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Diagnosis of late-onset congenital adrenal hyperplasia in clinical practice: current evaluation

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Aim. **Aim of the study was to investigate the need to perform the adrenocorticotropic hormone (ACTH) stimulation test by recognizing the importance of a second look at basal serum 17-alpha hydroxyprogesterone (17-OHP) levels and calculating new serum 17-OHP cut-off level.**

Methods. **A total of 142 patients who had hyperandrogenism symptoms and had basal serum 17-OHP levels of higher than 1.3 ng/mL were scheduled to have an ACTH stimulation test performed. Prior to ACTH stimulation, 17-OHP levels were recorded and as second-look levels.**

Results. **Patients were divided to two groups, late-onset congenital adrenal hyperplasia (LOCAH) (25/142), non-LOCAH (117/142). There were statistically significant results related to cycle length and menstrual irregularity between two groups (P=0.042, P=0.041, respectively). In the LOCAH group, basal serum 17 OHP levels were higher than non-LOCAH (P=0.001). When basal serum 17-OHP levels were measured a second time, the need for performing the ACTH stimulation test was decreased. According to cut-off levels of 1.3 ng/mL, 100% of patients needing to take the second serum 17-OHP decreased to 83.1%, a cut-off level of 2 ng/mL decreased numbers from 74.65% to 35.92% and for 2.25 ng/mL 58.42% of patients was decreased to 26.77%. In this study we established 2.25 ng/ml is a superior cut-off level for 17-OHP, its sensitivity is 84% and specifity is 50.4%.**

Conclusion. **The incidence of LOCAH is 1.35% among the patients with hyperandrogenism symptoms. We found a single measurement of serum 17-OHP level can be unreliable. Second 17-OHP test reduces the need of performing the ACTH stimulation test by approximately 30%.**

KEY WORDS: Adrenal hyperplasia, congenital - Hyperandrogenism - Adrenocorticotropic hormone.

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Late-onset congenital adrenal hyperplasia (LOCAH) is an autosomal recessive adrenocortical disorder that occurs in approximately 1% of the general population and is characterized by partial deficiency of steroidogenic enzymes essential for cortisol biosynthesis.[1, 2] LOCAH is most commonly due to an enzyme deficiency of 21-hydroxylase, 11-β-hydroxylase or 3-β-oxidoreductase, alone or in combination. LOCAH is also called non-classical congenital adrenal hyperplasia (NCCAH).

LOCAH patients do not present symptoms and clinical signs at birth and may not be detected in neonatal screening programs with 17-alfa-hydroxyprogesterone (17-OHP).[2] In women, LOCAH may cause menstrual disorders, obesity, short stature, infertility and skin manifestations (hirsutism,

seborrhea and/or acne) in the peripubertal period or in adulthood.[1-3] The screening test should be performed on women with these clinical features in order to differentiate the LOCAH from the polycystic ovary syndrome (PCOS) patients who may have similar clinical symptoms and laboratory findings.[4, 5]

The screening test of LOCAH can usually be made by measuring early morning serum 17-OHP levels. Basal 17-OHP levels of less than 2 ng/mL obtained during the follicular phase in patients with regular menses usually exclude the diagnosis of LOCAH. Basal 17-OHP levels greater than 8ng/ml are usually diagnostic for LOCAH. If the basal 17-OHP levels are between 2 ng/mL and 8 ng/ml, adrenocorticotropic hormone (ACTH) stimulation test should be performed. The levels of higher than 10 ng/mL 60 minute after ACTH stimulation indicates the positive results of LOCAH due to 21-hydroxylase deficiency.[6]

The ACTH stimulation test represents the hormonal gold standard test for the diagnosis of LOCAH due to 21-OHD.[7] It is an invasive, expensive and time consuming procedure. Some effort has been made to identify other specific diagnostic tools to avoid unnecessary ACTH stimulation tests. Many researchers try to determine a basal serum 17 OHP cut-off level to exclude LOCAH whereas others try to determine a cut-off level to diagnose LOCAH both without performing ACTH stimulation test. The determined cut-off levels of serum basal 17-OHP differentiate among researchers. So, the appropriate cut-off levels of basal serum 17OHP have not been found.

