Clinical Study

Analysis of the gene coding for steroidogenic factor 1 (SF1, NR5A1) in a cohort of 50 Egyptian patients with 46,XY disorders of sex development

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Abstract

Objective: Steroidogenic factor 1 (SF1, NR5A1) is a key transcriptional regulator of genes involved in the hypothalamic–pituitary–gonadal axis. Recently, SF1 mutations were found to be a frequent cause of 46,XY disorders of sex development (DSD) in humans. We investigate the frequency of NR5A1 mutations in an Egyptian cohort of XY DSD.

Design: Clinical assessment, endocrine evaluation and genetic analysis of 50 Egyptian XY DSD patients (without adrenal insufficiency) with a wide phenotypic spectrum.

Methods: Molecular analysis of NR5A1 gene by direct sequencing followed by in vitro functional analysis of the two novel missense mutations detected.

Results: Three novel heterozygous mutations of the coding region in patients with hypospadias were detected. p.Glu121AlafsX25 results in severely truncated protein, p.Arg62Cys lies in DNA-binding zinc finger, whereas p.Ala154Thr lies in the hinge region of SF1 protein. Transactivation assays using reporter constructs carrying promoters of anti-Müllerian hormone (AMH), CYP11A1 and TESCO core enhancer of Sox9 showed that p.Ala154Thr and p.Arg62Cys mutations result in aberrant biological activity of NR5A1. A total of 17 patients (34%) harbour the p.Gly146Ala polymorphism.

Conclusion: We identified two novel NR5A1 mutations showing impaired function in 23 Egyptian XY DSD patients with hypospadias (8.5%). This is the first study searching for NR5A1 mutations in oriental patients from the Middle East and Arab region with XY DSD and no adrenal insufficiency, revealing a frequency similar to that in European patients (6.5–15%). We recommend screening of NR5A1 in patients with hypospadias and gonadal dysgenesis. Yearly follow-ups of gonadal function and early cryoconservation of sperms should be performed in XY DSD patients with NR5A1 mutations given the risk of future fertility problems due to early gonadal failure.

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Introduction

Disorders of sex development (DSDs) are complex disorders with atypical chromosomal, gonadal or anatomical sex (1). DSDs are classified into three groups: i) DSD with numerical sex chromosome aberrations, ii) XY DSD and iii) XX DSD. Sex chromosome DSD consists mainly of disorders with gonadal dysgenesis due to sex chromosome imbalances such as Turner syndrome (45,X and mosaicism), Klinefelter syndrome (47,XXY), mixed gonadal dysgenesis (45,XO/46,XY) and chimeric DSD (46,XX/46,XY). XY DSD comprises mainly testicular dysgenesis, defects of androgen synthesis or action and hypospadias. XX DSD includes congenital adrenal hyperplasia, ovarian dysgenesis and uterine/vaginal malformations (1). In Egypt, the occurrence of DSD is rare with an incidence of 1:5000 (2) similar to 1:4500 worldwide (3). In Egypt, 46,XY DSD constitutes the majority of DSD cases (65.9%) (4) with androgen insensitivity syndrome and 5α-reductase deficiency being the two most frequent 46,XY DSD aetiologies due to high consanguinity. They constitute 64% among all reported cases (5). However, in many cases the pathogenetic cause is unknown.

During the last 10 years, steroidogenic factor 1 (SF1, NR5A1) has been found to play a pivotal role in human sex differentiation. SF1 is a key transcriptional regulator of many genes involved in the hypothalamic–pituitary–gonadal axis and adrenal cortex (6). Sf1 null mice demonstrate complete gonadal dysgenesis and adrenal failure (7). However, humans harbouring heterozygous NR5A1 mutations display a milder phenotype. The majority of cases display 46,XY DSD with ambiguous genitalia or hypospadias due to partial gonadal dysgenesis but no adrenal insufficiency. To date, about 60 different NR5A1 mutations have been reported in humans with DSDs. So far, there is no apparent genotype–phenotype correlation in patients with NR5A1 mutations. The phenotypic spectrum has been extended, involving not only ambiguous genitalia and hypospadias due to gonadal dysgenesis (8, 9, 10), but also vanishing testis syndrome (11), isolated hypoplastic penis (12) and male infertility (13, 14). Moreover, NR5A1 mutations were also found in 46,XX females with premature ovarian failure and primary ovarian insufficiency (15, 16, 17, 18, 19). Altogether, NR5A1 mutations have emerged as being the most frequent cause (6.5–15%) of different phenotypes of 46,XY DSD in Western countries. Our aim is to investigate whether NR5A1 mutations are also a cause of 46,XY DSD in Egypt.

