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17-Hydroxyprogesterone Response to Standard Dose Synacthen Stimulation Test in *CYP21A2* Heterozygous Carriers and Non-carriers in Symptomatic and Asymptomatic Groups: Meta-analyses

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What is already known on this topic?

Standard dose synacthen stimulation test (SDSST) is a gold standard biochemical-screening test for evaluating adrenal gland function. Despite studies investigating the use of SDSST to identify heterozygous *CYP21A2* mutation, the reliability of the test for this is still controversial.

What this study adds?

This meta-analysis was performed to determine if there were differences in 17-hydroxyprogesterone (17-OHP) response to SDSST (0.25 mg) in the identification of *CYP21A2* heterozygous carriers, with or without clinical sign of androgen excess disorders, to investigate the utility of the SDSST for this purpose and to determine the cut-off levels of 17-OHP for this purpose. The results support the hypothesis that stimulated 17-OHP level after SDSST had the potential to identify *CYP21A2* carriers, although basal 17-OHP level was not sufficiently informative. Additionally, the median level of stimulated 17-OHP was higher in symptomatic mutation-free controls than in asymptomatic mutation-free healthy controls and similarly, the median level of stimulated 17-OHP was higher in symptomatic mutation carriers than in asymptomatic mutation carriers. Clinical phenotype may affect the evaluation of the test.

Abstract

Objective: Standard dose synacthen stimulation test (SDSST) is a gold standard screening test for evaluating adrenal gland function. Despite studies using SDSST to identify heterozygosity in *CYP21A2*, the reliability of the test for this purpose is still controversial. Therefore, the meta-analyses were performed to determine the differences in 17-hydroxyprogesterone (17-OHP) responses to standard dose (0.25 mg) SDSST in the diagnosis of *CYP21A2* heterozygous individuals, with or without clinical signs of androgen excess disorders.
Methods: PubMed and MEDLINE databases were searched. A total of 1215 subjects (heterozygous carriers n = 669, mutation-free controls n = 546) were included in the meta-analyses.
Results: Basal 17-OHP median/mean levels were 4.156 (3.05-10.5)/5.241 (± 2.59) nmol/L and 3.90 (2.20-9.74)/4.67 (± 2.62) nmol/L in symptomatic heterozygous carriers and symptomatic mutation-free controls, respectively. Stimulated 17-OHP median/mean levels were 17.29 (14.22-37.2)/19.51 (± 7.63) nmol/L and 9.27 (7.32-15.9)/10.77 (± 3.48) nmol/L in symptomatic heterozygous carriers and symptomatic mutation-free controls, respectively. Basal 17-OHP median/mean levels were 3.21 (2.64-4.78)/3.33 (± 0.84) nmol/L and 3.12 (1.82-3.6)/2.83 (± 0.71) nmol/L in asymptomatic heterozygous carriers and asymptomatic mutation-free healthy controls, respectively. Stimulated 17-OHP median/mean levels were 14.16 (12.73-16.37)/14.16 (± 1.37) nmol/L and 6.26 (4.9-8.23)/6.48 (± 1.2) nmol/L in asymptomatic heterozygous carriers and asymptomatic mutation-free healthy controls, respectively. The cut-off levels for stimulated 17-OHP were 10.48 nmol/L and 13.48 nmol/L for asymptomatic heterozygous and symptomatic heterozygous, respectively.
Conclusion: The meta-analyses support the idea that stimulated 17-OHP level has potential for use in identifying *CYP21A2* carriers. Besides, considering differences in the basal and stimulated 17-OHP levels in symptomatic heterozygous individuals compared to those who were asymptomatic heterozygous could increase the accuracy of the test.
Keywords: Adrenal insufficiency, synacthen stimulation test, 17-OHP level, heterozygous, *CYP21A2*