The concentration of 17-OH-P in serum depends on age, daytime, gender, phase of the menstrual cycle and pregnancy. Normal values are also influenced by physical and psychological stress.[8] A single measurement of serum 17-OHP level in women may be unreliable.

In this study we have tried to find out the importance of repeating the 17-OHP levels before performing the ACTH stimulation test on patients with elevated 17-OHP levels in order to minimalize the ACTH stimulation test for the diagnosis. Also, we aim to detect LOCAH incidence and to determine a new cut-off 17-OHP level to perform ACTH stimulation test.

Materials and methods

Patients selection

We reviewed the records of 1848 consecutive women of reproductive age, who admitted with acne, hirsutism, androgenetic alopecia (hereafter termed alopecia) and menstrual dysfunction (oligomenorrhea and/or amenorrhea) complaintments suspected to be due to hyperandrogenism, to the Department of Gynaecology and Obstetrics, Cerrahpasa Medical Faculty, Istanbul, Turkey, between January 2009 and June 2012. According to basal serum 17-OHP levels named first basal serum 17-OHP levels, these patients were reevaluated. The patients who had higher than 1.3 ng/mL 17-OHP levels were accepted patients with elevated levels of 17-OHP. This retrospective study included 142 Turkish women with elevated levels of 17-OHP and the signs of hyperandrogenism. The presence of comedones on the face, neck, upper chest, upper back, or upper arms was classed as acne. Hirsutism was considered when a woman had a score 8 on the Ferriman- Gallwey scale. Women with androgenetic alopecia who normally exhibit a diffuse hair thinning over the top of their scalps was defined. Oligomenorrhea and amenorrhea were described as menstrual cycle greater than 40 days in length and the absence of a menstrual period for three consecutive months. Body mass index (BMI) was computed as kg/m^2. Criteria for inclusion were a patient age between 15 to 40 years old, body mass index of 18-30 kg/m^2, normal prolactin and thyroid stimulating hormone (TSH) values, normal gynecological ultrasound examination and cervical smear. All the patients did not use any drug which influenced hormonal parameters for at least six months. Criteria of exclusion were systemic illnesses, known

hypothalamic, pituitary and surrenal disorders. The local ethics committee approved the study.

ACTH stimulation test

No woman recevied hormonal medication for at least 6 months before the test. ACTH stimulation test was performed on all women in the fasting state and in the supine position between 08.00 and 09.00 am of their 3th-5th cycle day. A heparin lock was placed in the forearm. After baseline blood was sampled (this sample was performed to measure 17-OHP level named second basal serum 17-OHP levels), 0.25 mg synthetic ACTH (Synachten, Ciba-Geigy, Basel, Switzerland) was injected intravenously and blood samples were obtained in 1 hour. Then serum was separated and stored at -20 °C until it was assayed for 17-OHP determinations. An ACTH-stimulated 17-OHP level >10 ng/mL was considered a criterion for non-classical or "late-onset" CAH. After ACTH stimulation test, patients were divided into two groups, LOCAH (25/142) and non-LOCAH (117/142).

Hormone assay

17 OHP: all samples were assayed in competitive immunoenzymatic colorimetric method (Dia Metra S.r.I. Headquater, Segrate, Milano, Italy). The sensitivity of the assay was 0.009 ng/mL. The intra- and inter- assay variations were <7.4% and <13%, respectively. References values are between 0.2 and 1.3 ng/mL in follicular phase in women.

Dehydroepiandrosterone sulphate (DHEA-S), free testesterone, total testosterone: all samples were assayed in competitive immunoenzymatic colorimetric method (Dia Metra S.r.I. Headquater).

Follicle-stimulating hormone (FSH), luteinizing hormone (LH), TSH, estradiol (E2), prolactin (PRL): these were measured by chemiluminescent microparticle ımmunoassay (Architect Abbott Lab, IL, USA). 1-4 androstenodione were measured by enzym immunoassay with commercial kits (Biosource, Nivelles, Belgium).

Statistical analysis

Statistical analyses were performed using SPSS 18.0 for Windows (SPSS Inc., Chicago, IL, USA). For our statistical analyses, data were presented as mean±SD or number as appropriate. We compared age, BMI, third day of the mentrual cycle's FSH, LH, E2, PRL, TSH, 17-OHP, DHEAS04, free testosterone, inhibin B, 1-4 androstenedion of two groups, using the t-test. Receiver operating curve (ROC) analysis with area under curve (AUC), (ROC AUC) were used to determine the predictive value of the basal serum 17-OH-progesterone levels for diagnosis of LOCAH. We performed chi-square test for statistical significance of the nonparametric values and the result of the significant P values <0.05.