Subjects and methods

Cohort

A cohort of 50 Egyptian 46,XY DSD patients were recruited from the Pediatric Endocrinology Clinic of Cairo University and the Clinical Genetics and Endocrinology Departments of National Research Centre (NRC) in Cairo, Egypt. Patients’ chronological ages ranged from 2 months to 33 years at first presentation. The phenotypic spectrum included complete female external genitalia with/without uterus (n=8), ambiguous genitalia without uterus (n=5), vanishing/atrophic testes (n=10), hypospadias with normally descended testis (n=9), hypospadias with at least one undescended testis (n=14) and isolated hypoplastic phallus (n=4). Patients with syndromic forms of gonadal dysgenesis or chromosomal abnormalities were excluded. Among the 50 patients, 39 were reared as males, 11 as females and one was reared as female at birth then her sex was changed to male at 2 years of age. Written informed consent for genetic analysis was obtained according to institutional ethical guidelines from the patients and/or their parents.

Work-up included cytogenetic analysis by G-banding technique in 50 metaphases (20), and pelviabdominal ultrasound. Testosterone, precursors and dihydrotestosterone (DHT) were measured before and after human chorionic gonadotropin (hCG) stimulation. In some cases, pelvic laparoscopy, biopsy and histological analysis of the gonads were performed.

Molecular analysis of NR5A1 gene

Molecular analysis of exons 2–7 of the gene encoding SF1 (NR5A1) was performed as described previously (21). Numbering of the mutations is based on GenBank reference DNA sequence NM_004959.4, with the A of the ATG initiation codon designated +1 (www.hgvs.org/mutnomen).

A total of 100 Egyptian controls (200 alleles) were sequenced for both the mutations and the single-nucleotide polymorphisms (SNPs).

Functional analysis

Vector containing full-length mouse Nr5a1, pCNA3-NR5A1-myc and the mouse TESCO reporter were a gift from Dr Francis Poulat, Institut de Génétique Moléculaire de Montpellier. The reporters containing the minimal anti-Müllerian hormone (AMH) and CYP11A1 promoters (−269) are described previously (17).
analysed statistically using Student’s experiments, each performed in 12 replicates. The data were

The consanguinity rate among parents of all included 46,XY DSD patients in our study was 53%, where 34% were first cousins and 19% were second cousins or further relatives. In Egypt, the rate of total consanguineous marriages was 29.7% in 2008 (22) and reached 35.3% in 2011 (23).

Case histories

Patient 1 (p.Arg62Cys)  Patient 1 is a 1 six and a half-year-old male, born to non-consanguineous parents, presenting with severely hypospastic phallus (stretched penile length was 1 cm, < −2.5 SDS), penile hypospadias, hypoplastic scrotum and impalpable gonads. Pelviabdominal ultrasound revealed two small testes, each 8–9 mm in diameter, in medial ends of respective inguinal canals and no Müllerian structures. The patient was operated upon for orchiopexy, correction of hypospadias and circumcision. During mini puberty, basal testosterone level was low normal for age, while testosterone peak after hCG stimulation (5000 IU/m² divided over 3 days) was markedly low. Luteinising hormone-releasing hormone (LHRH) stimulation test showed slightly elevated FSH after stimulation. AMH and inhibin B were both diminished (Table 1). The patient showed no clinical signs or symptoms of adrenal affection and had normal cortisol and DHEAS levels. Mutational analysis revealed a heterozygous p.Arg62Cys (c.184 C>T) mutation which lies in the DNA-binding zinc finger region in exon 3 and

Results

NR5A1 analysis revealed three novel heterozygous mutations of the coding region in three patients with hypospadias: heterozygous p.Arg62Cys (c.184 C>T), heterozygous p.Glu121AlafsX25 (c.361delGAGACAGG) and heterozygous p.Ala154Thr (c.460 G>A) (Figs 1 and 2). No NR5A1 mutations were found in patients with other phenotypes such as complete gonadal dysgenesis, isolated micropenis or vanishing/atrophic testes.

