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ORIGINAL RESEARCH

# Lack of Influence of the Androgen Receptor Gene CAG-Repeat Polymorphism on Clinical and Electrocardiographic Manifestations of the Brugada Syndrome in Man

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**Abstract**

**Background:** Clinical studies suggest that testosterone (T) plays an important role in the male predominance of the clinical manifestations of the Brugada syndrome (BS). However, no statistically significant correlations have been observed between T levels and electrocardiogram (ECG) parameters in the BS patients. We investigated whether the hormonal pattern and the variation within CAG repeat polymorphism in exon 1 of the androgen receptor (AR) gene, affecting androgen sensitivity, are associated with the Brugada ECG phenotype in males.

**Methods and Results:** 16 male patients with BS (mean age 45.06 ± 11.3 years) were studied. 12-lead ECG was recorded. Blood levels of follicle-stimulating hormone, luteinizing hormone, prolactin, testosterone, free-T, dihydrotestosterone, 17-β-estradiol, estrone, 3-alpha-androstanediol-glucuronide, delta-4-androstenedione, dehydroepiandrosterone sulphate, progesterone, 17-hydroxyprogesterone, and sex hormone binding globulin were assayed. Genotyping of CAG repeats on DNA extracted from leukocytes was carried out. No relationship was found between hormone values and ECG parameters of BS. BS patients showed the CAG length normally recognized in the human polymorphism range and the number of CAG repeats did not correlate with the ECG pattern of BS.

**Conclusions:** The AR CAG repeat length does not correlate with the ECG features of the patients affected by BS. The search for genes downstream AR activation as possibly responsible for the increased risk of spontaneous arrhythmias in BS males after puberty is warranted.

**Keywords:** androgen receptor, CAG repeat polymorphism, Brugada syndrome

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## Introduction

Brugada syndrome (BS) is an autosomal dominant disease with low penetrance characterized by an electrocardiographic pattern of complete or incomplete right bundle branch block and ST segment elevation in leads V1–V3, complicated by ventricular tachycardia/ventricular fibrillation, sudden death, or syncope.[1] Roughly 11% to 28% of the patients with BS have mutations of the sodium channel gene (SCN5A) and mutations of genes that modulate sodium channel function are also associated with BS.[1] Although BS is inherited with equal frequency in men and women, a significant male predominance in BS clinical manifestations has long been reported[2] and the majority of carriers who actually develop arrhythmias are adults.[3] In contrast, no obvious male predominance exists among symptomatic or asymptomatic prepubertal children with BS.[4]

The prevailing explanation for the increased risk of spontaneous arrhythmias after puberty in males suffering for BS is that hormonal changes, and particularly the increase of testosterone (T), worsen the unbalanced flow of ion currents in BS heart.[5] In 2 reported cases of BS, the typical electrocardiogram (ECG) pattern disappeared following surgical castration for prostate cancer[6] and a case of BS showing a relationship between diurnal variations of T levels and ECG parameters has been described.[7] Nevertheless, although T was significantly higher in the Brugada males than in control males, no statistically significant correlations were observed between T levels and ST amplitude.[8] In addition, there were no significant differences in T values between symptomatic and asymptomatic Brugada males, between Brugada males with spontaneous ST elevation and those with sodium channel blocker-induced ST elevation, or between Brugada males with or without SCN5A mutation.[5] Additionally, although it has been reported that men with Brugada-like ECG have higher risk of prostate cancer, T or other sexual hormones were not measured in these patients.[9]

One possible explanation for the differences in the sensitivity to androgen resides in the CAG repeat length in exon 1 of the androgen receptor (AR). The number of AR CAG repeats is normally inversely associated with the transcriptional response to T in vitro.[10,11] Very large numbers of CAG repeats result in Kennedy's disease, a X-linked spinobulbar muscular atrophy.[12] A clinical correlation between CAG repeat number and biochemical markers of androgen insensitivity has been demonstrated in these patients.[13] In men whose CAG repeats are within the normal range, clinical indices of androgen action such as prostate disease[14–16] and androgen insensitivity, such as impaired spermatogenesis,[17,18] have been associated with differences in CAG repeat length. Several investigators have found an association between cardiovascular risk factors and CAG repeat length in ischemic heart diseases.[19–22]