Results

Twenty-five out of the 1848 (1.35%) patients who presented with signs of hyperandrogenism and 25 of the 142 patients (17.6%) who had basal serum 17 OHP levels of higher than 1.3 ng/mL were diagnosed as LOCAH. We performed ACTH stimulation test on 7.68% (142/1848) of the patients with hyperandrogenism signs.

The characteristics of the LOCAH and non-LOCAH patients were summarized in Table I.There are significant differences between the cycle length of LOCAH and non-LOCAH patients. The cycle length was 61.32±32.6 day, 47.12±23.9 day in non-LOCAH patients and LOCAH patients, respectively (P=0.042). In accordance with this result, the incidence of oligomenorrhea and amenorrhea was 76%, 56%, respectively (P=0.041).

The hormonal parameters of the LOCAH and non-LOCAH patients were summarized in Table II. There were no significant differences in FSH, E2, PRL, TSH, DHEA-S, free testesterone, total testosterone and 1-4 androstenodione levels between LOCAH and non-LOCAH patients. Mean LH levels were 3.79±2.30 mIU/mL, 5.79±3.46 mIU/mL in LOCAH and non-LOCAH patients,

TABLE I.—*Differences of demographic features and Clinical presentations between LOCAH and non-LOCAH.*

	LOCAH	non-LOCAH	P
Age (years)	21.08±3.62	22.36±4.40	0.178
BMI (kg/m²)	25.16±4.17	25.49±4.37	0.740
Cycle length (day)	47.12±23.9	61.32±32.6	0.042*
Hirsutism, n/N. (%)	21/25 (84%)	97/117 (82.9%)	0.895
Infertilite, n/N. (%)	0/25 (0%)	11/117 (9.4%)	0.110
Menstrual irregularity, n/N. (%)	14/25 (%56)	89/117 (76%)	0.041*
Acne, n/N. (%)	12/25 (48%)	46/117 (39.3%)	0.423
Alopecia, n/N. (%)	4/25 (16%)	7/117 (6%)	0.089

LOCAH: late-onset congenital adrenal hyperplasia, BMI: body mass index; * Statistically significant: P<0.05.

TABLE II.—*Hormonal parameters of the LOCAH and non-LOCAH patients.*

	LOCAH	non-LOCAH	P
FSH (mIU/mL)	4.69±1.63	4.92±1.38	0.467
LH (mIU/mL)	3.79±2.30	5.79±3.46	0.007*
E2 (pg/mL)	39.68±22.69	39.07±21.46	0.898
PRL (ng/mL)	19.64±6.96	22.66±15.29	0.335
TSH (mIU/L)	1.59±0,40	1.76±0,96	0.385
Basal serum 17-OHP (ng/mL)	3.57±1.48	2.55±1.32	0.001*
DHEA-S (nmol/L)	445.40±150.76	413.05±187.25	0.421
Free Testosterone (pg/mL)	2.52±1.25	2.82±3.17	0.638
Total Testosterone (ng/dL)	92.20±33.14	98.21±33.94	0.421
1-4 Androstenodione (ng/mL)	3.52±0.77	3.76±0.83	0.513
0.hour 17-OHP (ng/mL)	3.00±0.85	1.62±0.69	0.001*
1.hour 17-OHP (ng/mL)	11.69±2.45	4.11±1.23	0.001*

LOCAH: late-onset congenital adrenal hyperplasia. Values are means ± SD. FSH: follicular stimulating hormone; LH: luteinizing hormone; E2: estradiol; PRL: prolactin; TSH: thyroid stimulating hormone; 17-OHP: 17-alpha-hydroxyprogesterone; DHEA-S: dehydroepiandrosterone sulphate. * Statistically significant: P<0.05.

respectively (P=0.007). Mean LH/FSH ratio were 0.81±0.32, 1.17±0.41 in LOCAH and non-LOCAH patients, respectively (P=0.009). Mean basal serum 17-OHP levels (first basal serum 17-OHP levels) were 3.57±1.48 ng/mL and 2.55±1.32 ng/mL in LOCAH and non-LOCAH patients, respectively (P=0.001). Mean 0 hour basal serum 17-OHP level of the ACTH stimulation test (second basal serum 17-OHP levels) were 3±0.85 ng/mL and 1.62±0.69 ng/mL in LOCAH and non-LOCAH patients, respectively (P=0.001). And mean 1st hour serum 17-OHP level during ACTH stimulation test were 11.69±2.45 ng/mL and 4.11±1.23 ng/mL in LOCAH and non-LOCAH patients, respectively (P=0.001).