The mutated arginine residue in position 62 is highly conserved in human, chimp and rhesus but not in other species.

The NR5A1 expression vectors containing the coding sequence of all mutant plasmids was confirmed by direct sequencing prior to functional studies. Transient gene expression assays to assess NR5A1 function were performed in 96-well plates (TPP) using human embryonic kidney cells (HEK293-T), FuGENE6 and a Dual-Luciferase Reporter Assay System (Promega) with pCMV-RL. Cells were lysed 48 h later and luciferase assays were performed (Dual-Luciferase Reporter Assay system, Promega) of pCNA3-NR5A1-myc plasmid. The entire expression vectors containing the DNA-binding domain AF-2.
Table 1  Clinical, hormonal and molecular data of patients with NR5A1 mutations.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>External genitalia</th>
<th>Gonads</th>
<th>Uterus</th>
<th>Epididymis, vas deferens</th>
<th>Hormones</th>
<th>Gender assignment</th>
<th>Adrenal affection/investigations</th>
<th>Mutations (heterozygous)</th>
<th>Parents</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>6/12</td>
<td>Penile hypospadias, severely hypoplastic penis (1 cm) and hypoplastic scrotum</td>
<td>Bilateral small inguinal testes (each 8–9 mm in diameter)</td>
<td>No</td>
<td>Yes</td>
<td>At 4 months: testosterone, 0.1–1.8 ng/ml ((\downarrow)); FSH, 1.3–9.1 U/l ((\downarrow)) and LH, 0.5–2.2 U/l (N)</td>
<td>Cortisol, 9.9 (\mu)g/dl (N)</td>
<td>DHEAS, 12.4 (\mu)g/dl (N)</td>
<td>p.Arg62Cys (exon 3) + p.Gly146Ala (exon 4)</td>
</tr>
<tr>
<td>2</td>
<td>13</td>
<td>Hypospadias, severely hypoplastic penis (3.5 cm) and bifid scrotum</td>
<td>Right atrophic undescended testis</td>
<td>Left testis normal in scrotum</td>
<td>No</td>
<td>Yes</td>
<td>At 13 years: testosterone post-hCG, 0.75 ng/ml ((\downarrow)); inhibin B, 9.0 ng/l ((\downarrow)) and AMH, &lt;0.10 ng/ml ((\downarrow))</td>
<td>No symptoms of the adrenals being affected</td>
<td>p.Glu121AlafsX25 (exon 4)</td>
<td>Mother WT</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>Penile hypospadias, hypoplastic penis (2.5 cm) and anchored</td>
<td>Both testes normal in scrotum</td>
<td>No</td>
<td>Yes</td>
<td>At 4 years: testosterone, 0.18–1.6 ng/ml ((\downarrow)); FSH, 1.2–8.7 U/l (N); LH, 0.6–2.1 U/l (N); inhibin B, 70.4 ng/l (N) and AMH, &gt;20 ng/ml (N)</td>
<td>No symptoms of the adrenals being affected</td>
<td>p.Ala154Thr (exon 4)</td>
<td>Mother WT</td>
<td>Father p.Ala154Thr</td>
</tr>
</tbody>
</table>

