

Establishing a new screening 17 hydroxyprogesterone cut-off value and evaluation of the reliability of the long intramuscular ACTH stimulation test in the diagnosis of nonclassical congenital adrenal hyperplasia

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Abstract. – **OBJECTIVE:** Nonclassical congenital adrenal hyperplasia (NCAH) is a common genetic transmitted endocrinological disease. The validity of screening by using a 17 hydroxyprogesterone (17 OH-P) cut-off level of 2 ng/ml is controversial due to the frequent overlap with the polycystic ovary syndrome (PCOS). The availability of the standard intravenous cosyntropin (ACTH) formula is a problem in many countries including our country and the diagnostic test is performed by using the intramuscular depot form. In this study, we aimed to determine our own cut-off value for screening and to test the reliability of long intramuscular ACTH stimulation test in our patients.

PATIENTS AND METHODS: One hundred and seventy-five fertile age women whose basal follicular phase 17 OH-P level above 2 ng/ml were included in the study. All of the patients underwent an intramuscular long cosyntropin (ACTH) stimulation test.

RESULTS: 17 OH-P levels were above 10 ng/ml in 16 of 175 (9.14%) patients who were compatible with the diagnosis of NCAH. There was no significant difference between NCAH, PCOS and idiopathic hyperandrogenism (IH) groups in terms of hirsutism and hyperandrogenemia. In ROC analysis, 3.19 ng/ml was found to be a reliable cut-off value (AUC: 0.698, 95% GA: 0.540-0.855, $p < 0.05$). In the extended intramuscular ACTH stimulation test, sensitivity increased from 56.2% to 91.6% at 180th minute

CONCLUSIONS: Our study gives a perspective about the detection of screening threshold value for the diagnosis of NCAH and the availability of the intramuscular long ACTH stimulation test.

Key Words:

Nonclassical congenital adrenal hyperplasia, ACTH stimulation test, 17 Hydroxy-progesterone.

Introduction

Congenital Adrenal Hyperplasia (CAH) is a genetic endocrinological disease. The pathogenic mechanism involves the decrease in the amount of final product of steroid hormones due to the reduced activity of one or more enzymes in the steroid hormone synthesis cascade in the adrenal glands and ACTH stimulation occurs with negative feedback. As a result, there is an increase in intermediate products that are the substrate of the defective enzyme. CAH is the final clinic picture of these mechanisms¹⁻³.

In nonclassical form, patients may not have any obvious clinical findings until adulthood, since the enzyme activity is around 20-50%. General prevalence varies between 1/1000 - 1/2000, but it is more common in some races such as Turks, Croats and Jews. Its frequency is about 1-6% in hirsute women, but in some races, frequency can reach to 10-20%^{4,5}. In Turkish studies, prevalence varies from 1.3% to 3.1%⁶⁻⁸.

Nonclassical congenital adrenal hyperplasia usually presents with symptoms such as oligomenorrhea, hirsutism and acne, which are indistinguishable from PCOS. An average of 40-50% of these patients have classical polycystic ovarian appearance on pelvic ultrasonography, and they are generally diagnosed as PCOS^{1,4,7,8}. This diagnosis is made by determining a basal follicular phase 17 Hydroxy-Progesterone (17 OH-P) level of ≥ 2 ng/ml (6 nmol/L), and these patients undergo an ACTH stimulation test. If the level of 17 OH-P is above 10 ng/ml (30.3 nmol/L) at any

sampling time, the diagnosis is confirmed. If the basal follicular phase 17 OH-P level is already 10 ng/ml and above, stimulation test is not required. However, a study reported high false positivity rates when the stimulated 17 OH-P level cut-off value was taken at 10 ng/ml. Therefore, the definitive diagnosis is made when the 17 OH-P level is above 15 ng/ml, and genetic confirmation is recommended for values between 10 ng/dl and 15 ng/ml^{4,5,9,10}.

Although the diagnostic criteria is generally established in NCAH, follicular phase 17 OH-P levels can be detected at high levels in patients with PCOS and these two diseases overlap frequently^{1,13}. Therefore, it is a matter of curiosity whether it is correct to use the same screening 17 OH-P cut-off value in our country.

In the standard ACTH stimulation test, cosyntropin (ACTH) 250 mcg is administered in intravenous (IV) bolus form and 17 OH-P response at 60 minutes is measured. However, the IV form is not always available in many countries, including our country. Therefore, the tests are also performed by using cosyntropin 1 mg intramuscular (IM) depot form. The IM form has a longer transition time to the circulation and a longer half-life. It is, thus, widely believed that the sampling time should be extended.