ROC curves for 17-OHP levels were depicted in Figure 1. The ROC-AUC for first basal 17-OHP was 0.746 with a 95% CI (0.693-0.799). The cut-off value for AMH, 2.25 ng/mL was determined with the highest sensitivity (84%) and specificity (50.4%) for predicting LOCAH. The ROC-AUC for second basal 17-OHP was 0.905 with a 95% CI (0.876-0.934). The cut-off value for AMH, 2.25 ng/mL was determined with the highest sensitivity (84%) and specificity (87.2%) for predicting LOCAH (Table III).

When the second 17-OHP levels were measured the number of patients with 17-OHP levels over the cut-off values for 2 ng/mL decreases from 74.65% to 35.92% and for 2.25 ng/mL from 58.42% to 26.77%. If we take the cut-off value as 1.3 ng/mL and take the blood sample a second time, 16.9% of patients would have normal values of 17-OHP therefore the need for performing ACTH stimulation test on these patients would be eliminated (Table IV).

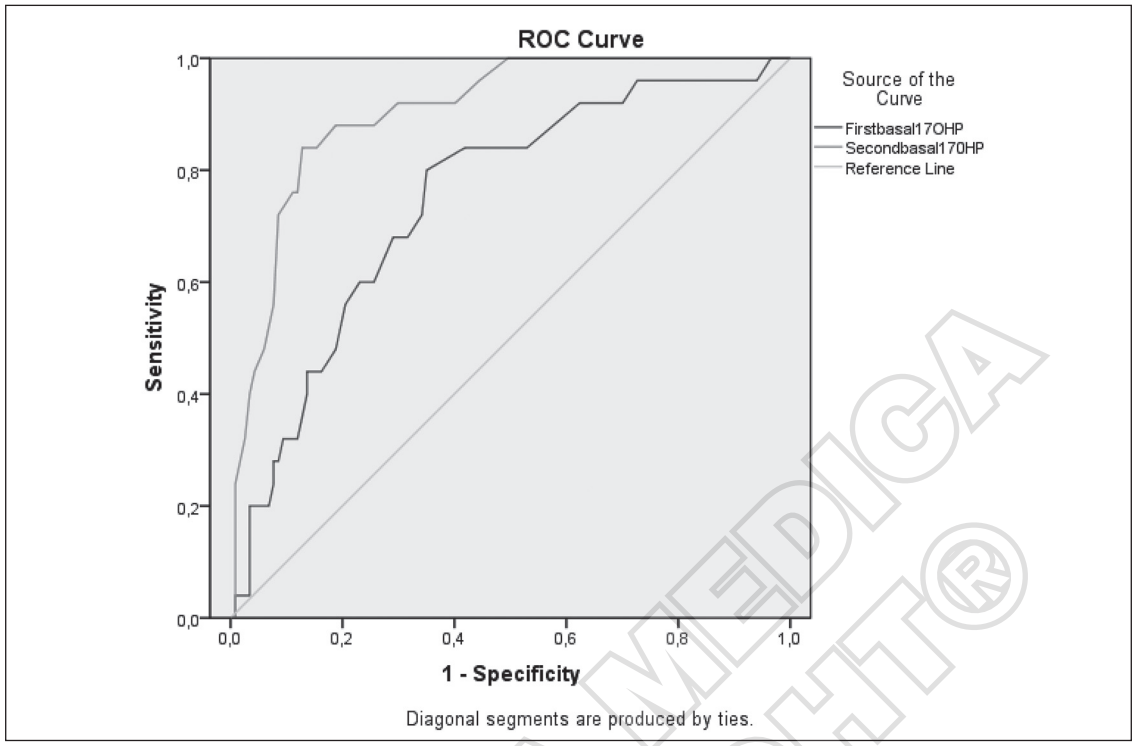

Figure 1.—ROC curve for 17-OHP levels.

