

Test with 5 mcg ACTH for Diagnosis of Non-classic Congenital Adrenal Hyperplasia

Chagay NB¹, Fadeyev VV², Melnichenko GA³, Ivanova ON³, Bakulina EG⁴ and Kotelnikova NYu⁴

¹Department of Endocrinology, Stavropol State Medical University, Stavropol, Russia

²Department of Endocrinology, I. M. Sechenov First Moscow State Medical University, Moscow, Russia

³Endocrinology Research Centre, Moscow, Russia

⁴Center of Family Healthcare and Reproduction, Stavropol, Russia

*Corresponding author: Chagay NB, Department of Endocrinology, Stavropol State Medical University, Stavropol, Switzerland, Russia, Tel: +79624063171; E-mail: chagay2006@rambler.ru

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Abstract

Background: Short test with 250 mcg corticotropin (Tetracosactide acetate, a substance of first 24 amino acids from 39-acid chain of endogenous ACTH) is a diagnostic standard of non-classic form of congenital adrenal hyperplasia (CAH). However, it is well known that morning ACTH levels in healthy people fluctuate between 10 and 60 pg/ml, and peak cortisol levels can be achieved with 1-24 ACTH dose of 1 mcg or 5 mcg.

Objective: To study the sensitivity and specificity of the test with 5 mcg ACTH to diagnose non-classic form of congenital adrenal hyperplasia (NCAN).

Materials and Methods: During from 2006 to 2011, we screened 435 women aged from 16 to 35 (25 (21; 29) years) for CAH after exclusion of neoplastic hyperandrogenism.

Protocol of low-dose (5 mcg) and standard (250 mcg) 1-24 ACTH tests: Blood sampling for basal 17-OHP and cortisol levels; blood sampling for 17-OHP and cortisol at 30 and 60 minutes after 5 mcg or 250 mcg 1-24 ACTH stimulation. Molecular genetic analysis for most prevalent *CYP21* mutations was performed using allele specific polymerase chain reaction.

Results: Diagnosis of non-classic CAH with 21-hydroxylase deficiency was proved in 5.3% (23/435) of patients.

Conclusion: Low dose test with Tetracosactide 5 mcg can identify 21-hydroxylase deficiency in patients with stimulated 17-OHP concentrations over 14 ng/ml. Test with Tetracosactide 5 mcg had sensitivity of 72.7% and specificity of 100% with positive prognostic value (+PV) of 100%, and negative prognostic value (-PV) of 89.3%.

Keywords: Nonclassic adrenal hyperplasia; Low dose test; ACTH

Introduction

Short test with 250 mcg corticotropin (Tetracosactide acetate, a substance of first 24 amino acids from 39-acid chain of endogenous adrenocorticotrophic hormone (ACTH)) is a diagnostic standard of non-classic form of congenital adrenal hyperplasia (CAH). This ACTH dose results in serum ACTH concentrations of up to 2000 pg/ml. However, it is well known that morning ACTH levels in healthy people fluctuate between 10 and 60 pg/ml, and peak cortisol levels can be achieved with 1-24 ACTH dose of 1 mcg or 5 mcg [1]. Thus, standard ACTH tests may result in false-positive results due to over stimulation of adrenal glands.

Patients and Methods

During from 2006 to 2011, we screened 435 women aged from 16 to 35 (25 (21; 29) years) for CAH.

Study inclusion criteria were following:

1. Dermatological or biochemical signs of hyperandrogenism in women with normal gonadotropins anovulatory and ovulatory cycle.

2. Normal gonadotropins anovulatory infertility without symptoms of hyperandrogenism.

First group of patients comprises of 234 patient with clinically and/or biochemically established non-neoplastic hyperandrogenism; 201 women without hyperandrogenism were included in the second group. Ovulation absented in 75.2% (176/234) of hyperandrogenic women and in 85.6% (172/201) of patients without hyperandrogenism. In our study we also included some women with recurrent pregnancy loss (12 in first group and 29 in second group).