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Introduction

Adrenal insufficiency is caused by a failure of the adrenal cortex to produce cortisol. The most common cause of adrenal insufficiency is autosomal recessive inherited congenital adrenal hyperplasia (CAH) (OMIM 201910), characterized by excess adrenal androgen production resulting from impairment of the adrenal 21-hydroxylase (21OH) enzyme. Androgen excess affects approximately 10% of women (1). Disorders that result from hyperandrogenism include polycystic ovary syndrome (PCOS) (OMIM 184700) and 21OH-deficient non-classic adrenal hyperplasia (21OHD-NCAH) (OMIM 201910). The etiology of PCOS is not fully understood but it is a familial disorder that appears to be inherited as a complex genetic trait with a risk to siblings of ~50%. There is no accepted precise mode of inheritance (2,3,4) but it is one of the most common endocrine disorders, affecting 6-10% of reproductive-age women (5). It may be difficult to distinguish PCOS from NCAH clinically (6). Of hyperandrogenic women, 1-10% is reported to be affected by NCAH due to 21OHD and NCAH may even be asymptomatic (7). In childhood, hyperandrogenism may present with premature pubarche (PP) and 5-20% of PP cases were diagnosed with NCAH, mainly due to 21OHD-NCAH (8,9,10,11,12).

Standard dose synacthen stimulation test (SDSST) is the gold standard screening test to evaluate adrenal gland function (13). It is the principal challenge test to estimate the relative activity of adrenocortical enzymes (14) and it has been widely used for the biochemical diagnosis of NCAH, due to various adrenocortical enzyme deficiencies including 21OHD. A consensus is not available about whether heterozygous individuals with *CYP21A2* mutations have a higher risk of developing clinical hyperandrogenism. In some selected populations, being heterozygous for *CYP21A2* seems to be related to irregular menses, hirsutism, PCOS, premature adrenarche (PA), acne, and central precocious puberty (12,15,16,17,18,19,20,21). In contrast, other investigators concluded that heterozygosity for *CYP21* mutations did not increase the risk of clinical androgen excess above that expected in the general population (22,23). The prevalence of asymptomatic carriers for the disease in the general population was estimated to range from 1:50 to as high as 1:16 (24) and even higher among Ashkenazi Jews, according to a single report (8). The frequency of mutation carriers of *CYP21A2* was almost 1 in 4 in PP and hirsute groups (21,25).

The objectives of this meta-analysis were to determine if there was a difference in 17-hydroxyprogesterone (17-OHP) response to standard dose (0.25 mg) SDSST in the diagnosis of *CYP21A2* heterozygous individuals, with or without clinical androgen excess, to investigate the utility of the SDSST to identify heterozygous *CYP21A2* carriers and to determine the cut-off levels of 17-OHP for this purpose.

Methods

Search Strategy

PubMed and MEDLINE databases were searched for relevant literature. The search strategy was kept broad, included several synonymous expressions, and performed using the keywords ("21-hydroxylase" OR "*CYP21A2*" OR "21 α-hydroxylase" OR "CYP21") AND "heterozygous" AND ["hirsutism" OR "hyperandrogenemia" OR "polycystic ovary syndrome" OR "PCOS" OR "acne" OR "alopecia" OR "oligomenorrhea" OR "adrenal hyperplasia" OR "adrenocorticotropic hormone (ACTH)]". Only peer-reviewed, original articles were included in the study. Additional publications from the references of the included studies were manually searched by the investigators to identify the articles that may be missed by the electronic search.

Study Selection

Human studies, published between January 1995 and May 2020, were considered further. Studies without control groups were excluded, as well as those written in languages other than English. Since genetic mutation screening performed before 1995 was based on human leukocyte antigen (HLA) typing in most cases, studies performed before this date were excluded from the search criteria.

In the publications included in the study, mutation analyses were performed using a range of methods, including amplification-refractory mutation system, allele-specific oligonucleotide hybridization, Sanger sequencing, single-strand conformation polymorphism, multiplex ligation-dependent probe amplification, Southern Blot, sequence-specific oligonucleotide probes, real-time quantitative reverse transcription-polymerase chain reaction and multiplex mini-sequencing. 17-OHP measurement was done using radioimmunoassay (RIA), enzyme-linked immunosorbent assay, and liquid chromatography with tandem mass spectrometry (LC-MS/MS) methods.

