Author's response to reviews

Title: Novel p.Cys65Tyr mutation in NR5A1 gene in three 46,XY siblings with normal testosterone levels and signs of late adrenal insufficiency and their mother with primary ovarian insufficiency

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Author's response to reviews: see over
The novel p.Cys65Tyr mutation in NR5A1 gene in three 46,XY siblings with 46,XY disorders normal testosterone levels and signs of sex development late adrenal insufficiency and their mother with primary ovarian insufficiency

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**Background:** Disorders of sex development (DSD) is the term used for congenital conditions in which development of chromosomal, gonadal, or phenotypic sex is atypical. **There are several genes that participate in both sex determination and differentiation processes.** Nuclear receptor subfamily 5, group A, member 1 gene (NR5A1) encodes steroidogenic factor 1 (SF1), a transcription factor, is involved in that regulates gonadal development and regulates adrenal steroidogenesis. Mutations in the NR5A1 gene may lead to different 46,XX or 46,XY DSD phenotypes with or without adrenal failure. We report a Brazilian family with a novel NR5A1 mutation causing ambiguous genitalia in 46,XY affected individuals and signs of premature ovarian failure. They had apparently normal Leydig and Sertoli function. Currently, their laboratory data suggest late-onset adrenal insufficiency, and their heterozygous mother presents evidences of primary ovarian insufficiency. **Case presentation:** Three siblings presenting 46,XY DSD. A family with three siblings with 46,XY DSD, ambiguous genitalia and normal testosterone production was included in the study. Molecular analyses were carried out for AR, SRD5A2 and NR5A1 genes. **Sequencing AR and SRD5A2 gene sequencing did not reveal any mutation.** However, NR5A2 sequence analysis indicated that all three siblings were heterozygous for p.Cys65Tyr mutation which was inherited from their mother. **In silico analysis was carried out to elucidate the role of the amino acid change on the protein function.** After the mutation was found, all sibs and the mother had been reevaluated. Basal hormone dosages were normal except ACTH levels that were slightly elevated. After 1 mcg ACTH stimulation test, only the older sib showed subnormal cortisol response. **Conclusion:** The p.Cys65Tyr mutation located within the second zinc finger of DNA binding domain was considered deleterious upon analysis with predictive algorithms. The identification of heterozygous individuals with this novel mutation may bring additional knowledge on structural modifications that may influence NR5A1 DNA-binding ability, and may also contribute to genotype-phenotype correlations in DSD since hormone dosages in the three sibs with. Considering that basal ACTH levels were slightly elevated for all three patients with 46,XY DSD and in their mother suggest that the proband responded with subnormal cortisol dosage after ACTH stimulation, it may be inferred that this mutation may lead to late-onset adrenal
disorder and ovarian abnormalities, respectively. A long-term follow-up is essential for these patients. Our data reinforce that NR5A1 analysis must also be performed in 46,XY DSD patients with normal testosterone levels without AR mutations.

**Keywords:** disorders of sex development, NR5A1 mutation, premature ovarian failure, primary ovarian insufficiency

**Background**

Steroidogenic factor 1 (SF1), denominated as nuclear receptor subfamily 5 group A member 1 (NR5A1 [OMIM +184757]), is a protein that regulates several steps of adrenal and gonadal development [1,2]. It is encoded by NR5A1 gene, which is an autosomal gene mapped to 30 kb within 9q33. NR5A1 gene sequence includes one non-translated exon (exon 1), six coding exons (exon 2-7) and six introns [3,4]. The SF1 protein contains 461 amino acid divided into a two zinc-finger DNA-binding domain (DBD), a ligand-binding domain (LBD), two functional activation domains (AF-1 and AF-2), an accessory region, and a hinge region [5]. SF1 protein is extremely conserved among species, presenting and presents 95% overall amino acid homology/identity between human and mouse sequences [6].