Here we investigated whether hormonal pattern and the variation within the gene that code for the CAG repeats in AR exon 1 might influence the specific clinical and ECG expression of the BS patients, with the hypothesis that BS individual expression variability may be influenced by androgen sensitivity.

## Materials and Methods

### Study population

Sixteen consecutive male patients (mean age 45.06 ± 11.3 years, range 18–64 years) with proven BS were included in the study. Diagnosis of BS was based on the criteria of the BS consensus report.[3] All the patients had type 1 ECG Brugada pattern either spontaneously or after a provocative challenge with a sodium channel blocker administration (either intravenous ajmaline, 1 mg/kg body weight, or flecainide, 2 mg/kg body weight). A type 1 ECG was defined as a prominent coved ST-segment elevation ≥ 2 mm or 0.2 mV at its peak, followed (without isoelectric separation) by a negative T wave in ≥2 right precordial leads.[3] ECG parameters of interest were heart rate, PQ interval, QRS duration, maximal ST elevation (among the precordial leads), and QTc duration calculated using Bazett's formula for heart rate correction.[23] Eight patients had implantable cardioverter defibrillator (ICD) implantation. Clinical data consisted of sex, date of birth, age and circumstances at diagnosis, presence/absence of symptoms, and treatment. Moreover, investigation of family history for the presence of BS and for occurrence of sudden cardiac death (SCD) in family members was performed for all patients. The diagnostic workup included physical examination, chest x-ray

and 2-dimensional echocardiography.[24,25] None of the patients had arrhythmogenic right ventricle cardiomyopathy or overt myocarditis. Laboratory tests were done to exclude electrolyte or metabolic disturbances at the time of ECG recording. Baseline electrophysiological study (EPS) was performed in 4 patients. A maximum of 3 ventricular extrastimuli with a minimum coupling interval of 200 ms were delivered from 2 right ventricular sites unless ventricular fibrillation or a sustained ventricular tachyarrhythmia.

The study was approved by the local ethics committee. Informed consent was obtained from each patient.

## Anthropometric measurements

All anthropometric measurements were taken in the morning by a trained staff. Body weight (BW) was measured to the nearest 0.1 kg and height was measured to the nearest 0.5 cm. Body mass index (BMI) was calculated as weight/height$^2$ (kg/m$^2$).

## Blood sampling and processing

Venous blood samples obtained between 8:00 and 9:00 am after an overnight fast, processed and stored according to standard protocols, were used for the DNA and hormone measurements. DNA was extracted from leukocytes using standard phenol:chloroform extraction after differential lysis of erythrocytes. DNA and blood serum samples were stored at −80 °C until analyzed.

## Hormone assays

Follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin (Prl), T, dehydroepiandrosterone sulphate (DHEAS), 17-β-estradiol ($E_2$), and progesterone (P) were assayed using an enzyme chemiluminescence immunoassay (ECLIA; Roche Products Ltd, Penzberg, Germany); 3-alpha-androstanediol-glucuronide (3α-Adiol-G) was assayed using an ELISA assay (DRG International, Inc. Mountainside, NJ 07092 USA); free-testosterone (fT), dihydrotestosterone (DHT), 17-hydroxyprogesterone (17OHP), sex hormone binding globulin (SHBG), delta-4-androstenedione (Δ-4A) (Cisbio Bioassays, Bagnols/Cèze, France), and estrone ($E_1$) (BIOCODE S.A., Liege, Belgium), were assayed with radioimmunoassay.[26]

## CAG repeat determination

Genomic DNA was extracted from the peripheral blood leukocytes using DNA purification kit (Wizard Genomic DNA purification kit, Promega). The AR exon 1 region encoding the polyglutamine repeat was amplified using PCR. The primers used in this study were AR1 5′-GCC TGT TGA ACT CTT CTG AGC-3′ (1039-1060) and AR2 5′-GCT GTG AAG GTT GCT GTT CCT C-3′ (1470-1445).