Serum levels of LH, FSH, prolactin, testosterone, sex-binding globulin (SBG), dehydroepiandrosterone sulfate (DHEA-S) and other hormones were evaluated by solid chemiluminescence immunoassay (IMMULITE I-DPC, Beckman Coulter DxI). Bioactive testosterone (bioTs) concentration estimated by on-line calculator ISSAM [2] using SBG level, constant SA [albumin concentration 4.3 g/dl]. Androgens

levels compared to age-matched healthy women: Ts (50.8 (38.0; 60.9) ng/l); bioTs (13.2 (9.5; 19.6) ng/dl); DHEA-S (228 (180; 310) mg/dl).

For differential diagnostic of non-classic CAH with 21-hydroxylase deficiency we performed low-dose (5 mcg) 1-24 ACTH test with Tetracosactide acetate solution (250 mcg per 1 ml, NOVARTIS, Switzerland). Immediately before the test, 1 ml Tetracosactide acetate solution (250 mcg) was diluted by 49 ml of 0.9% NaCl solution to get concentration of 1-24 ACTH of 5 mcg per 1 ml. For intravenous bolus 1 ml of the final solution (5 mcg 1-24 ACTH) injected with 4 ml of 0.9% NaCl solution. In 20 women, standard test with 250 mcg 1-24 ACTH was additionally needed for further diagnosis.

Protocol of low-dose (5 mcg) and standard (250 mcg) 1-24 ACTH tests:

- 1) Blood sampling for basal 17-OHP and cortisol levels.
- 2) Intravenous bolus of 5 mcg or 250 mcg 1-24-ACTH.
- 3) Blood sampling for 17-OHP and cortisol at 30 and 60 minutes after 1-24-ACTH stimulation.

Molecular genetic analysis (MGA) for most prevalent *CYP21* mutations was performed using allele specific polymerase chain reaction [3].

For sensitivity and specificity analysis of low-dose ACTH test we compared results of low-dose (5 mcg) and standard (250 mcg) Tetracosactide tests with data of molecular genetic analysis for *CYP21*.

Statistical data analysis was run using software STATISTICA 6.0 and MedCalc Version 7.4.2.0. We used Shapiro-Wilk test to assess normality of distribution, and relevant t-test for comparison of two depending or non-depending samples. Descriptive non-parametrical statistics included Median (Me), Mean (M), standard deviation (SD), quartiles [25; 75] and Min-Max range. To compare quantitative parameters of non-related samples, Mann-Whitney (U) test was

applied. Wilcoxon test (W) and Friedman variance analysis (F) was used when comparing two or more related samples. Kendall's coefficient of concordance (nonparametric correlation coefficient between two variables) was used to assess the relationship between multidimensional variables. Chi-squared test (χ^2) was done to analyze difference in distribution of categorical parameters in two groups. Correlation between numerical parameters was tested by Spearman (r) and Kendall's Tau criteria. Sensitivity, specificity and accuracy of 5 mcg 1-24 ACTH test were calculated using standard formulas. Significance level was chosen at $p < 0.05$.

Results

Anovulation in women from both groups was diagnosed as abnormally prolonged menstrual cycle (over 35 days up to amenorrhea, Me=52 days) and/or negative ovulation tests in at least 5 cycles. In group 1 hyperandrogenism was proven clinically (at least 8 scores of Ferriman-Gallwey scale in 62.4% (146/234), acne in 29.5% (69/234) of patients) and biochemically (Table 1).

According to New et al. [4] and Moran et al. [5], non-classical deficiency of 21-hydroxylase should be considered in patients with elevated 17-OHP levels (basal as well as ACTH stimulated) >10 ng/ml.