NR5A1 is expressed in the developing urogenital ridge, steroidogenic tissues (as gonads, adrenals, and placenta), hypothalamus and anterior pituitary [7-9]. In general, it activates the expression of AMH in Sertoli cells leading to the regression of Müllerian structures [1,2,9]; in Leydig cells, it activates the expression of several enzymes involved in steroidogenesis, resulting in virilization of external genitalia and testicular descent [1,2,9]; and, in ovaries, NR5A1 is expressed in the granulosa and theca cells where it regulates genes required for ovarian steroidogenesis and follicle growth maturation [8,9]. Genes such as: CYPs, HSD3B2, StAR, SOX9, NR0B1, and others are among the gene targets for NR5A1/SF1 regulation [5]. As an essential transcription regulator for adrenal and gonadal development NR5A1, SF1 is very important in the sex differentiation processes, although it also plays important physiological roles in the central nervous system [10]. Therefore, mutations in NR5A1 may lead to Disorders of Sex Development (DSD) defined as incomplete or disordered
gonadal or genital development, causing divergences between genetic sex, gonadal sex and phenotypic sex [11,12].

p.Gly35Glu and p.Arg92Gln were the first two mutations described in human NR5A1. They had been identified in patients with primary adrenal insufficiency, complete gonadal dysgenesis and Müllerian duct persistence [13,14]. After those, over 50 mutations have been reported in a large number of 46,XY DSD individuals with apparently normal adrenal function, in 46,XX individuals with premature ovarian failure and normal female phenotype and also with male individuals with infertility [15-19]. In addition, several reports demonstrated that NR5A1 variations might also be associated with hypospadias, anorchia, and with some cases of adrenal tumors and endometriosis [20,21].

The findings in the literature indicate a complex phenotype expressivity of NR5A1 mutations. Therefore, it is difficult to establish a direct phenotype-genotype correlation [22]. Recently, some authors have reported heterozygous loss-of-function NR5A1 mutations in patients with clinical features of androgen insensitivity syndrome (AIS) and apparently normal Leydig and Sertoli cell function but without androgen receptor gene (AR) mutations [23-25].

In this report, we describe the novel c.195G>A NR5A1 gene mutation identified in three siblings with 46,XY DSD. The siblings were born of non-consanguineous parents, and had been brought to medical care due to genital ambiguity. All of them had normal testosterone levels in first months of life and the proband had normal male puberty. Current laboratory data suggest late-onset adrenal insufficiency. Molecular analyses showed that the putative p.Cys65Tyr missense was inherited from the mother, who presented with signs of premature ovarian failure and primary ovarian insufficiency.

Case Presentation

Case report

The study was undertaken under an institutionally approved ethic protocol and informed consent was obtained from all subjects and relatives.
Three affected siblings with 46,XY DSD had been evaluated (Fig. 1A). The index case, now aged 14-15, was born at term to healthy non-consanguineous parents, after an uneventful pregnancy. He had received a female sex assignment at birth and was first seen with referred to our service due to genital ambiguity, at the age of 2-3 months when he still had a female sex assignment. Physical examination revealed a 2-cm phallus, a single perineal opening, and palpable gonads were palpable in the labioscrotal folds. Laboratory dosages indicated high levels of FSH but normal levels of LH, and a normal testosterone response to hCG test. The karyotype was 46,XY and pelvic ultrasound showed absence of mullerian derivatives. The medical team presented such results to parents, who also received support from a Psychologist. They decided for a female to male sex reassignment a few months later. Therefore, hypospadias repair was done at the age of 1 year and 4 months, when he underwent hypospadias repair. Spontaneous puberty, Puberty began spontaneously when he was 11 years, with sustained high levels of old. Hormone dosages have been performed every year. Testosterone and FSH, initially normal levels remained normal and high, respectively; whereas, LH levels, that were initially normal, progressively elevated with time, and normal levels of testosterone (Table 1). Currently, he is now at Tanner stage G4P5 without sex hormone replacement. His height is near the target. Recent evaluation of the adrenal function revealed slightly elevated and normal basal levels for ACTH and cortisol, respectively (Table 1). However, cortisol response was subnormal after stimulation with 1 mcg ACTH (Table 1).