The PCR amplification was performed in a total volume of 50 μL reaction mixture containing 25 mM of $MgCl_2$, dNTP 10 mM, 1 μg of DNA template, 50 pmol of each primer and 1U of Exact Taq polymerase (5Prime, Hamburg). The cycling profile consisted of 35 cycles, denaturation at 94 °C for 1 min, annealing at 60 °C for 30 sec, and extension at 72 °C for 1 min. The amplified products (431 bp) were sequenced in both the sense and antisense orientations using AR1 or AR2 primers, by an ABI PRISM DyeDeoxy Terminator cycle Sequencing kit and an ABI 3100 Genetic Analyzer (Applied Biosystem, Warrington, UK).

## Statistical analysis

Data was analyzed with the use of STATISTICA software, version 6.1 (Stat Soft, Inc, Tulsa, Oklahoma). Results are expressed as mean ± SD. Pearson correlation test was used to measure a linear association between variables. The roles of age, BMI, ECG parameters and hormone values as associated variables with CAG repeats length were tested by linear regression with the use of univariate and multivariate models. In the multivariate analysis the CAG repeat number was the dependent variable and the regression model included age, BMI, ECG parameters, and hormone levels as independent variables. A $P$ value of $<0.05$ was considered to be statistically significant.

# Results

## Demographic, clinical and genetic characteristics

Demographic and clinical characteristics of the patients are summarized in Table 1. The study population consisted of 16 individual belonging to 13 different families with a mean age of 45.0 ± 11.9 years (range 18–64). The mean age at diagnosis was 35 ± 12 years. 11 patients had a BMI in the normal range (BMI 18.5–24.99), 5 were overweight (BMI 25.0–29.99).

**Table 1.** Clinical and electrocardiographic features of the patients.

| Patient n. | Age (yrs) | Height (cm) | Weight (kg) | BMI (kg/m²) | Basal ECG* | Syncope | Aborted SCD | Family history SCD | ICD | PR interval (ms) | QRS duration (V2, ms) | Qtc interval (ms) | ST (mm) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 52 | 170 | 84 | 29.06 | **1** | Yes | No | No | No | 170 | 110 | 390 | 1.0 |
| 2 | 42 | 175 | 70 | 22.87 | 1 | No | No | No | No | 136 | 106 | 411 | 3.0 |
| 3 | 48 | 172 | 83 | 28.13 | 1 | Yes | No | No | No | 154 | 118 | 402 | 2.5 |
| 4 | 28 | 174 | 62 | 20.52 | 1 | Yes | No | Yes | Yes | 120 | 110 | 375 | 1.5 |
| 5 | 33 | 175 | 70 | 22.87 | 2 | Yes | No | Yes | Yes | 104 | 92 | 382 | 3.5 |
| 6 | 42 | 168 | 67 | 23.78 | 2 | No | No | No | No | 180 | 96 | 384 | 0.5 |
| 7 | 49 | 172 | 74 | 25.08 | 1 | Yes | No | No | No | 148 | 92 | 444 | 4.0 |
| 8 | 57 | 184 | 90 | 26.62 | 1 | No | No | No | Yes | 248 | 122 | 386 | 1.5 |
| 9 | 64 | 173 | 70 | 20.23 | 2 | Yes | No | No | Yes | 144 | 94 | 398 | 1.0 |
| 10 | 50 | 178 | 80 | 22.47 | 1 | No | No | No | No | 166 | 96 | 405 | 1.0 |
| 11 | 56 | 175 | 67 | 19.14 | 2 | Yes | Yes | Yes | Yes | 171 | 97 | 398 | 1.5 |
| 12 | 49 | 178 | 70 | 19.66 | 1 | Yes | Yes | Yes | Yes | 168 | 95 | 410 | 2.0 |
| 13 | 33 | 160 | 60 | 23.43 | 1 | Yes | Yes | Yes | Yes | 140 | 96 | 401 | 2.5 |
| 14 | 18 | 175 | 55 | 17.97 | 2 | No | No | Yes | No | 144 | 97 | 396 | 3.0 |
| 15 | 46 | 170 | 83 | 28.71 | 1 | No | No | Yes | No | 162 | 95 | 399 | 3.0 |
| 16 | 54 | 176 | 77 | 24.91 | 1 | No | No | Yes | Yes | 156 | 100 | 406 | 2.0 |