The selection criteria were: 1) case-control studies; 2) studies that evaluated the relationship between basal and/or stimulated 17-OHP levels after SDSST in *CYP21A2* carriers and non-carriers with one of the clinical hyperandrogenic symptoms including hirsutism, and/or oligo/amenorrhea

and/or acne and/or elevation of at least one serum androgen; 3) useable data on 17-OHP levels to identify 21OH deficiency in patients with PP, PA and premature thelarche (PT); and 4) studies focused on differential diagnosis between NCAH and PCOS. The exclusion criteria were: 1) case or family reports; 2) studies that evaluated genetically confirmed CAH and NCAH patients; 3) studies focused on diagnosis, treatment, review, method and general information; and 4) studies related to other diseases including Cushing syndrome, acromegaly, adrenal tumor, 11-hydroxylase, cytochrome P450 oxidoreductase (*POR*), 3-beta (β)-hydroxysteroid dehydrogenase (*HSD3B2*), and 17α-hydroxylase/17,20-lyase (*CYP17A1*) deficiencies (Figure 1).

Data Extraction and Analysis

Two authors independently screened the title, abstract and full text of potentially eligible studies twice at two different time points. Any disagreements were resolved by discussion or by seeking an independent third opinion. The titles and abstracts of the articles were examined and irrelevant ones were excluded. The full texts of the remaining articles were reviewed to find relevant studies that met the inclusion criteria. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses guideline is aimed at improving systematic reviews and formed the basis for the selection protocol used in the current study (Figure 1).

Statistical Analysis

For all meta-analyses, Review Manager (2014) version, 5.3 was used. A random-effects model and fixed-effect model were used while performing the meta-analyses. Due to the large degree of heterogeneity, a random-effects model was applied, which does not adjust heterogeneity but it is a more conservative approach when the heterogeneity exists. Summary statistics were reported as standardized mean difference (SMD) and mean difference (MD) with 95% confidence intervals (CI). SMD levels of <0.2, >0.2 and <0.7, or >0.8 were considered small and moderate, or large effects, respectively (26). Study-level mean (standard deviation; SD) and median (minimum-maximum) levels were reported. Study-level mean levels were used to generate receiver operating characteristic (ROC) curve using Statistical Package for the Social