In this study, we aimed to determine the follicular phase screening 17 OH-P cut-off level with a sufficient degree of sensitivity and specificity, and to investigate the reliability of the extended sampling (up to three hours) IM ACTH stimulation test in the diagnosis of NCAH.

Patients and Methods

Patients

Fertile age women whose basal follicular phase 17 OH-P levels ≥ 2 ng/ml and underwent a cosyntropin (ACTH) stimulation test were included in the study. The demographic data, history of menstrual irregularity and infertility, the presence of polycystic ovary on ultrasonography and hirsutism were determined. Oligomenorrhea was defined as the presence of menstrual intervals above 35 days and amenorrhea was defined as the condition of not having menstruation for more than 6 consecutive months⁷. Hirsutism was determined as 8 or higher score according to the Modified Ferriman Gallwey Scoring System¹². PCOS diagnosis was based on the Rotterdam and Androgen Excess Society diagnostic criteria^{13,14}. If there was

no oligo-anovulation findings or polycystic ovary on ultrasonography, it was defined as idiopathic hyperandrogenism (IH)¹⁵.

Patients with Cushing disease, virilizing adrenocortical and ovarian tumours, hyperprolactinemia, hypothyroidism, active malignancy, pregnancy, postmenopausal, chronic liver disease and chronic kidney failure, and those using medications such as oral contraceptives, glucocorticoids and antiandrogen pills that may affect the hormonal panel were excluded from the study.

Informed consent was obtained from all patients participating in the study. The Local Ethics Committee approval was obtained from the Sakarya University Local Ethics Committee (Approval date and No: 21.11.2019-71522473/050.01.04/211).

Laboratory Parameters

Biochemistry and follicular phase hormonal parameters were sampled in the follicular phase of the menstrual cycle. In oligomenorrhic patients, it was sampled at any time. The basal blood tests were taken between 08.00-10.00 in the morning following an eight-hour fasting period. Routine biochemistry thyroid function tests, follicular phase hormonal panel, total testosterone, dehydroepiandrosterone sulfate (DHEASO₄) and basal 17 OH-P levels at diagnosis were recorded. Cosyntropin (ACTH) stimulation tests were performed in patients who had a 17 OH-P level ≥ 2 ng/ml in the follicular phase hormonal panel. The tests were started at 08.00 am after an eight-hour fasting. After 0-minute blood test was taken in supine position, Synacten Depot 1 mg IM amp. 1/4 (Tetracosactrin β 1-24 Ciba Geigy Switzerland) was injected intramuscularly. For 17 OH-P determination, an intravenous catheter was used, and 0-30-60-90-120-180th minutes blood samples were collected in to the dry plain tube and sent to the laboratory immediately. Basal and stimulated 17 OH-P measurements were done by Radioimmunoassay (RIA) method *via* a DIA Source Kit (Berthold LB2111 Autoanalyzer[®]). Intraassay variability was <10%.

Patients with 17 OH-P value 10 ng/ml or above in any sampling time in the stimulation test were considered as NCAH. All patients whose test results were compatible with NCAH were referred to the genetics department and diagnoses were genetically confirmed.

Statistical Analysis

Statistical analyses were performed by using SPSS for Windows 24 program (Statistical Package for Social Science, SPSS Inc. Chicago IL,

USA®). The variables were investigated using visual (histograms, probability plot) and analytical methods (Kolmogorov-Smirnov/Shapiro-Wilk's test) to determine whether they were normally distributed. We performed analyses to describe and summarize the distributions of variables. Continuous variables were reported as the median and interquartile range (IQR) and as whole number and percentages for categorical variables. Modified Ferriman Gallwey scores and Total Testosterone levels did not fit the normal distribution, whereas DHEASO₄ levels were observed to fit the normal distribution. Variables that were not normally distributed were compared using the Kruskal-Wallis test. Binary comparisons were made by using the Mann-Whitney U test and evaluated by Bonferroni correction. Variables with normal distribution were compared using one-way ANOVA. The homogeneity of the variances was evaluated by Levene test. Type 1 error level <5% ($p < 0.05$) was accepted as statistically significant. The predictive value of basal 17 OH-P levels for the diagnosis of NCAH was analyzed via "Receiver Operating Characteristics (ROC)" curve analysis. In the presence of significant limit values, the sensitivity and specificity values of these limits were calculated. In the evaluation of the diagnostic value of the test, statistical significance was accepted at $p < 0.05$ by calculating the area under the curve (AUC).