Conversion to SI units: testosterone (ng/mL) \(\times 3.47 = \text{nmol/L}\); FSH and LH (mIU/ml) \(\times 1 = \text{IU/L}\); AMH (ng/ml) \(\times 7.14 = \text{pmol/L}\) and DHEAS (\(\mu\)g/dl) \(\times 0.027 = \text{nmol/L}\). Serum testosterone levels are basal and after hCG stimulation. FSH and LH levels are basal and 90 min after LHRH stimulation. Normal values: basal testosterone level in males with; Tanner stage 1 (<7 years), <0.11–0.65; Tanner stages 2–3, 0.62–2.26 ng/mL and Tanner stages 4–5, 1.68–6.0 ng/mL. Normal testosterone peak after stimulation with 5000 IU/m² hCG at 72 h: 3.0–10.0 ng/mL. Basal FSH in males: Tanner stage 1 (0.5–10 years), <1–1.3 IU/L; Tanner stages 2–3 (>10 years), <1–4.0 IU/L and Tanner stages 4–5 (>10 years), 1.4–5.1 IU/L. After LHRH stimulation, FSH in males: Tanner stage 1, <1.1–6.3 IU/L; Tanner stages 2–3, <4 IU/L and Tanner stages 4–5, 4–15.2 IU/L. Basal LH in males: Tanner stage 1, <1–1.5 IU/L; Tanner stages 2–3, 1.4–4.1 IU/L and Tanner stages 4–5, 3.4–7.5 IU/L. After LHRH stimulation, LH in males: Tanner stage 1, <1.4–4.1 IU/L; Tanner stages 2–3, 1.5–4.1 IU/L and Tanner stages 4–5, 19–37 IU/L. Inhibin B reference values: see reference (25). AMH (26); in childhood, median = 105.7 ng/ml and range = 55.3–186.9 ng/ml; prepubertal <9 years =97.58 (44.94–170.52); prepubertal >9 years =81.9 (41.58–155.82); Tanner G2 =35.42 (6.44–156.8); Tanner G3 =9.24 (3.08–102.76); Tanner G4 =6.02 (2.1–15.68) and Tanner G5 =6.86 (3.22–17.92). NA, not available; \(\uparrow\), high; \(\downarrow\), low; N, normal.


is predicted to result in conformational change of the protein. The arginine residue in position 62 is highly conserved in all species (Fig. 1). DNA of the parents was not available for genetic analysis.

**Patient 2 (p.Glu121AlafsX25)** Patient 2 is a male born to non-consanguineous parents. He presented at 13 years of age with severely hypoplastic phallus (stretched penile length was 3.5 cm, <−2.5 SDS) and impalpable right testis. The left testis was descended and of average size. Pelvic laparoscopy revealed atrophic right testis and no Müllerian structures. He was operated upon for correction of hypospadias and bifid scrotum. Serum post-hCG testosterone level was low for age. AMH and inhibin B levels were very low for age and Tanner stage (Table 1). The patient showed no signs or symptoms of the adrenals being affected. Mutational analysis revealed a heterozygous p.Glu121AlafsX25 (c.361delGAGACAGG) which is an 8 bp deletion mutation causing frameshift resulting in a premature stop codon in exon 4 (Fig. 1), predicted to produce either a severely truncated protein or no protein at all through nonsense-mediated mRNA decay. It is a *de novo* mutation as none of the parents harbour it.

**Patient 3 (p.Ala154Thr)** Patient 3 is a male patient, born to consanguineous parents, who presented at 4 years of age with hypoplastic anchored phallus (stretched penile length was 2.5 cm, <−2.5 SDS) and penile hypospadias. Both testes were descended and normal. Hypospadias was surgically corrected. Serum basal testosterone level was normal for age, while post-hCG testosterone level was slightly decreased. LHRH stimulation test revealed slightly elevated FSH level. Inhibin B and AMH levels were normal for age, while post-hCG testosterone level was slightly decreased. LHRH stimulation test revealed slightly elevated FSH level. Inhibin B and AMH levels were normal (Table 1). The patient showed no clinical signs or symptoms of the adrenals being affected. Mutational analysis revealed a heterozygous p.Ala154Thr (c.460 G>A) mutation which lies in hinge region in exon 4. The hinge region is important for transcriptional capacity of SF1 as it contains a phosphorylation site and a synergy control motif with sumoylation sites and hence is predicted to reduce the transcriptional capacity of the protein (24). Both the nucleotide and the amino acid in this position are conserved in human, chimp and rhesus but not in other species (Fig. 1). There were no SNPs found in the altered region as well as no abrogation of potential splice sites.

The heterozygous p.Ala154Thr mutation was inherited from the unaffected father. He had normal male genitalia (G5, P5, testes 25 ml) and a history of normal sexual activity and fathered two other children. The mother showed a normal WT sequence. All 100 Egyptian controls did not harbour this mutation.

