisional diagnoses of 248 patients with primary amenorrhea referred to Elite Genetics Center.

Provisional diagnosis	Physical examination	Clinical findings		Genetic workup		N (%)
		Imaging	Hormone profile	karyotype	SRY gene	
46,XX Gonadal dysgenesis	Underdeveloped secondary sexual characteristics, normal female genitalia	Hypoplastic uterus, streak ovaries	↑FSH and LH ↓ estrogen ↔ testosterone	46,XX	-ve	63 (25.4%)
XY-DSD	Underdeveloped breasts, Clitoromegaly, Undescended testes +/- Inguinal hernia	undescended testes	↔ FSH, LH and estrogen ↔↑testosterone ↓ DHEAS	46,XY	+ve	21 (8.6%)
CAIS	well-developed breast, normal external female genitalia	Absent uterus, undescended Testes	↔ FSH, LH and estrogen ↑ testosterone	46,XY	+ve	8 (3.2%)
Swyer's syndrome	Female phenotype, Clitoromegaly	Present Uterus and gonads.	↑FSH and LH ↓ estrogen ↔ testosterone	46,XY	-ve	7 (2.8%)
Ovotesticular DSD	Unilateral Palpable gonads, ± big phallus and vagina	Small uterus, intra-abdominal gonad	↔ FSH, LH and estrogen ↑ testosterone	46,XY/46,XX	+ve	4 (1.6%)
Mayer-Rokitansk -Küster- Hauser syndrome	Normal secondary sexual characteristics, normal external genitalia	Absence of uterus, cervix, and vagina, with normal Ovaries	↔ FSH, LH, estrogen and testosterone	46,XX	-ve	5 (2%)
Triple X syndrome	Tall, well-developed breast, normal femal genitalia	Normal uterus, cervix, vagina, and ovaries	↔ FSH, LH, estrogen	47,XXX		3 (1.2%)
Turner's syndrome	Short stature, Wide neck, short, low hairline, broad chest, underdeveloped breast with wide distance nipples, increased carrying angle, absence of secondary sexual characteristics, normal external female genitalia	Hypoplastic uterus, streak ovaries.	↑FSH and LH	45,X 46,X,del(X)(p10) 46,X,i(X)(q10) 46,XX/45,X 46,XX/46,X,del(X)(p10) 46,XX/46,X,i(X)(q10) 45,X/46,X,i(X)(q10) 46,XX/46,X,del(X)(p10)/ 46,X,i(X)(q10) 46,XX/45,X/ 46,X,i(X)(q10)		137(55.2%)
Total						248

DISCUSSION

Primary amenorrhea (PA) is a common gynecological disorder that indicates a potential future fertility issue. A normal karyotype and/or genome is necessary for normal female sexual development.¹¹ Worldwide, genomic alterations, including sex chromosomal abnormalities, are repeatedly reported as etiological causes of PA.^{12,13} Many studies have investigated the frequency and types of chromosomal abnormalities in PA, but few have been conducted in Sudan. A previous survey in Sudan suggested that some physicians were hesitant to refer patients for genetic investigation, and many patients were unwilling to seek medical help or refused chromosomal analysis due to shame or cultural issues. As a result, our present study may not accurately reflect the true extent of the problem. The possibility of Muslim males entering into multiple marriages leads to female concerns about detecting potential genetic disorders that may affect female fertility and could justify having a second wife.¹⁴

Previous studies estimated the frequency of chromosomal abnormalities to range from 24% to 63%, with most falling between 20-30%.^{15,16} This aligns with our results, which showed 58.1% abnormal numerical and/or structural karyotypes. However, the data showed an even higher percentage (70.1%) of abnormal genetic profiles, considering the molecular results of the SRY gene. The complementary molecular analysis in our study adds valuable information for the clinical diagnosis of cases with normal karyotypes, such as Swyer's syndrome and Androgen Insensitivity Syndrome (AIS). The elevated occurrence of abnormal karyotypes within our cases could potentially be attributed to the notably higher prevalence of Turner syndrome in our study, accounting for 55.2% of cases, compared to the average of 20% observed in most other studies.¹⁷ The disparity in findings may be attributed to the predominant failure to diagnose Turner syndrome during childhood in our region, potentially resulting in a higher number of identified cases in our study. Furthermore, our research revealed a marked increase in the number

of cases with disorders of sexual development compared to the findings of other studies.¹⁸

This study indicates the importance of including genetic workup for patients with PA, even in countries with limited health resources. The genetic workup can eventually help reveal the etiology of PA and assist in delivering proper genetic counseling for the potential infertility problem.³