In our study 17-OHP after ACTH-stimulation ranged from 0.96 to 323.7 ng/ml (Me 3.29 (2.33; 4.79) ng/ml) at 30 minutes, and from 0.99 to 113.23 ng/ml (Me 3.62 (2.59; 5.86) ng/ml) at 60 min. In 81.8% (356/435) of patients maximal 17-OHP response to ACTH stimulation occurred at 60 min (F-variance analysis χ^2 - test for 0-30-60 min, W-test for 30-60 min, $p=0.000$). Differences in changes of 17-OHP concentrations reflects the clear dependence on the stimulation (Kendall's coefficient of concordance=0.77524; rank correlation mean (r)=0.77460), but not similar sensitivity to ACTH in one person. Therefore, one can get false-negative result if 17-OHP is measured only at 60 min.

Group	Ts (ng/dl) Me (25; 75)	bioTs (ng/dl) Me (25; 75)	17-OHP (ng/ml) Me (25; 75)	DHEA-S (mg/dl) Me (25; 75)	U-тест (p) HA+ vs HA-
HA+ (n=234)	88.5 (68; 105)	27.9 (21.6; 33.5)	1.82 (1.27; 2.77) Min 0.4 Max 89.6	245.0 (187; 347) Min 46 Max 1000	bioTe p=0.000 17-OHP p=0.000042 DHEA-S p=0.718456
HA- (n=201)	56.6 (38.9; 64.9)	14.75 (9.8; 18.0)	1.43 (0.98; 2.2) Min 0.2 Max 6.52	259.0 (185; 274) Min 48 Max 518	

HA: Hyperandrogenism

Table 1: Adrenal corticosteroids levels in study groups before ACTH-5 mcg stimulation test.

For the proper interpretation of the 5 mcg ACTH-test results in diagnostics of 21-hydroxylase deficiency we formulated two tasks:

- 1) To estimate diagnostic ratio of 17-OHP increase.
- 2) To evaluate the relevance of absolute values of stimulated 17-OHP levels.

Diagnosis of non-classic CAH is probable

Only 2 patients (0.95%) with hyperandrogenism had basal 17-OHP level >10 ng/ml and stimulated levels >17 ng/ml. Twenty-five women (23-with hyperandrogenism and 2 without it), with basal 17-OHP concentration between 2.58 ng/ml and 7 ng/ml showed four-fold

increase in 17-OHP levels up to >10 ng/ml after stimulation with Tetracosactide 5 mcg: 18 patients had stimulated 17-OHP concentrations >14 ng/ml, and 7 patients showed stimulated 17-OHP between 10 and 14 ng/ml. These results made the diagnosis of non-classic CHA probable in 27 women.

Non-classic CAH is unlikely

To evaluate relevance of increase ratio BUT NOT absolute stimulated levels of 17-OHP for the diagnostics of 21-hydroxylase deficiency, we compared increase rate of 17-OHP and cortisol concentrations. Such approach prevents underestimation of maximal 17-OHP concentrations <10 -14 ng/ml due to «low dose» of ACTH.

We found that cortisol rises in significantly lower degree than 17-OHP in response to 5 mcg Tetracosactide (variance analysis χ^2 $p < 0.00000$). There was a correlation between basal cortisol or 17-OHP levels and elevation degree after stimulation with Tetracosactide-5 mcg ($r = 0.265034$; $p = 0.000001$ for cortisol; $r = 0.477788$; $p = 0.0000$ for 17-OHP). 334 patients had less than 2.5-fold rise of 17-OHP levels: 32 women (9.5%) reached 17-OHP maximal concentrations of 4.0-6.2 ng/ml and other 332 women-under 4 ng/ml. We suggested that 76.8% ($n = 334$) patients with less than 2.5-fold 17-OHP increase and maximal 17-OHP concentrations < 6.2 ng/ml were unlikely to have non-classic CAH. MGA of *CYP21*-gene in 14 women with hirsutism and relatively high simulated 17-OHP levels showed no mutations.

Test results are ambiguous and further standard test with 250 mcg of ACTH is needed for differential diagnosis of CAH

Further search for 21-hydroxylase deficiency was suggested in 48 patients with basal 17-OHP level over 1.7 ng/ml (recommended basal

17-OHP level for the diagnosis of 21-hydroxylase deficiency [5]) and documented > 3 -fold increase of 17-OHP.