**Abbreviations:** SCD, sudden cardiac death; ICD, implantable cardioverter-defibrillator; *1, type 1 ECG spontaneously; 2, type 1 ECG after drug challenge.

Diagnosis of BS was made under the following circumstances: aborted SCD (n = 1), syncope probably related to arrhythmia (n = 7), ECG recorded for other medical reasons that were suspicious for BS (n = 5), and family screening for BS (n = 3).

All the patients in the present study showed a type 1 ECG either spontaneously (n = 11) or after drug challenge (n = 5). Among the 11 patients with a spontaneous type 1 ECG, 6 experienced syncope or aborted SCD, whereas 3 of the 6 patients with a drug-induced type 1 ECG were symptomatic. Eight patients had a family history of sudden cardiac death (SCD) and 8 had ICD implantation.

The mean value for spontaneous ST-segment elevation was 2.09 ± 1.02 mm (range 0.5–4.0 mm), QRS duration in V2 lead was 101.00 ± 9.33 ms (range 92.00–122.00 ms), the QTc interval was 399.418 ± 15.78 ms (range 382–444 ms) and the PR interval was 156.93 ± 31.34 ms (range 104–248 ms).

Genetic screening for mutations in the SCN5A gene was performed in 2 of 16 patients (patient 5 and patient 8). An SCN5A mutation was found in both patients (data not shown).

Table 2 shows the values of the hormonal parameters evaluated and the individual CAG repeats length of each patient. Figure 1 shows the representation of the CAG genotyping in a single Brugada patient. The mean value of triplets expression among the 16 patients was 22.25 ± 2.62. None of the patients had CAG length outside the normal range.[27] Gonadotropins were normal except for patients 8 and 10, both with low levels of FSH and LH. Patient 8 had low normal levels of T, fT, and E1 while FSH, LH, 17OHP and E2 were below the normal range, a hormonal asset compatible with a central hypogonadal hypogonadism. Patient 10 was under excessive T replacement therapy that caused the abnormal increase of T, fT, E1 and E2. Abnormally high DHT values were seen in patients 9 and 15. No further hormonal abnormalities with any clinical significance were recorded including Δ-4A and 3α-Adiol-G, evaluated in order to have indications on peripheral tissue androgen metabolism, and estrogens and SHBG, evaluated as counterbalancing the relative biological effect of androgens were in the normal range. No significant correlations were observed between hormone concentrations, BMI values and ECG parameters or between CGA repeats and

ECG parameters by linear regression analysis (data not shown). Analogously, multivariate linear regression analysis between CGA repeat length and all the covariates including ECG parameters, age, BMI and hormones failed to reach statistical significance (not shown).

## Discussion

Data on androgen sensitivity in BS are lacking and to the best of our knowledge, the present investigation is the first study evaluating the AR gene locus in men with BS. We studied a group of BS male patients first analyzing the entire spectrum of androgenic action along with the peripheral metabolites of T, T precursors, SHBG and gonadotropins. Our patients had normal values of all the hormones measured including DHT and 3α-Adiol-G, both indicators of peripheral tissue androgen metabolism, with the notable exception of one patient (subject 10) whose T, fT, E2 and E1 values were clearly higher than normal due to an excess of T replacement therapy. No significant correlations between ECG parameters and hormonal parameters were found.