Cytogenetic analysis showed loss of the p arm of chromosome X (45,X/46,X,i(Xq)/46,X,del(Xp)) in the vast majority (55.2%) of the cases being the most common genetic disorder foreseen in the phenotype diagnosis. Such karyotypic abnormalities are mainly due to initial X chromosome breakage and fusion, leading to unipolar segregation.¹⁶ Turner syndrome is the most common disease, occurring in approximately one in 1500 newborn female infants. Endocrine, cardiac autoimmune, and other structural complications pose considerable risks.¹⁹ Identifying these patients as early as possible may allow them to benefit from a variety of existing fertility preservation options and pre-puberty hormonal therapy to overcome their short stature.²⁰

Eight patients (3.2%) showed male karyotypes (46,XY) despite their female phenotype. Such patients need further investigation, including ultrasonography and PCR analysis, to assess the integrity of the SRY gene and other genes in the testes determining pathway. Patients with female phenotype and male karyotypes (positive SRY gene) are clinically known as Androgen Insensitivity Syndrome (AIS). Usually, they represent a significant counseling challenge for treating physicians if they present complaining of failure to conceive after getting married. In a society like Sudan, counseling is even more difficult for the couple and their families. Moreover, such patients are also at high risk of testicular tumors due to the undescended testicles; thus, surgical interventions (bilateral orchiectomy) aiming to reduce this risk are recommended.²¹

Triple X syndrome is characterized by the presence of an extra X chromosome. It may show non-mosaic karyotypes in most cases. Mosaicism cases are seen in 10% of cases with variable combinations such

as 46,XX/47,XXX or 47,XXX/48,XXXX, and rarely in combinations with karyotypes consistent with Turner syndrome cell lines (45,X/47,XXX; 45,X/46,XX/47,XXX). The phenotypes of this syndrome are variable, including primary or secondary amenorrhea, late menarche, and menstrual irregularities.²²

In this study, 7 cases were diagnosed as reverse syndrome, Swyer syndrome, or 46 XY gonadal dysgenesis. All patients present with a female phenotype and normally developed Mullerian ducts, streak gonads, poorly developed breasts, and primary amenorrhea. The chromosomal analysis showed a male karyotype (46, XY) with a negative SRY gene in the complementary PCR analysis. The SRY mutation might be either de novo during spermatogenesis or deletion due to unexpected cross-over of the SRY gene to the X chromosome during the meiotic process of spermatogenesis, eventually giving rise to a sperm with a Y chromosome devoid of the SRY gene or a mutated gene. Fertilization of this Y-bearing sperm (mutated SRY or deleted SRY) with a normal X-bearing ovum gives rise to a sex-reversed female with a karyotype of 46, XY. Even though the sex-reversed females carry both X and Y sex chromosomes, the undifferentiated gonad fails to develop into a testis due to the absence of the SRY gene. Testosterone and anti-Mullerian hormone (AMH) are not produced, leading to decreased virilization and the development of a female phenotype. As the Wolffian duct fails to develop, the internal male organs are not formed. The absence of AMH allows the development of the uterus, fallopian tubes, cervix, and vagina. In 10%-15% of cases of female sex reversal, an SRY gene mutation was seen. The remaining cases may be due to mutations of other genes involved in sex differentiation pathways, such as the autosomal genes SOX9 and WT1 and the DAX1 gene on the X chromosome. Gonadal dysgenesis is associated with an increased risk of gonadal tumors. Hence, early diagnosis is crucial, given the risk of developing germ cell tumors at an early age.²⁴

In some of our cases, the clinical diagnosis of Ovotesticular DSD (OT-DSD) was revealed in patients with normal male karyotypes (46, XY), female karyotypes (46, XX), or mosaic ones (46, XX/46, XY). The clinical tool to differentiate OT-DSD from mixed gonadal dysgenesis lies in the asymmetrical gonadal dysgenesis in cases of OT-DSD, i.e., one well-developed gonad that can be identified as a testis or ovary, whereas the other is a rudimentary streak gonad. It is essential to differentiate between the two distinct conditions for genetic counseling and gender assignment. Moreover, patients with mixed gonadal dysgenesis are at risk of developing malignant germ cell tumors and, therefore, need preventive gonadectomy.^{25,26}

CONCLUSION

The present study showed a high number of abnormal karyotypes among patients with PA. This implies the critical role of chromosomal analysis in the workup of patients with PA. Patients with normal karyotypes may need complementary PCR tests for the SRY gene to help identify other disorders with no karyotypic abnormalities.

DECLARATIONS

Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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Data Availability

All data from this study are available upon request.

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