According maximal stimulated 17-levels in the tests with low and standard 1-24 ACTH doses (5 mcg and 250 mcg Tetracosactide) we differentiated three different types of adrenal response:

-Dose-independent 87.5% (42/48)-differences of peak 17-OHP levels are not significant;

-Dose-dependent 12.5% (6/48)-peak 17-OHP levels between 6.7-7.3 ng/ml after low ACTH dose and > 10 ng/ml after standard ACTH dose (Table 2).

There was a subgroup of patients (6/48 (12.5%)) with dose dependent adrenal response to ACTH who needed further search for non-classical CAH. All these 6 women had maximal stimulated 17-OHP concentrations after 5 mcg Tetracosactide test ≥ 6.7 ng/ml and dermatological signs of hyperandrogenism (hirsutism).

Response to ACTH, M \pm m	17-OHP, basal, ng/ml	17-OHP, ACTH 5 mcg	17-OHP, ACTH 250 mcg
Dose-independent (n=42)	5.4 (1.9; 9.6)	16.4 (7.3; 39.5)	19.3 (6.4; 44.4) * $p = 0.39$
Dose-dependent (n=6)	2.99 \pm 0.97 (CI 1.98; 4.01)	7.104 \pm 1.35 (CI 5.69; 8.52)	17.306 \pm 4.7 (CI 12.36; 22.25) * $p = 0.0025$

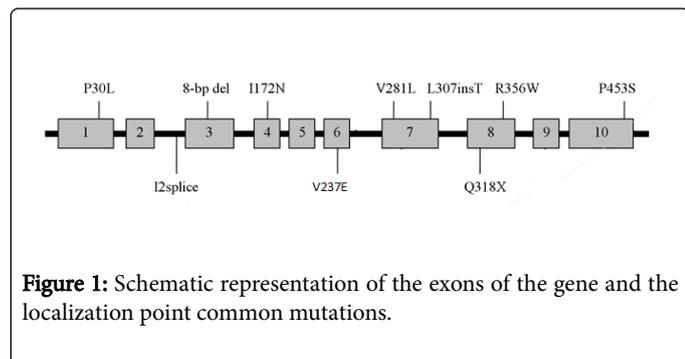
*t-test for maximal 17-OHP levels after stimulation with Tetracosactide 5 mcg and Tetracosactide 250 mcg

Table 2: Dose-independent and dose-dependent 17-OHP response to Tetracosactide 5 mcg and Tetracosactide 250 mcg.

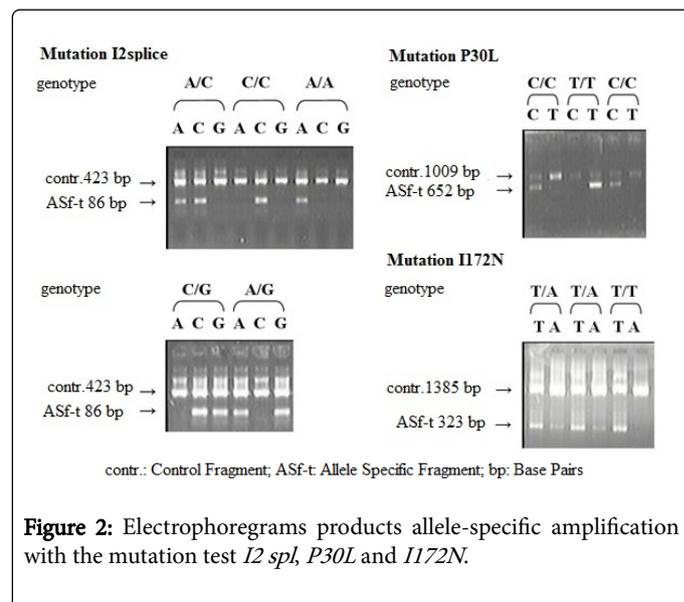
Molecular genetic analysis of *CYP21* gene

Twenty-seven patients had basal or stimulated 17-OHP concentrations over 10 ng/ml, and 17 of them had molecular genetic analysis of *CYP21* gene. Patients with stimulated 17-OHP levels between 10 ng/ml and 14 ng/ml were found to be heterozygous carriers of most common *CYP21* mutations. Women with maximal 17-OHP levels ≥ 14 ng/ml had homozygous mutations or combinations of different heterozygous mutations (Table 3) (Figures 1 and 2).