The basis for this intriguing sex-related distinction in Brugada syndrome is substantially unknown. In animal models, experimental findings demonstrate that $I_{to}$ density and time constant for inactivation is respectively 26% and 17% smaller at 40 mV in female versus male RV epicardial cells.[28] Moreover, T induces more prominent outward currents and reduces inward currents, thereby accentuating the pathophysiological alteration causing Brugada ECG phenotype.[29–31] Despite this interesting data, T values do not help in predicting the severity of the clinical and electrocardiographic phenotype of the BS patients. These findings are in line with and extend to peripheral metabolites of previous studies demonstrating that, in spite of higher T, no significant correlations are observed between T levels and clinical and ECG parameters in BS patients.[8] We did not find any association between BMI and ECG parameters of our patients. This is in partial contrast with what found by other authors that reported that BS male patients were thinner, had lower BMI and lower visceral fat mass as measured by bioelectrical impedance analyses (BIA) compared to normal controls.[8,32] However, BMI cannot represent a reliable measure of adiposity as it is strongly influenced by the height of the subject. Additionally, the analysis of fat mass by BIA does not separate visceral fat from subcutaneous fat, which has a different pathogenetic significance. We think that the assessment of fat distribution by dual energy X-ray absorpiometry might allow a reliable measurement of visceral fat providing a better methodological approach for upcoming studies.

ARs have been identified in the atria and ventricles,[33,34] whereas estrogen receptors appear to be largely confined to atrial myocytes.[35] Several reports indicate that longer CAG repeat length in the human AR results in a linear decrease of transactivation function.[10,11] Polymorphic CAG repeat sequence normally ranges from 8 to 31 and averages about 20 repeats in length.[24] Alteration in length of CAG triplets gives rise to a number of X-linked diseases.[36] However, also in men whose CAG repeat number is within the normal range, clinical indices of androgen action[14–16] and insensitivity[17,18] have been associated with differences in CAG repeat length. Recently, several investigators have found an association between cardiovascular risk factors and CAG repeats in ischemic heart diseases.[19–22] A low number of CAG repeats implies a greater risk for coronary heart disease through reduction of HDL cholesterol and endothelial response to ischemia.[19] Furthermore, CAG polymorphism modulates body fat mass and concentrations of leptin and insulin in men, suggesting a role of CAG repeats in modulating androgen effects on cardiovascular risk factors.[20] No association was observed between AR CAG repeat number and risk of cardiovascular disease (CVD) in women.[37] We found a number of CAG repeats within the normal human polymorphism range and no correlation between CAG length and ECG manifestations of the patients. We conclude that the AR CAG repeats do not influence the extent of ECG manifestation in the BS, and whether androgens accentuate the Brugada phenotype; this is unlikely due to the CAG repeats length.

Thus, it is difficult to explain arrhythmogenesis and gender differences in BS only by differences in gonadal steroids and, unfortunately, T does not seem to be of help, despite clinical observations suggesting an involvement of sex hormones in sex disparity.

Genotype-phenotype relationships in BS are more complex and serve to underscore our incomplete