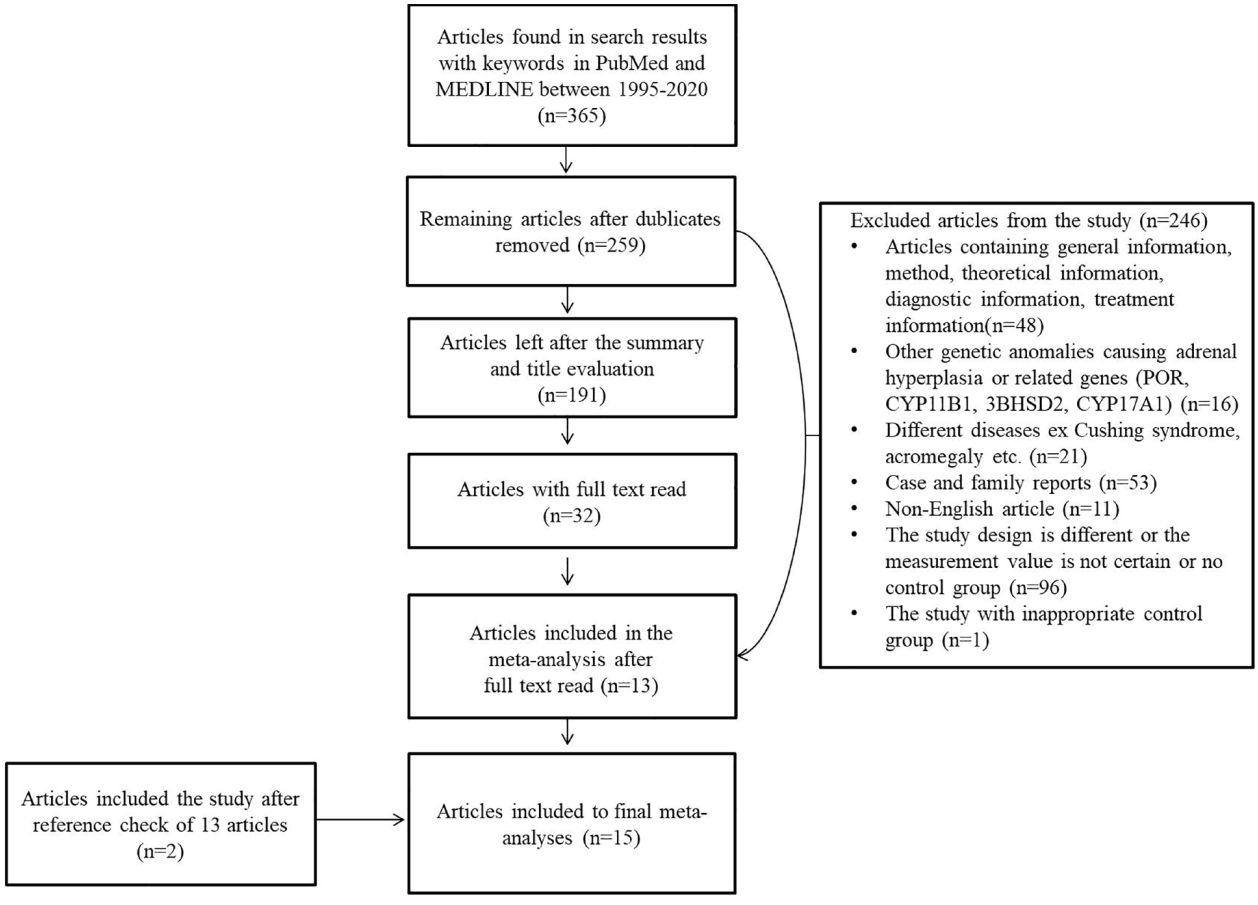

Figure 1. Flow chart to illustrate the process by which articles were selected or rejected based on the inclusion and exclusion criteria of the study

Sciences, version 22 (IBM Inc., Armonk, NY, USA) (27). When cut-off values were determined, Youden's index was used and diagnostic accuracy measures are reported.

To convert all reported results to a standard units of measurement (nmol/L) in all the included articles, the 17-OHP values were multiplied by 0.0303 and 3.0261 to convert ng/dL to nmol/L and ng/ml to nmol/L, respectively.

Patients

Individuals from both "female and male" gender, who were *CYP21A2* heterozygous mutation carriers and non-carriers and aged between 0.7-65 years, were included in the study (Table 1). The study groups consisted of females and/or males with PCOS, PP, PA, PT and clinical hyperandrogenism, relatives of patients with CAH or NCAH, and healthy controls.