TABLE III.—*ROC AUC curve for estimate the value of the basal serum 17-OH-progesterone levels for performing the ACTH stimulation test for diagnosis.*

	ROC AUC	95%CI	Significance	Cut-off	Sensitivity	Specifity
First basal 17-OHP (ng/mL)	0.746	0.693-0.799	0.001	≥2.250	84	50.4
Second basal 17-OHP (ng/mL)	0.905	0.876-0.934	0.001	≥2.250	84	87.2

17-OHP: 17-alpha-hydroxyprogesterone.

TABLE IV.—*17-OHP cut-off levels triggering need for ACTH stimulation tests.*

	>1.3 ng/mL	>2 ng/mL	>2.25 ng/mL
First basal serum 17-OHP, n/N. (%)	142/142 (100%)	106/142 (74.65%)	83/142 (58.42%)
Second basal serum 17-OHP, n/N. (%)	118/142 (83.1%)	51/142 (35.92%)	38/142 (26.77%)
Advantage of the measurement of second time, n/N. (%)	24/142 (16.9%)	55/142 (38.73%)	45/142 (31.75%)

17-OHP: 17-alpha-hydroxyprogesterone.

Discussion

The symptoms of LOCAH and PCOS in adolescents and adults (*i.e.*, hirsutism and irregular menses) are similar so a differential diagnosis is between PCOS and LOCAH needs to be done and treatment needs to be individualized for each affected individual.

In this study, there were no difference clinical presentations between LOCAH and non- LOCAH patients, except cycle length, oligomenorrhea and amenorrhea. Non-LOCAH patients had longer cycle length and higher incidence of oligomenorrhea and amenorrhea. The majority of non-LOCAH patients may be possible PCOS because of oligomenorrhea and amenorrhea, one of the diagnostic criteria of PCOS.

There were significant differences between LOCAH and non-LOCAH patients in regards to LH, LH/FSH rate, basal serum 17-OHP. Although LH levels and LH/FSH rate in non-LOCAH patients compared with higher than LH levels and LH/FSH ratio of LOCAH, LH levels in both patients were in normal ranges. The majority of non- LOCAH patients may be possible PCOS patients who should have been higher LH levels and LH/FSH ratio. There were no significant differences between other hormonal parameters.

Incidence of LOCAH among the hyperandrogenemic women varies between 1% to 33% in literature. We found that the prevelance of LOCAH (25/1848) was 1.35% among the patients who had the signs of hyperandrogenism. We diagnosed 25 patients as LOCAH with ACTH stimulation test. None of the patients had serum basal 17-OHP levels greater than 8 ng/mL so we could not diagnose LOCAH without the ACTH stimulation test. We previously reported that the incidence of LOCAH due to 21-hydroxylase deficiency was found to be as 3% among hirsute Turkish women.[9]

The gold standard diagnosis of LOCAH is the ACTH stimulation test. There are established normograms for the diagnosis of congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency. The normograms use stimulated 17-OHP levels in response to ACTH to differentiate the cases into normal, heterozygous, nonclassical, or classical CAH.[6] ACTH stimulation test is a strong tool in the diagnosis of LOCAH, however, it is costly and invasive. Clinical and laboratory clues for the diagnosis of LOCAH is an effort to escape performing the test have been previously analyzed by other researchers.

Researchers are still trying to determine a cut-off level for basal 17-OHP to exclude the diagnosis of LOCAH and avoid testing of ACTH unnecessarily. Azziz *et al.*[10, 11] demonstrated that all LOCAH females investigated for hyperandrogenism had basal 17-OHP >2 ng/mL, and suggested 2 ng/mL as a cut-off level in female hyperandrogenism. Dewailly *et al.*[12] recommended testing ACTH when the basal 17-OHP level was >2 ng/mL, because LOCAH is unlikely with lower basal 17-OHP.

However, there are several conflicting data indicating LOCAH cases with lower basal 17-OHP levels. Bachega *et al.*[13] reported that 4 of 58 patients with LOCAH had basal 17-OHP levels <2 ng/mL. Similarly, if the basal 17-OHP level of 2 ng/mL had been used 13-14% of patients with LOCAH would have been missed.[14, 15] Escobar-Morreale *et al.*[16] proposed a cut-off level of 1.7 ng/mL with 100% sensitivity and 88.6% specificity.