Results

One hundred and seventy-five patients were included to the study. The median age of the study

population was 23 years (IQR: 21-26). There were no significant differences between the NCAH and other groups in terms of demographic characteristics of the patients.

The median Modified Ferriman Gallwey (FG) score for hirsutism evaluation was 10 (IQR: 10-14). According to the ACTH stimulation test, 16 of 175 patients (9.14%) had 17 OH-P levels above 10 ng / ml and were diagnosed as NCAH. The remaining 122 (69.7%) patients were evaluated as PCOS and 37 (21.1%) patients were evaluated as IH. There was no significant difference between the three groups in terms of biochemical values, follicular phase hormone levels and thyroid function tests.

According to ROC analysis that basal 17 OH-P levels had a statistically significant value in predicting the diagnosis of NCAH (AUC: 0.698, 95% CI: 0.540-0.855, $p = 0.009$). When the 3.19 level was taken as the cut-off (threshold value) for the basal 17OH-P level, the sensitivity was calculated as 75% and the specificity was 51.6% (Figure 1).

Patients with a diagnosis of NCAH according to the ACTH stimulation test were compared to PCOS and IH groups in terms of follicular phase DHEASO₄ and total testosterone levels, menstrual irregularity and FG hirsutism scores. There was no statistically significant difference between the groups in terms of FG scores, DHEASO₄ and total testosterone levels. However, menstrual irregularity was significantly higher in NCAH and PCOS groups compared to IH ($p < 0.05$) (Table I).

The cut-off 17 OH-P value in the ACTH stimulation test used for the diagnosis of NCAH is accepted as 10 ng/ml and above. In our study, the

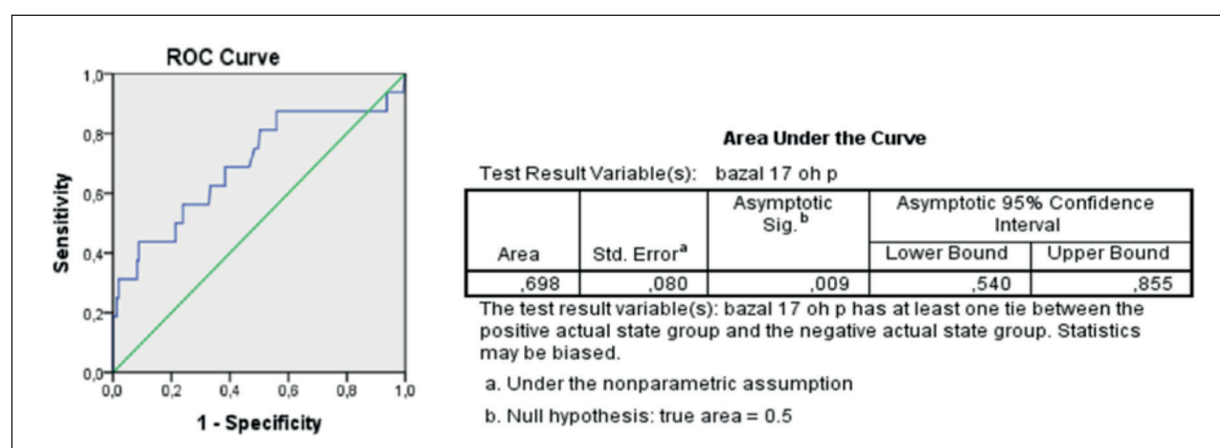


Figure 1. Examination of 17-OH-P value by ROC analysis in predicting the diagnosis of NCAH and summary of analysis results. The ROC curve and reference diagonal line are shown together on the graph.

Table I. Summary of differences between NCAH and other groups (IH and PCOS groups) in terms of DHEASO₄, total testosterone, oligomenorrhea and Modified Ferriman Gallwey Scores.

		Other groups	Average difference	p
NCAH	DHEASO ₄ (n: 175)	IH	-43.5	0.608
		PCOS	-89.2	0.096
	Total Testosterone (n: 175)	IH	4.4	0.71
		PCOS	2.2	0.90
	FG score (n: 175)	IH	1.8	0.11
		PCOS	-1.0	0.46
	Menstrual Irregularity (n: 174)	IH		0.008
		PCOS		0.604

diagnostic sensitivities for 17 OH-P levels at 30, 60, 90, 120 and 180 minutes after IM ACTH were 37.5%, 56.2%, 71.4%, 71.4% and 91.6%, respectively (Table II).