Table 1. Main characteristics of the included studies

Author	Year	Nationality	Age range (year)	Gender	Case (21-HTZ)	Control (mutation free)	Mutation analyses method	17-OHP measurement method
Oriolo et al (34)	2020	Italy	HTZ: 22.2 + 7.2 Control: 24.0 + 9.2	F	15 (PCOS diagnosis)	32 (PCOS diagnosis)	Sanger sequencing	RIA
Polat et al (21)	2019	Turkey	Range: 18-45	F	14 (PCOS diagnosis)	40 (PCOS diagnosis)	Sanger sequencing	RIA
Grodnitskaya and Kurtser (40)	2017+	Russia	HTZ: 26.4 ± 5.3 Control: 26.1 ± 7.2	F	7 (one of the clinical androgenic symptoms (oligo/amenorrhea, hirsutism or acne) and/or elevation of at least one serum androgen)	43 (one of the clinical androgenic symptoms (oligo/amenorrhea, hirsutism or acne) and/or elevation of at least one serum androgen)	ARMS, MLPA	ELISA
Neocleous et al (30)	2017	Greek Cypriot	Not stated	F	52 (clinical hyperandrogenism)	52 (clinical hyperandrogenism)	Sanger sequencing, MLPA	RIA
Settas et al (31)	2013+	Greece	HTZ: 23.4 ± 5.4 Control: 29.5 + 5.5	F	15 (PCOS diagnosis)	68 (PCOS diagnosis)	ARMS + Sanger sequencing	RIA
Napolitano et al (35)	2011*	Italy	Not stated	F + M	161 (relatives of patients with CAH and NCAH)	73 (relatives of patients with CAH and NCAH and healty volunteers)	Southern Blot, Multiplex minisequencing, LR-PCR	RIA
Costa-Barbosa et al (37)	2010*	Brazil	HTZ: 23-62 Control: 23-65	F + M	61 (parents of affected patients with 21OHD)	27 (healthy volunteers)	ARMS, MLPA	LC-MS/MS
Paris et al (38)	2010	France	HTZ: 6.8 + 0.7 Control: 6.7 + 1.3	F	8 (PP diagnosis)	25 (PP diagnosis)	Sanger sequencing	RIA
Bidet et al (39)	2009*	France	23.4 + 8.8 (range: 13-52)	F	211 (relatives of patients with CAH and NCAH)	36 (relatives of patients with CAH and NCAH)	Sanger sequencing, Southern Blot, RT-qPCR	RIA
Admoni et al (41)	2006	Israel	Not stated	F + M	24(PP and/or hirsutism, and/or premature telarche)	43 (hirsutism, PP, precocious puberty, menstrual irregularity)	SSOP	RIA
Bachega et al (36)	2000	Brazil	Not stated	F + M	13 (precocious pubarche, acne, hirsutism and/or menstrual irregularities)	8 (precocious pubarche, acne, hirsutism and/or menstrual irregularities)	ARMS	RIA
Dacou-Voutetakis and Dracopoulou (15)	1999	Greece	Not stated	F + M	18 (premature adrenarche)	26 (premature adrenarche)	ARMS, Southern Blot	RIA

Table 1. Continued

Author	Year	Nationality	Age range (year)	Gender	Case (21-HTZ)	Control (mutation free)	Mutation analyses method	17-OHP measurement method
Witchel and Lee (33)	1998*	USA	Not stated	F + M	28 (relatives of patients with 21-hydroxylase deficiency)	23 (healthy control and relatives of patients with 21-hydroxylase deficiency)	ASOH, SSCP	RIA
Witchel et al (32)	1997[a]	USA	Not stated	F + M	28 (relatives of patients with 21OHD)	22 (healthy control and relatives of patients with 21-hydroxylase deficiency)	ASOH, SSCP	RIA
Witchel et al (18)	1997[b#]	USA	Not stated	F + M	10 (PP diagnosis)	18 (PP diagnosis)	ASOH, SSCP	RIA
Witchel et al (18)	1997[b#]	USA	Not stated	F + M	4 (clinical hyperandrogenism)	10 (clinical hyperandrogenism)	ASOH, SSCP	RIA

*Shows the study included in the analysis group formed from asymptomatic heterozygous carriers and asymptomatic mutation-free controls, [+]shows the study that only had basal 17-OHP levels [#]shows the same study used two times in the same analyses with two different data set.
F: female, M: male, RIA: radioimmunoassay, LC-MS/MS: liquid chromatography with tandem mass spectrometry, ELISA: enzyme-linked immunosorbent assay, SSCP: single-strand conformation polymorphism, ASOH: allele-specific oligonucleotide hybridization, SSOP: sequence-specific oligonucleotide probes, ARMS: amplification-refractory mutation system, MLPA: multiplex ligation-dependent probe amplification, RT-qPCR: quantitative polymerase chain reaction, LR-PCR: long range PCR, 21-HTZ; CYP21A2 heterozygous, HTZ: heterozygous, PCOS: polycystic ovary syndrome, CAH: congenital adrenal hyperplasia, NCAH: non-classic CAH, PP: premature pubarche, USA: United States of America

ΩMutation analysis of the *CYP21A2* gene and SDSST were applied to all volunteers participating in the study.