**Single-nucleotide polymorphisms**

Six different SNPs were detected in our patients in both heterozygous and homozygous forms: p.Gly146 Ala, p.Pro125Pro, IVS4-13, c.871-20bp C>T, p.Leu451Leu and c.*82 C>T. The p.Gly146Ala (c.437G>C, rs1110061) polymorphism, was found in 17 patients (34%) and 31 controls in this study. We report a frequency of p.Gly146Ala in Egypt (31%), which is synonymous with the global minor allele frequency (27.2%) in the 1000 Genome phase 1 population (http://www.1000genomes.org) from 1094 worldwide individuals, making it a common polymorphism in Egypt as well (27). We detected the heterozygous form of this SNP (Ala/Gly genotype) in 34% of our patients (17/50) and 26% of controls (26/100). Moreover, the homozygous form (Ala/Ala genotype) was found in 5% of controls (5/100) and in none of our patients. In previous studies, the SNP p.Gly146Ala was described as having slightly reduced transcriptional activity and to be more common in cryptorchidism (28, 29).

Furthermore, two novel heterozygous intron 6 mutations (c.1137+105b C>T and c.1137+129b C>A) were detected 105 and 129 bp, respectively, following exon 6. Neither of them was reported in the SNP databases (Ensembl, Exome Variant Server). Neither were detected in the control cohort.

**Functional analysis**

Both p.Ala154Thr and p.Arg62Cys show an aberrant transactivation of the known SF1-responsive reporters (human AMH promoter, human CYP11A1 promoter and mouse TESCO core enhancer of Sox9) in human embryonic kidney, HEK293-T, cells (Fig. 3). A previously published loss of function p.Gly35Glu mutation was used as a control. The p.Arg62Cys mutant protein shows a significant reduction, whereas p.Ala154Thr shows a significant increase in its ability to stimulate either of the reporter genes’ activity. The activities of the mutant proteins were calculated relative to that of the WT NR5A1, which was designated as 100%.

**Discussion**

A growing number of heterozygous mutations in NR5A1 have been identified in patients displaying the most
common SF1 phenotype of 46,XY DSD with ambiguous genitalia due to severe partial gonadal dysgenesis at birth (9, 16, 21, 28, 29, 30, 31, 32, 33, 34, 35). Recent studies have reported several cases with NR5A1 mutations and the milder 46,XY DSD phenotype of hypospadias (8, 9, 10, 16) (Fig. 2). We report three novel NR5A1 mutations with variable degrees of hypospadias from an Egyptian cohort of patients with 46,XY DSD. An estimated frequency of 3–5% has been reported in patients with hypospadias in Europe (8, 10). We found two novel NR5A1 mutations with reduced transcriptional function in 23 patients with hypospadias (8.5%). This rather high frequency might be due to preselection of more severe cases (Fig. 2).

In 46,XY DSD patients with severe underandrogenisation (female or severely ambiguous external genitalia and cryptorchidism with or without uterus), a frequency of NR5A1 mutations of 8–15% (8, 10, 16) has been reported in Europe. In this study, we investigated 13 patients with a similar phenotype and could not detect any NR5A1 mutations.

All three patients with NR5A1 mutations were presenting with penile hypospadias and hypoplastic phallus. Patients 1 and 2 (p.Arg62Cys and p.Glu121A-lafsX25) also displayed maldescended testes and partial gonadal dysgenesis with impaired Leydig cell and Sertoli cell functions (low testosterone, low inhibin B and AMH). Patient 3 (p.Ala154Thr) showed normally descended testes, only mildly impaired Leydig cell and normal Sertoli cell function (slightly decreased testosterone, normal inhibin B and AMH) (Table 1).

The results of the functional in vitro assays of the mutations of patients 1 and 3 were in accordance with their phenotypes. The p.Arg62Cys mutation of patient 1 showed significantly reduced transcriptional activity of the CYP11A1 and AMH promoters and TESCO, while the p.Ala154Thr mutation of patient 3 did not result in reduction of transcriptional activity of any of the three reporters (Fig. 3).