The second sib, now currently aged 6-7 years, was born at term after an uneventful pregnancy. At birth he presented with a 2-cm phallus, penoscrotal hypospadias and palpable gonads in the labioscrotal folds. His karyotype was 46,XY. Pelvic ultrasound did not show mullerian derivatives. Hormone investigation was performed with the age of 2 month, with at 2 month of age indicated high levels of LH level and normal levels of FSH and testosterone (Table 1). He had At that time, he was assigned as male and had hypospadias repair. In a male sex assignment and hypospadias repair. Recent recent evaluation of the adrenal function
revealed, basal levels for ACTH and cortisol were, respectively, slightly elevated and normal (Table 1). Upon 1 mcg ACTH stimulation, cortisol response remained normal (Table 1).

The third sib, currently aged 56 years, was born at term after an uneventful pregnancy with a 1.3-cm phallus, a single perineal urogenital opening, and both palpable gonads were palpable in the inguinal region. He also had a normal 46,XY karyotype and a. Pelvic ultrasound examination did not show mullerian derivatives. At the age of 2 months, normal hormone levels for FSH, LH, testosterone and dihydrotestosterone were normal (Table 1). He was assigned as male and underwent further hypospadias repair and orchidopexy. Recent evaluation of the adrenal function revealed slightly elevated ACTH with normal cortisol levels (Table 1). He also had a normal cortisol response after 1 mcg ACTH stimulation (Table 1).

The 33-year-old mother was subject to a thorough hormone evaluation after identifying NR5A1 mutation was detected (see below). Hormonal results revealed high FSH, normal to high LH and normal to low estradiol levels with normal adrenal function (Table 1). She also referred irregular menses as a clinical symptom and hot flushes, suggesting premature primary ovarian failure insufficiency.

**Methods**

Genomic DNAs from patients and parents were purified from peripheral leukocytes by proteinase K lysis, phenol/chloroform extraction, and ethanol precipitation, according to standard techniques. Sequencing of both AR (androgen receptor) and SRD5A2 (5α-reductase) genes had been performed as described elsewhere [23,24,26,27]. The NR5A1 exons and 5' and 3' untranslated flanking regions were amplified by polymerase chain reaction (PCR) using specific primers designed based on the normal gene sequence (ENSG00000136931, www.ensembl.org). Independent PCR fragments were purified in 1% agarose gel electrophoresis with the Wizard SV Gel and PCR clean-up system (Promega, Madison, WI, USA), and both sense and antisense strands were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Grand Island, NY, USA) with the same primers used in PCR reactions. The Chromas Lite 2.0 (Technelysium Pty Ltd) and CLC Sequence Viewer
v.6.8.1 free software (CLC bio) were used to analyze and compare sequences with the reference NR5A1 sequence. Structural analyses were performed using PDB ID: 2FF0 – chain A as template. The native and mutant models were constructed by SWISS MODEL web-served program. Internal contacts were evaluated by STING Millenium (http://www.cbi.cnptia.embrapa.br) and visualized by PyMol®.

**Results**

DNA sequence analyses of AR and SRD5A2 genes did not show any mutation. However, NR5A1 gene sequencing revealed a novel heterozygous transition G>A within exon 3 in all three heterozygous siblings as well as in their mother (Fig. 1B). The nucleotide change c.195G>A is predicted to cause the substitution of a cysteine by a tyrosine at the amino acid residue 65 (p.Cys65Tyr).

Residue 65 in the NR5A1 protein corresponds to a highly conserved cysteine in mammal corresponding proteins (Fig. 2A). It is located at the second zinc finger of DNA binding domain, as illustrated by Little *et al.* [28] (Fig. 2B,C). Structural analyses demonstrated that C65 in the native protein, besides binding directly to zinc atom, it makes a hydrogen bond with R69 and hydrophobic interaction with C68 (Fig. 2D). The mutant Y65 maintains both interactions. However, a new hydrophobic interaction by 3.42 Ångstrons is established with C55 (Fig. 2E).