Heterogeneity and Publication Bias

Between-study variability was compared for within-study variability (i.e., heterogeneity of effect size) using the I^2 statistic, which measures the percentage of variation due to heterogeneity (28). An I^2 level less than 25% indicated low heterogeneity, whereas levels between 35 to 50% showed moderate heterogeneity and those above 50% showed high heterogeneity (28). Publication bias was assessed using contour-enhanced funnel plots (29).

Results

Search Results

Three hundred and sixty-five relevant publications were found after the screening of studies published between 1995 and 2020. After excluding repetitive and irrelevant publications, fifteen high-quality, peer-reviewed publications that met inclusion criteria were included in the meta-analysis. These studies were carried out in Europe, the United States of America, and Russia. Three studies were carried out in Greece (15,30,31), three in the United States of America (18,32,33), two in Italy (34,35), two in Brazil (36,37), two in France (38,39), one in Turkey (21), one in Russia (40) and one in Israel (41). *CYP21A2* mutation analysis and basal and/or stimulated 17-OHP measurements were available in all study subjects. The SDSST was performed in patients by administration of a single, intravenous dose of 0.25 mg synacthen (synthetic ACTH). Measurements of the basal and stimulated serum 17-OHP levels were done after 30 or 60 minutes of synacthen administration.

Among the included studies, two were added to the relevant sub-analysis since they only included basal 17-OHP measurements (31,40). Two different control groups were identified in the included studies; a mutation-free asymptomatic healthy volunteer group (asymptomatic heterozygous vs. asymptomatic mutation-free healthy control), and a mutation-free but clinically symptomatic volunteer group (symptomatic heterozygous vs. symptomatic mutation-free control). Therefore, these two control sub-groups were analysed in two separate analyses to make clear discrimination. In a study, since 17-OHP levels were given separately based on two different clinical findings, the same study was included twice with different data sets (18). Whereas 17-OHP level measured in a publication was remarkably high in both groups compared to given 17-OHP levels in other publications used in the meta-analysis, the study was included in the analysis since it met the inclusion criteria (36). One thousand, two hundred and fifteen subjects including 21OH-heterozygous carriers (n = 669) and mutation-free controls (n = 546) were included in the meta-analysis (Table 1).

Comparison of Basal and Stimulated 17-OHP Levels

Symptomatic Heterozygous vs. Symptomatic Mutation-free Control

The fixed-effects model was used with basal 17-OHP levels because the heterogeneity of the studies was low ($I^2 = 0\%$, $p = 0.620$). In the symptomatic heterozygous carriers, the level of the MD was found to be higher than symptomatic mutation-free controls (MD: 0.70 nmol/L, 95% CI: 0.21-1.18, $Z = 2.81$, $p = 0.005$). When the MD was standardized, the difference was determined to have a medium effect size (SMD: 0.33 nmol/L, 95% CI: 0.14-0.51, $Z = 3.42$, $p < 0.001$) (Figure 2).

When comparing the stimulated levels of 17-OHP in symptomatic heterozygous carriers and symptomatic mutation-free controls, the random-effects model was used due to significant heterogeneity ($I^2 = 72\%$, $p < 0.001$). The MD were found to be higher in heterozygous carriers (MD: 7.20 nmol/L, 95% CI: 5.15-9.25, $Z = 6.87$, $p < 0.001$). The SMD (SMD: 0.9 nmol/L, 95% CI: 0.46-1.34, $Z = 4.01$, $p < 0.001$) shows that the difference has a large effect size (Figure 2).

The ROC curves for symptomatic heterozygous and symptomatic mutation-free controls are shown in Figures 3A, 3B, demonstrating that stimulated 17-OHP provides good discrimination (area under ROC curve = 0.80, $p = 0.034$) between symptomatic heterozygous and symptomatic mutation-free controls, with an optimal cut-off of 13.41 nmol/L, yielding a sensitivity of 100% and a specificity of 66.7%. Basal 17-OHP level was not found to be capable of discriminating heterozygous from wild type in the symptomatic group (Table 2).