Molecular genetic analysis in 14 patients with maximal 17-OHP concentrations between 4 and 6.2 ng/ml revealed no *CYP21* mutations using allele-specific polymerase chain reaction ($n = 10$) or direct *CYP21* gene sequencing. Thus, 21-hydroxylase deficiency was excluded in these women. 334 women with non-significant 17-OHP rise (< 6.2 ng/ml) did not need further investigation for the non-classical CAH and were excluded from the study. In other 320 patients 21-hydroxylase deficiency could be surely excluded only after either standard test with 250 mcg ACTH or direct *CYP21* gene sequencing.



We found no *CYP21* mutations in 21 patients with dose independent adrenal response to 5 mcg tetracosactide. There were 13 women with maximal 17-OHP concentrations > 6.7 ng/ml. However, non-classical CAH was not proved in them during standard test and allele specific PCR of *CYP21* gene.



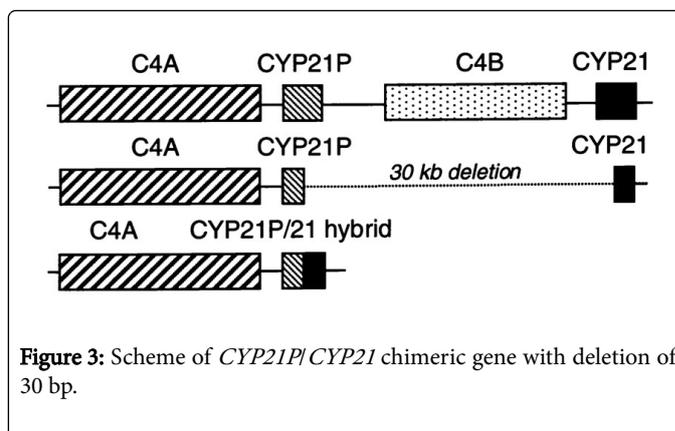
Molecular-genetic analysis in 6 women with dose-dependent response to Tetracosactide 5 mcg and Tetracosactide 250 mcg revealed 2 cases of homozygous *CYP21* mutations: *V281L/V281L* [6], *V237E/V237E* and one case of chimera gene *CYP21P1/CYP21* [7] (Figure 3).

Non-classical CAH was proved only in women with maximal 17-OHP concentration ≥ 6.7 ng/ml after stimulation with 5 mcg Tetracosactide and >14 ng/ml after stimulation with 250 mcg tetracosactide.

Three patients with maximal 17-OHP levels under 6.0 ng/ml after stimulation with 5 mcg Tetracosactide and under 10 ng/ml after 250 mcg Tetracosactide, had heterozygous carrier state of *V281L* mutation.

We evaluated sensitivity and specificity of the main hyperandrogenism symptoms, such as hirsutism and anovulation to define those women who need examination for 21-hydroxylase deficiency.

Hirsutism seems to be the main symptom which disturbs women with NC-CAH. However regular and ovulatory menstrual circle cannot rule out NC-CAH.