**Table 2.** CAG repeat length and hormone values of the BS patients.

| Patient n. | CGA | FSH | LH | PRL | T | fT | DHT |
|---|---|---|---|---|---|---|---|
| | 8–31 repeat n. | 1–12 mU/mL | 0.80–8.6 ng/mL | 4–15 ng/mL | 200–900 ng/mL | 9–40 pg/mL | 100–850 pg/mL |
| 1 | 19 | 5.4 | 4.6 | 4.0 | 478.4 | 5.8 | 501 |
| 2 | 21 | 5.1 | 5.1 | 6.5 | 442.3 | 7.4 | 391 |
| 3 | 22 | 6.8 | 4.1 | 5.8 | 449.4 | 9.1 | 692 |
| 4 | 20 | 2.9 | 2.6 | 7.1 | 462.8 | 10.3 | 503 |
| 5 | 28 | 1.1 | 3.2 | 7.8 | 399.7 | 8.9 | 523 |
| 6 | 22 | 2.9 | 4.3 | 15.2 | 466.8 | 12.2 | 683 |
| 7 | 25 | 2.9 | 6.6 | 5 | 464.6 | 14.8 | 504 |
| 8 | 23 | 0.2 | <0.1 | 4.7 | 339 | 9 | 536 |
| 9 | 26 | 10.8 | 6.2 | 4.5 | 642.5 | 10.4 | 990 |
| 10 | 19 | 0.6 | <0.1 | 8.7 | 1366.0 | 51.4 | 649 |
| 11 | 23 | 5 | 5.2 | 12.7 | 773.8 | 17.1 | 145 |
| 12 | 20 | 5.9 | 3.9 | 15.3 | 450 | 14.2 | 815 |
| 13 | 21 | 1.8 | 3.4 | 8.3 | 514.0 | 10.3 | 715 |
| 14 | 25 | 4.0 | 2.1 | 8.3 | 386.2 | 10.5 | 542 |
| 15 | 20 | 4.6 | 3.2 | 7.1 | 518.0 | 12.1 | 1065 |
| 16 | 22 | 4.4 | 2.8 | 3.3 | 355.5 | 8.9 | 693 |

knowledge of the pathogenesis of this inherited arrhythmogenic disease.

A limitation of our study is the small number of patients, which allows only a partial conclusion. BS is a clinically vague syndrome and might include similar diseases association with different genetic/non-genetic physiopathology. AR's biological determinants of heart androgen sensitivity, including the AR tissue distribution, as well as non-genomic mechanisms of action, remain to be better defined in order to understand androgens integrated effects on heart. Furthermore, in light of the lack of association between ECG indexes and CAG repeat length found here, the increased risk of spontaneous arrhythmias in BS after puberty predominantly in males might be due to the activation of genes downstream AR. Studies are needed to test this hypothesis.

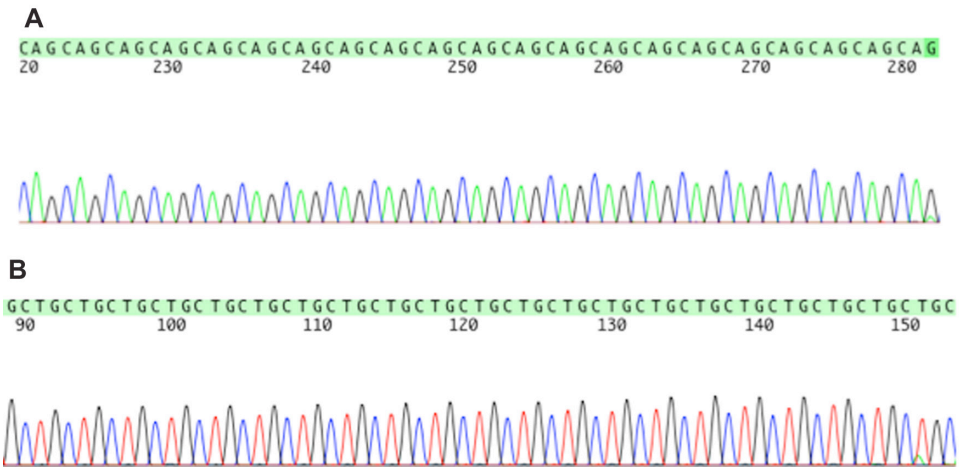


**Figure 1.** Representative gene sequencing of the CAG repeats in androgen receptor exon 1 of patient 13. A polymorphic sequence of 21-repeat length sequencing (forward, **A**; reverse, **B**) is shown.