Asymptomatic Heterozygous vs. Asymptomatic Mutation-free Healthy Control

The fixed-effects model was used in the analysis of basal 17-OHP levels in asymptomatic heterozygous carriers with the asymptomatic mutation-free healthy controls due to the low heterogeneity of the studies ($I^2 = 0\%$, $p = 0.53$). Basal 17-OHP level was higher in asymptomatic heterozygous carriers than in asymptomatic mutation-free healthy controls (MD: 0.62 nmol/L, 95% CI: 0.20-1.04, $Z = 2.92$, $p < 0.001$). The SMD in the groups was 0.27 nmol/L (SMD: 0.27, 95% CI: 0.10-0.45, $Z = 3.04$, $p = 0.002$), and this difference was determined to have a medium effect size (Figure 4).

When comparing the stimulated level of 17-OHP in asymptomatic heterozygous carriers and asymptomatic mutation-free healthy controls, the random-effects model was used due to significant heterogeneity ($I^2 = 85\%$, $p < 0.001$). The 17-OHP level was higher in asymptomatic

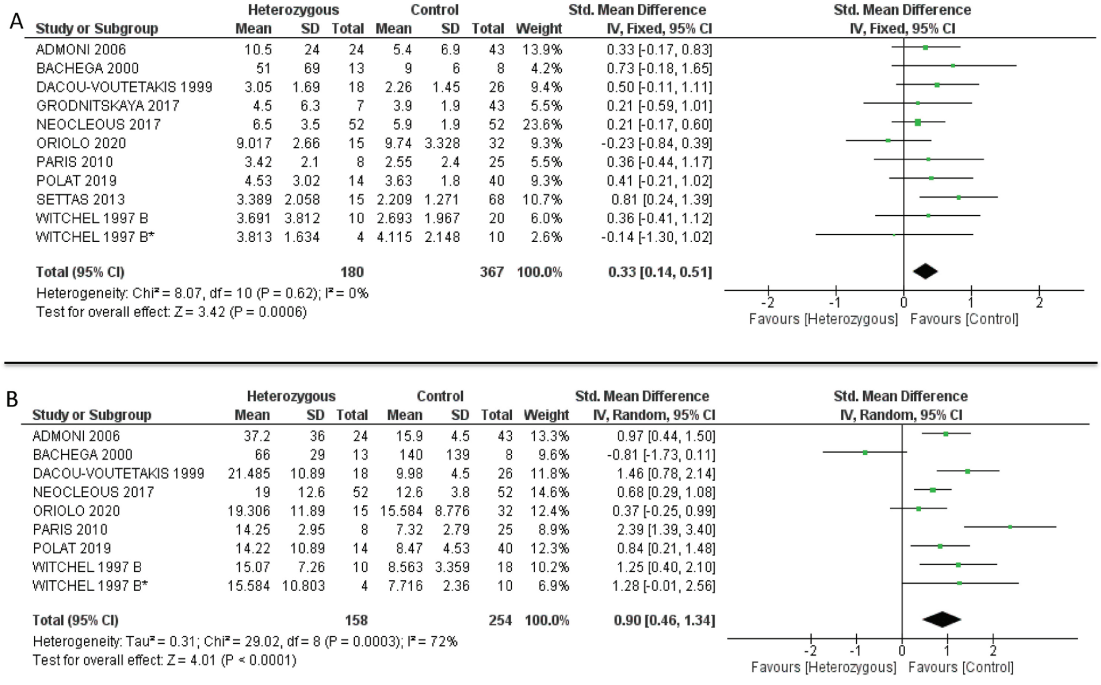

Figure 2. Forest plot of 17-hydroxyprogesterone response to standard dose synacthen stimulation test of symptomatic heterozygous vs. symptomatic mutation-free volunteers

**Shows the same study used two times in the same analyses with two different data set*

CI: Confidence interval, SD: Standard deviation

heterozygous carriers than in asymptomatic mutation-free healthy controls (MD: 7.57 nmol/L, 95% CI: 6.82-8.32). The SMD in the groups was 1.34 nmol/L (SMD: 1.34, 95% CI: 0.81-1.87, Z = 4.99, p < 0.001), and the effect size for this difference was similar to the symptomatic heterozygous vs. symptomatic mutation-free controls comparison (Figure 4).