Three predictive methods to evaluate the effect of the amino acid substitution were used: PolyPhen (Polymorphism Phenotyping) that gives scores ranging from 0 (neutral) to a positive (damaging) number; SIFT (Sorting Intolerant From Tolerant) whose scores range from 0 (damaging) to 1 (neutral); and Aling GV-GD that classifies the amino acid change into classes ranging from C0 to C65, where C0 is considered tolerant and C65 deleterious [29,30]. The p.Cys65Tyr mutation resulted in PolyPhen score of 1.0, SIFT score of 0 and Aling GV-GD put it into class C65 indicating a protein damage, probably leading to patients’ phenotypes. In order to discard the possibility of the nucleotide variation being a frequent polymorphism, 86 healthy controls (172 alleles) were analyzed and c.195G>A was not identified in any allele.
Discussion

We present here the follow-up of patients with 46,XY DSD in a Brazilian family. The three siblings described here presented with different hormone profiles at minipuberty in the first year of life: all of them with normal testosterone levels, but associated with isolated elevation of FSH (sib 1), isolated elevation of LH (sib 2); or normal FSH, LH, and testosterone levels (sib 3) (Table 1). Initially, AR and SRD5A2 gene sequence analyses were performed due to the characteristics such as: 46,XY karyotype, genital ambiguity without Müllerian derivatives and normal testosterone production. Nevertheless, As mutations in those genes had not been identified, patients remained idiopathic, and the etiologic diagnosis was not defined, even though the severity of genital ambiguity and the familial recurrence clearly indicated a genetic origin. After long-term follow-up, the etiology of 46,XY DSD in these cases had been clarified due to the identification of p.Cys65Tyr mutation on the in NR5A1 gene which was investigated based on the recent knowledge on description of variable phenotypic expression of phenotypes as results of different NR5A1 mutations, and on the possibility of the mutation inheritance from a fertile mother, which mimicked an X-linked recessive pattern [23-25].

Several mutations have already been described in NR5A1 in different cases of 46,XY patients with NR5A1 loss-of-function mutations have had biological markers that evidenced gonadal dysgenesis, i.e.: LH and FSH levels were usually elevated, AMH levels, if measured, were low, and Leydig cell function was invariably defective, indicated by low levels of testosterone [23]. Here we report three sibs with normal testosterone levels during the first year of life. Puberty development of the proband was also indicative of normal Leydig cell function. In addition, Müllerian derivatives were not found suggesting that Sertoli cell function was also normal. As discussed by Tantawy et al. [31], there are very few reports on 46,XY DSD cases that had puberty development and normal male testosterone production for spontaneous virilization, however long-term follow-up of such cases indicated a progressive gonadal failure with elevated FSH. Although the proband described here developed normal puberty, he
persisted with high levels of FSH from first months of life till puberty suggesting some degree of tubular defect that might cause fertility impairment.

Recently, Wu et al. [25] speculated if SF1 disruption caused by NR5A1 loss-of-function mutations may be associated with functional androgen resistance or alter Leydig cell maturation leading to hyper-responsiveness to postnatal LH stimulation. Our data also suggest such a mechanism and reinforce that, in the absence of AR mutations, NR5A1 gene analysis must be performed in 46,XY DSD despite normal testosterone levels.

Several mutations have been described in NR5A1 in different cases of 46,XY DSD so far [15,17,32,33], however a mutation in the C65 residue has been here described here for the first time. The nucleotide change c.195G>A results in the p.Cys65Tyr missense which is predicted as damaging by in silico tools. It is well known that cysteine influences the overall three-dimensional structure of proteins. Its sulfur group reacts quite readily with other sulfur groups, forming disulfide bonds that play important role in the folding and stability of proteins. Cysteine residues also play a valuable role in crosslinking proteins, which increases protein rigidity and also confers proteolytic resistance [34]. Conversely, tyrosine is an aromatic and partially hydrophobic amino acid [34]. The aromatic side chain is usually involved in stacking interactions with other aromatic side chains [34]. Tyrosine can also be involved in phosphorylation within intracellular protein [34]. In the structural analyses, a previously inexistent hydrophobic interaction with C55 was observed for Y65 residue. In addition, this novel mutation is located within the second zinc finger of DNA binding domain and where C65 itself binds directly to the zinc atom [28], therefore the change to tyrosine may influence NR5A1 DNA-binding ability by destabilizing zinc-finger conformation. Although further functional analyses will be necessary for the formal demonstration of a deleterious effect for p.Cys65Tyr mutation, both predictive and structural analyses indicate that it might correlate with the DSD phenotype in the three heterozygous siblings. Their fertile 32,33-year-old mother who is also heterozygous for the mutation referred irregular menses and hot flushes, after molecular diagnosis. Upon endocrine investigation, levels for gonadotropin and estradiol levels suggest premature primary ovarian failure insufficiency. Taking together, those
data indicate that p.Cys65Tyr mutation may also compromise the ovarian functional maintenance similar to other NR5A1 mutations described in the literature [15-17].