We found the predictive cut-off value as 2.25 ng/mL. This value's sensitivity is 84% and the specifity is 50% in the first basal serum 17-OHP levels for ACTH stimulation test. When we take the cut-off value as 2 ng/ml for our test group, the sensitivity was 92% and the specifity was 37.6%. The cut-off value that we found (2.25 ng/mL) is similar to the world accepted cut-off value. However, this cut-off value will not exclude all the non-LOCAH patients.

Is it possible to set a cutoff level for basal 17-OHP in order to diagnose LOCAH without performing the ACTH test? Leite *et al.*[17] showed that a basal level of 17-OHP >3 ng/mL was sufficient to diagnose LOCAH, however, the study group was small in that report making it difficult to reach a conclusion. The most commonly used cut-off in this respect is 5 ng/mL.[12, 13, 18, 19] Gonc *et al.*[20] reported 5 cases of premature adrenarge whose basal 17-OHP levels were >5 ng/mL. Three (60%) of those cases had stimulated 17-OHP level <10 ng/mL; thereby, if a cut-off level of 5 ng/mL is accepted, 60% of cases were ultimately misdiagnosed with LOCAH. One patient (with a basal 17-OHP level of 6 ng/mL). After stimulation, this patient had a lower stimulated level than basal level. A probable cause for this phenomenon can be explained by the fact that, at times, the stress of blood sampling can be a stronger stimulus than the ACTH test itself. Therefore, individual factors possibly altering basal levels of steroids should be taken into account when diagnosing LOCAH. In this study, the basal serum 17-OHP in all LOCAH patients have been detected lower than 5 ng/mL. So, there is no an appropri-

ate cut-off level for basal 17-OHP in order to diagnose LOCAH without performing the ACTH test.

The level of 17-OH-P in serum may vary depending on age, daytime, gender, phase of the menstrual cycle and pregnancy. The screening test of LOCAH can usually be made by measuring early morning serum 17-OHP levels during follicular phase in female patients with regular menses. In additionally, the levels of serum 17-OHP are also influenced by physical and psychological stress. A single measurement of serum 17-OHP level can be unreliable. When the second 17-OHP levels were measured the number of patients with 17-OHP levels over the cut-off values for 2 ng/mL decreases from 74.65% to 35.92% and for 2.25 ng/mL from 58.42% to 26.77%. If we take the cut-off value as 1.3 ng/mL and take the blood sample a second time, 16.9% of patients would have normal values of 17-OHP, therefore, the need for performing ACTH stimulation test on these patients would be eliminated. These results may reflect that the stress factor and stress level of the patient at time of the blood sampling may be a stronger stimulus than the ACTH itself. Stress factors have significant effect on basal serum 17-OHP levels. So we believe that prior to performing the ACTH stimulation test, blood samples of 17-OHP levels should be performed a second time.

When we repeat to test basal serum 17-OHP levels for performing ACTH stimulation test, the sensitivity and specificity for the 2.25 is 84% and 87.2% respectively and the sensitivity and specificity for 2 ng/ml is 88% and 81.2%. The specificity and the sensitivity became greater when we look at the basal serum 17-OHP levels for a second time. As a result, the need for performing ACTH stimulation tests decreased when the second samples were taken as the number of the patients who had high basal serum 17-OHP levels decreased.

Conclusions

The gold standard of diagnosis of LOCAH is the ACTH stimulation test. The incidence of LOCAH is 1.35% among the patients with hyperandrogenism symptoms. A total of 17.6% of the patients who were performed ACTH stimulation test was diagnosed LOCAH. We found the predictive cut-off value of basal serum 17-OHP levels as 2.25 ng/mL. for performing ACTH stimulation test. A single measurement of serum 17-OHP level can be unreliable. When we examine that these second blood samples it reduces the need of performing the ACTH stimulation test by approximately 30%. Therefore, before performing ACTH stimulation tests in hyperandrogenemic patients with high serum 17-OHP from cut-off levels, we suggested to repeat basal serum 17-OHP levels in order to decrease the requirement of performing the ACTH stimulation test by about 30%.

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Conflicts of interest.—The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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