Discussion

Serious clinical forms of NCAH manifest immediately after birth; however, NCAH begins to give symptoms in the peripubertal period or adulthood. The clinic findings cannot be distinguished from PCOS in young women, and very rarely infertility can be seen due to adrenal rest tumours¹⁷.

In our study, the percentage of NCAH was found to be 9.14% among hyperandrogenemic hirsute women. This rate is correlated quite well with the studies previously conducted in hyperandrogenic women in Turkey^{4,5,7}. Also, no significant difference was found between PCOS and NCAH patients in terms of hirsutism and adrenal and ovarian androgen hormone levels.

ACTH stimulation test is performed if the basal follicular phase 17 OH-P value is 2 ng/ml (6 nmol/L) or above. In the test, if the 17 OH-P value at 60 minutes is above 10 ng/ml (30.3 nmol/L), NCAH is considered^{9,16}. In a study conducted in Turkey, the diagnostic threshold value for stim-

ulated 17 OH-P was determined as 13.4 ng/ml. Also, according to another important study, when the stimulated 17 OH-P threshold was taken as 10 ng/ml or above, the false positivity rate was found to be much higher^{7,10}. Therefore, according to the current recommendations, levels of 15 ng/ml or above are defined as compatible with NCAH. Genetic confirmation is recommended in values between 10 and 15 ng/ml¹⁶.

Plasma 17 OH-P levels can fluctuate due to many factors such as menstrual cycle time, hours of the day, etc. The recommended 2 ng/ml 17 OH-P level is a suitable threshold value for morning basal follicular phase measurements in women with regular menstruation; however, it is not always possible to catch the follicular phase in these women, most of whom are oligomenorrhic^{4,5}. In addition, PCOS also leads to increased follicular phase 17 OH-P levels which may cause overlaps^{1,11,16}.

For these reasons, we tried to determine whether a higher determinant cut-off value may be more reliable associated with this genetic hormonal disease which is common in our community. A study conducted from Turkey found that 2.25 ng/ml value can be accepted as a suitable cut-off value with a 50.4% sensitivity and 84% specificity⁶. In another study, while the sensitivity and specificity for a cut-off value of 2.07 ng/ml

Table II. Interpretation of ACTH stimulation test results of patients with NCAH according to the time tested.

17 OH-P (ACTH stim.)	Patient number (n)	Assessed patient percent (%)	Positive result	Sensitivity (%)
30.min	16	16 (100)	6	37.5
60.min	16	16 (100)	9	56.2
90.min	16	14 (87.5)	10	71.4
120.min	16	14 (87.5)	10	71.4
180.min	16	12 (66.7)	11	91.6

was 83.3% and 93.9%, respectively, the sensitivity was 66.7% and the specificity increased up to 98.9% when the cut-off value was increased to 3.03 ng/ml¹⁷. Similarly, in our study, we calculated a 75% diagnostic sensitivity and 51.6% diagnostic specificity for 3.19 ng/ml cut-off value for basal 17 OH-P level. Considering that ACTH stimulation test is both costly and invasive test cannot be performed in every center, the value of a more sensitive and specific diagnostic cut-off value cannot be denied.

The availability of IV ACTH formula which used in the standard stimulation test is limited. Therefore, tests are usually done with IM formulation. We attempted to test the accuracy of this view in our study. In a study comparing IV and IM ACTH stimulation in dogs, target cortisol levels were obtained independently from the route of administration and the peak mean values were observed at 60-90 minutes¹⁸. In a study by Azziz et al¹⁹, IV and IM ACTH stimulation tests were compared. It was determined that the maximal adrenal response was obtained at 60-90 minutes, but it was recorded that the maximal response shifted towards 90 to 120 minutes in the IM test group.

In another study, 250 mcg Synacthen® IV form and 1 mg Synacthen® IM depot forms were compared, and sampling times were planned up to 120 minutes. It was observed that the maximal adrenal response extending to 120 min. was significantly higher in the IM stimulation group than the IV group²⁰. Based on these data, we extended the sampling times up to 180 minutes in our IM stimulation test and observed that the test sensitivity and the rate of diagnosis was increased especially at 120th and 180th minutes.

Conclusions

Although the recommended screening cut-off values are now generally well accepted in adults, it is still a matter of debate whether the same threshold values can be used for each community because of the differences for the diagnosis of NCAH. Our study gives some clues on this issue, but larger randomized controlled comparison studies are needed.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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