Maximal simulated 17-OHP level	Homozygous mutations or combinations of heterozygous mutations	Carrier state of heterozygous mutations
NC-CAH is very likely		
Test with ACTH 5 and 250 mcg: 10 ng/ml<17-OHP<14 ng/ml (n=9)	Not found	<i>V281L</i> <i>V281L</i> <i>P30L</i> <i>Q318X</i> <i>I2 spl</i> <i>V281L</i> <i>V281L</i> Common mutations not found (n=2)
Test with ACTH 5 and 250 mcg: 17-OHP >14 ng/ml (n=8)	<i>V281L/V281L</i> <i>V281L/V281L</i> <i>V281L/V281L</i> <i>V281L/V281L</i> <i>P453S/P453S</i> <i>I172N/V281L</i> <i>I2 spl/V281L</i> <i>V281L/V281L</i>	Not found
NC-CAH is unlikely		
Test with ACTH 5 mcg 17-OHP 4-6.2 ng/ml (n=14)	Not found	Not found
NC-CAH is ambiguous		
Dose independent response Test with ACTH 5 mcg and Test with ACTH 250 mcg: 17-OHP ≥ 6.7 ng/ml (n=21)	Not found	Not found
Dose-dependent response (n=3) Test with ACTH 5 mcg: 6.7<17-OHP<10 ng/ml; Test with ACTH 250 mcg: 10<17-OHP<14 ng/ml	Not found	<i>V281L</i> <i>I2 spl</i> <i>V281L</i>
Test with ACTH 5 mcg (n=3): 6.7<17-OHP<10 ng/ml; Test with ACTH 250 mcg: 17-OHP >14 ng/ml	<i>V237E/V237E</i> <i>V281L/V281L</i> Chimeric gene	Not found

Table 3: Results of molecular-genetic analysis of *CYP21* gene.

Sensitivity and specificity analysis of low-dose test with 1-24 ACTH-5 mcg included data from 36 patients who run through low-dose and standard ACTH test as well as genetic analysis of *CYP21*. These were patients with 17-OHP concentration over 14 ng/ml and homozygous or mixed heterozygous mutations of *CYP21* gene. Other

patients with heterozygous mutations and 17-OHP under 14 ng/ml (n=10), and 2 patients with dose-independent reaction to ACTH, max 17-OHP level >7 ng/ml and no proven mutations of *CYP21* gene (PCR) were excluded from this analysis. Unfortunately, 10 clinically hyperandrogenic patients and high Tetracosactide 5 mcg-stimulated

17-OHP levels (over 14 ng/ml) withdrew from the study. This fact may have influenced the results of sensitivity analysis.

Test with Tetracosactide 5 mcg had sensitivity of 72.7% and specificity of 100% with positive prognostic value (+PV) of 100%, and negative prognostic value (-PV) of 89.3%.

According to the results of the test with 5 mcg Tetracosactide, absolute stimulated 17-OHP levels but not grades of its increase are relevant for the diagnostics of 21-hydroxylase. Steroidal response to Tetracosactide may be dose dependent in 12.5% of patients, and they have higher peak 17-OHP levels after stimulation with Tetracosactide-250 mcg. Lack of low-dose 1-24 ACTH stimulation can be probably explained by an adaptation of hyperplasia adrenal glands to greater pituitary stimuli.

Diagnosis of non-classic CAH with 21-hydroxylase deficiency was proved in 5.3% (23/435) of patients reported hirsutism and/or anovulation. 21-hydroxylase deficiency was verified in 8.5% (20/234) of hyperandrogenic patients and in 1.5% (3/201) of women without clinical signs and symptoms of hyperandrogenism.

Conclusion

1. Low dose test with Tetracosactide 5 mcg can identify 21-hydroxylase deficiency in patients with stimulated 17-OHP concentrations over 14 ng/ml.

2. Standard stimulation test with Tetracosactide 250 mcg and MGA of *CYP21* gene is indicated for patients with dermatological signs of hyperandrogenism and maximal 17-OHP levels \geq 6.7 ng/ml after stimulation with Tetracosactide 5 mcg.

It should be noted, that clinical relevance of mild non-classic CAH which cannot be detected with low-dose but not with standard-dose Tetracosactide test is still uncertain. It is also known, that most women with heterozygous mutation of *CYP21* gene are symptoms free and have normal fertility. Further studies of the cases with discordant results of tests with Tetracosactide 5 mcg and 250 mcg may bring useful information.

Conflict of Interest and the Source of Funding

The authors deny financial support from the drug manufacturers. Conflict of interest in determining the structure of the study, in the collection, analysis and interpretation of missing data.

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