Considering the slightly elevated basal ACTH levels in all three patients with 46,XY DSD and the subnormal cortisol response after stimulation test with 1 mcg ACTH in the proband, it may be inferred that p.Cys65Tyr mutation might probably present could have a late-onset effect upon adrenal function, justifying a long term follow-up on such patients.

In conclusion, based on recent knowledge concerning the phenotypic expression of NR5A1 mutations, the analysis of this gene becomes an important tool not only for diagnosing patients with DSD including the cases with normal testosterone secretion without AR mutations, but also for identifying their female relatives at risk of developing primary ovarian insufficiency and allowing reproductive counseling as well as potentially assisted reproductive techniques.

Consents
Written informed consent was obtained from each member of the family for publication of this Case Report and any accompanying images. A copy of the written consent is available for review by the Series Editor of this journal.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
HCF carried out the molecular genetic studies with NR5A1 gene, participated in the NR5A1 sequence alignment and drafted the manuscript. JGRA carried out the clinical genetic studies and contributed with writing clinical description of the cases for the manuscript draft. FCS contributed with the structural analysis of normal and mutant proteins. FLC conducted the molecular genetic studies with SRD5A2 gene and sequence alignment investigation. RJP was responsible for the molecular genetic studies with AR gene and sequence alignment comparison.
ATM-G participated in the design of the study and was responsible for the clinical genetic evaluation of the patients. GG-J participated in the design of the study and was responsible for the endocrine evaluation of the patients. MP-de-M conceived of the study, and participated in its design and coordination and helped to draft the manuscript.

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References


Legend of the Figures

**Figure 1.** A) Pedigree of the family. The three siblings and the mother carry the mutation c.195G>A. B) Electropherogram showing part of NR5A1 exon 3 sequence where the c.195G>A heterozygous transition leading to p.Cys65Tyr mutation occurred.

**Figure 2.** A) Multiple alignment of NR5A1 protein family using ClustalW: the conserved residue C65 is shown in red. B) Scheme of the two zinc fingers from the DNA-binding domain (DBD). Red circle denotes the C65 residue (adapted from Little *et al.* [25]). C) Structural complex of NR5A1 bound to DNA showing the C65 residue ligated to the zinc atom at the zinc-finger binding site within the DNA binding domain. D) Structural model of the native protein showing internal contacts. The C65 interacts by hydrogen bond with R69 and hydrophobic interaction with C68. E) Mutant protein internal contacts. The Y65 establish new hydrophobic contact with C55.
Table 1: Hormonal values for the three patients.