Our patients demonstrated moderate clinical underandrogenisation at birth showing that Leydig cell function was already disturbed during prenatal life. In contrast, embryonic Sertoli cell function seemed to have been sufficient for adequate AMH production and subsequent Müllerian duct regression prenatally. In patients 1 and 2, AMH and inhibin B levels were diminished at 3 and 13 years of age respectively, revealing progressive Sertoli cell hypofunction as described previously in 46,XY patients with NR5A1 mutations and progressive deterioration of the Sertoli cell function with age (13, 36). Patient 3 displayed normal Sertoli cell function at 4 years of age.
To date, two other mutations were reported with isolated hypospadias and normal testes (9, 10). Warman et al. reported a family with six members harbouring a heterozygous NR5A1 hinge region mutation (p.Tyr183X) and displaying a wide range of different phenotypes. Three affected members had isolated hypospadias, two of whom were severe perineal and one was mild glandular (9). Another case of isolated distal hypospadias carrying a de novo heterozygous missense mutation (p.Arg313Cys) located at the end of the ligand-binding domain of the NR5A1 gene was reported by Allali et al. (10) (Table 2).

So far, no clear genotype–phenotype correlation could be detected in patients with NR5A1 mutations. However, in patients with severe forms of 46,XY DSD, previously described mutations are mostly missense mutations in the DNA-binding region (including its accessory DNA-binding domain) or in the ligand-binding domain as well as nonsense mutations leading to severe changes of the protein (8, 10, 16, 17, 21, 30, 31, 32, 33, 36, 37, 38, 39).

We hypothesise that the mild clinical picture of patient 3 might be attributed to the less critical mutation in the hinge region. However, so far, the p.Ala154Thr mutant did not show reduced transcriptional activity in vitro. But, interaction of the mutant with modifying factors affecting testosterone production through protein interaction might play a role in the development of hypospadias in this patient. Further studies are needed to support this hypothesis. So far, almost only nonsense mutations in the hinge region have been reported in the DSD phenotypes (9, 10, 16, 17, 21, 30, 34), hypospadias (8, 9), 46,XX primary ovarian insufficiency (16, 17) and premature ovarian failure (9). In contrast, only missense mutations in the hinge region were detected in the minor DSD phenotype of male infertility (13, 14) (Table 2).

Interestingly, the unaffected father of patient 3 harboured the same mutation. Previously, another case was reported with a heterozygous NR5A1 mutation (p.Arg281Pro) transmitted by the unaffected father to his son with hypospadias and testicular dysgenesis (40). Mosaicism was suggested to be the reason for the different phenotypes in the father and son as the mutation was detected in the father’s DNA at a low copy number through direct sequencing and high-resolution melting assay (40). In our case, the father’s and son’s mutations showed similar strength in the sequences (data not shown). However, genetic modifier, thresholds or dosage effects of SF1 might contribute to the different phenotypes of father and son.

The p.Gly146Ala polymorphism, which was described previously as having reduced transcriptional activity and being associated with micropenis or cryptorchidism, was found to be very frequent in our Egyptian cohort with 46,XY DSD and the controls in this study. We detected the heterozygous form of this SNP (Ala/Gly genotype) in 34% of our patients and 26% of controls. Moreover, the SNP was found in a homozygous form (Ala/Ala genotype) in 5% of controls. The high frequency of p.Gly146Ala in our controls suggests no pathogenicity compared with the previous studies (28, 29).

This is the first work searching for NR5A1 mutations in patients from the Middle East and Arab region with high consanguinity suggesting a high frequency of NR5A1 mutations as in European cohorts. The type and location of the mutation might be the cause of some differences of the phenotypes. However, most probably, other additional modifying factors or digenic mutations should be considered especially in familial cases (35).

### Table 2 SF1 mutations described previously in the hinge region and their phenotype.

<table>
<thead>
<tr>
<th>Phenotype group</th>
<th>SF1 mutations in hinge region</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete/severe gonadal dysgenesis female external genitalia with (or NA) Müllerian structures</td>
<td>c.424_427dupCCCA c.666delC</td>
<td>(8) (17)</td>
</tr>
</tbody>
</table>

*None of the patients had adrenal failure.*
In conclusion, we also recommend the screening of NR5A1 in patients with hypospadias and gonadal dysgenesis as well as yearly follow-ups of gonadal function beginning at puberty due to the risk of future fertility problems. As the clinical consequences are not yet clear, more outcome data are needed before recommending general screening of NR5A1 in isolated hypospadias. However, future mild progressive effects on the gonads resulting in male infertility cannot be excluded in this case, as also infertile males with normal male gonadals resulting in male infertility cannot be excluded.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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