**Table 2.** (*Continued*)

| 3α-Adiol-G | Δ-4-A | DHEA-S | $E_2$ | $E_1$ | P | 17OHP | SHBG |
|---|---|---|---|---|---|---|---|
| **3.40–22 ng/mL** | **0.30–3 ng/mL** | **<50aa 80–500 µg/dL; >50aa 20–220 µg/dL** | **15–50 pg/mL** | **10–60 pg/mL** | **<1.7 ng/mL** | **0.87–3.12 ng/ml** | **9–60 nmol/L** |
| 7.89 | 1.11 | 150 | 21 | 20.1 | 0.4 | 0.98 | 34 |
| 5.54 | 2.13 | 337 | 14 | 21 | 0.95 | 1.13 | 36 |
| 6.61 | 1.65 | 238 | 16 | 16.2 | 0.62 | 0.87 | 42 |
| 2.88 | 0.89 | 200 | 7 | <10 | 0.34 | 0.23 | 29 |
| 8.34 | 1.15 | 183 | 23 | 15 | 0.41 | 0.88 | 10 |
| 5.09 | 1.66 | 345 | 17 | 24 | 0.77 | 1.35 | 6.3 |
| 9.94 | 1.01 | 185 | 28 | 29 | 0.52 | 1.03 | 14 |
| 4.01 | 0.44 | 14 | 8 | 10 | 0.06 | <0.1 | 9 |
| 7.86 | 1.51 | 212 | 18 | 35 | 0.49 | 0.74 | 36 |
| 12.3 | 2.49 | 98 | 70 | 65 | 0.41 | 0.13 | 7.5 |
| 5.21 | 1 | 220 | 31 | 56 | 0.53 | 0.56 | 17 |
| 6.51 | 1.67 | 206 | 13 | 11 | 0.53 | 1.12 | 11.8 |
| 15.5 | 1.49 | 200 | 17 | 12.9 | 0.28 | 0.49 | 28 |
| 7.0 | 1.84 | 220 | 15 | <10 | 0.28 | 0.45 | 24 |
| 6.9 | 2.21 | 241 | 21 | 17.1 | 0.34 | 0.51 | 33 |
| 7.8 | 1.20 | 205 | 11 | 59 | 0.26 | 0.23 | 37 |

**Abbreviations:** FSH, follicle-stimulating hormone; LH, luteinising hormone; PRL, prolactin; T, total testosterone; fT, free-testosterone; DHEA-S, dehydroepiandrosterone sulphate; $E_2$, 17-β-estradiol; $E_1$, estrone; P, progesterone; 17OHP, 17-hydroxyprogesterone; 3α-Adiol-G, 3-alpha-androstanediol-glucuronide; Δ-4-A, delta-4-androstenedione; DHT, dihydrotestosterone; SHBG, Sex Hormone Binding Globulin.

## Author Contributions

Conceived and designed the experiments: SM, BM, CA, MV, LG. Analysed the data: SM, BM, SB, DF, PF, AP, CA, SU, LG. Wrote the first draft of the manuscript: SM, BM, LG. Contributed to the writing of the manuscript: SM, BM, CA, LG. Agree with manuscript results and conclusions: SM, BM, SB, DF, PF, AP, MV, CA, CM, SU, LG. Jointly developed the structure and arguments for the paper: SM, BM, SB, DF, PF, AP, MV, CA, CM, SU, LG. Made critical revisions and approved final version: SM, BM, SB, DF, PF, AP, MV, CA, CM, SU, LG. All authors reviewed and approved of the final manuscript.

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## Competing Interests

Author(s) disclose no potential conflicts of interest.

## Disclosures and Ethics

As a requirement of publication author(s) have provided to the publisher signed confirmation of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this section. The external blind peer reviewers report no conflicts of interest.

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