<table>
<thead>
<tr>
<th>Patient, Age (yr)</th>
<th>Clinical Presentation</th>
<th>Testosterone (nmol/L)</th>
<th>FSH (IU/L)</th>
<th>LH (IU/L)</th>
<th>Estradiol (nmol/L)</th>
<th>Cortisol (nmol/L)</th>
<th>ACTH (pmol/L)</th>
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<tbody>
<tr>
<td><strong>Patient 1</strong></td>
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<tr>
<td>0.75</td>
<td>micropenis and perineal hypospadia</td>
<td>1.04 (pre hCG)</td>
<td>11.90</td>
<td>2.80</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>11</td>
<td>Started spontaneous puberty</td>
<td>6.59 (after hCG)</td>
<td></td>
<td></td>
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<tr>
<td>13</td>
<td>Tanner IV</td>
<td>12.87</td>
<td>27.25</td>
<td>11.96</td>
<td>-</td>
<td>376.46</td>
<td>46.17</td>
</tr>
<tr>
<td>14</td>
<td>Tanner IV-V</td>
<td>15.89</td>
<td>23.51</td>
<td>10.95</td>
<td>-</td>
<td>261.80</td>
<td>16.10</td>
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<tr>
<td>15</td>
<td>Tanner IV-V</td>
<td>16.79</td>
<td>21.60</td>
<td>16.13</td>
<td>-</td>
<td>421.60*</td>
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<tr>
<td><strong>Patient 2</strong></td>
<td></td>
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<tr>
<td>0.17</td>
<td>micropenis and penoscrotal hypospadia</td>
<td>7.63</td>
<td>4.85</td>
<td>10.00</td>
<td>-</td>
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<tr>
<td>6</td>
<td>Tanner I</td>
<td>0.66</td>
<td>1.15</td>
<td>&lt;0.10</td>
<td>-</td>
<td>576.01</td>
<td>14.87</td>
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<tr>
<td>7</td>
<td>Tanner I</td>
<td>0.66</td>
<td>1.51</td>
<td>&lt;0.10</td>
<td>-</td>
<td>195.61</td>
<td>14.87</td>
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<tr>
<td><strong>Patient 3</strong></td>
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<tr>
<td>0.17</td>
<td>micropenis, perineal urogenital opening and bilateral cryptorchidism</td>
<td>0.66 (pre hCG)</td>
<td>4.62</td>
<td>0.20</td>
<td>-</td>
<td>-</td>
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<tr>
<td>5</td>
<td>Tanner I</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>820.27</td>
<td>12.61</td>
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<tr>
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<td>Tanner I</td>
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<td>2.60</td>
<td>&lt;0.10</td>
<td>-</td>
<td>300.21</td>
<td>12.61</td>
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<tr>
<td><strong>Mother</strong></td>
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<tr>
<td>32</td>
<td>Irregular menses</td>
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<td><strong>Normal Range</strong></td>
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<td></td>
<td></td>
<td>48.88-13.81</td>
<td>19.82-7.05</td>
<td>109.11-176.22</td>
<td>218.31</td>
<td>9.66</td>
<td>7.37</td>
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<tr>
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<td></td>
<td>Male: 2.42-12.48</td>
<td>Male: 1.50-12.40</td>
<td>Male: 1.70-8.60</td>
<td>218.31</td>
<td>9.66</td>
<td>7.37</td>
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<tr>
<td></td>
<td></td>
<td>Female: &lt;5.80</td>
<td>Female: &lt;6.80</td>
<td>Female: &lt;6.80</td>
<td>218.31</td>
<td>9.66</td>
<td>7.37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 a.m.: 138.00-690.00</td>
<td>388.70</td>
<td>9.66</td>
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</tbody>
</table>

For the patients, testosterone values were measured before (pre hCG) and after (after hCG) human chorionic gonadotropin (hCG) stimulation. For the mother, values were measured without stimulation.
<table>
<thead>
<tr>
<th>Tanner III</th>
<th>Tanner IV</th>
<th>Tanner V</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.25-27.07</td>
<td>6.47-26.37</td>
<td>6.59-30.54</td>
</tr>
<tr>
<td>Male 18-49 yrs: 8.64-29.01</td>
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**Normal Range**

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Value 1</th>
<th>Value 2</th>
<th>Value 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newborn</td>
<td>2.43-13.88</td>
<td>5.89</td>
<td>645.33*</td>
</tr>
<tr>
<td>Children</td>
<td>&lt;5.89</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Puberty:</td>
<td>Tanner I - &lt;0.69</td>
<td>Tanner II - &lt;14.92</td>
<td>Tanner III – 2.25- 27.07</td>
</tr>
<tr>
<td></td>
<td>Tanner IV – 6.47-26.37</td>
<td>Tanner V – 6.59-30.54</td>
<td></td>
</tr>
<tr>
<td>Male 18-49 yrs: 8.64-29.01</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Female:

- Male: 1.50-12.40
- Female (menopause): 1.70-8.60
- Female (menopause): <200.75
- 8 a.m.: 138.00-690.00
- *after 1 mcg ACTH test: ≥ 551.80

*<10.12