The ROC curves for asymptomatic heterozygous and asymptomatic mutation-free healthy controls are shown in Figures 3C, 3D, demonstrating that stimulated 17-OHP provided good discrimination (area under ROC curve = 1.0, p = 0.009) between asymptomatic heterozygous and asymptomatic mutation-free healthy control with an optimal cut-off of 10.48 nmol/L, yielding a sensitivity of 100% and a specificity of 100%. Basal 17-OHP level was not found to be capable of discriminating heterozygous from wild type in the asymptomatic group (Table 2).

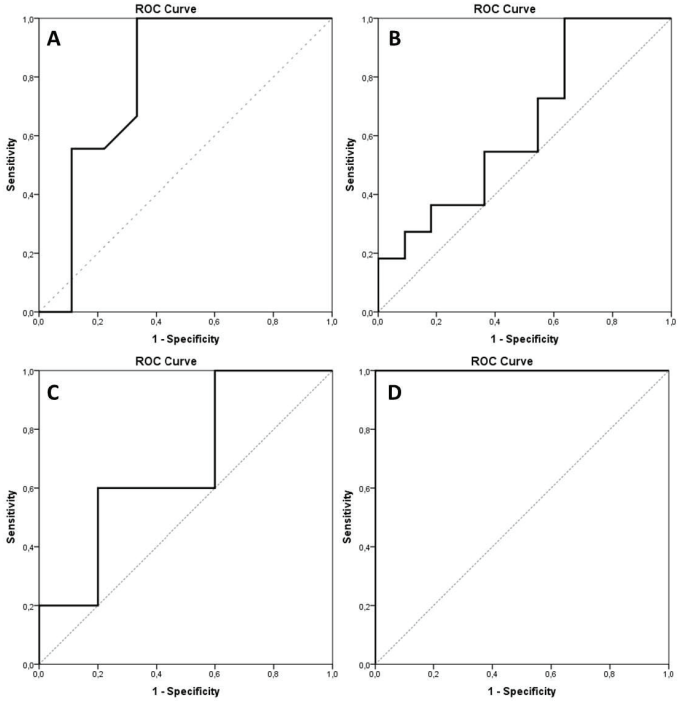

Figure 3. Receiver operating characteristic-curve analysis for basal and stimulated 17-hydroxyprogesterone (17-OHP) levels in symptomatic and asymptomatic groups. A: Basal 17-OHP levels of symptomatic heterozygous vs. symptomatic mutation-free volunteers B: Stimulated 17-OHP levels of symptomatic heterozygous vs. symptomatic mutation-free volunteers C: Basal 17-OHP levels of asymptomatic heterozygous vs. asymptomatic mutation-free healthy volunteers D: Stimulated 17-OHP of asymptomatic heterozygous vs. asymptomatic mutation-free healthy volunteers

Median and Mean of Basal and Stimulated 17-OHP Levels

Study-level median and mean were calculated, after elimination of the publication with extreme basal and stimulated 17-OHP levels (36). Basal 17-OHP median (range) levels were 4.156 (3.05-10.5) nmol/L and 3.90 (2.20-9.74) nmol/L in symptomatic heterozygous carriers and symptomatic mutation-free controls, respectively. Basal 17-OHP mean ± SD levels were 5.24 ± 2.59 nmol/L and 4.67 ± 2.62 nmol/L in symptomatic heterozygous carriers and symptomatic mutation-free controls, respectively. Stimulated 17-OHP median levels were 17.29 (14.22-37.2) nmol/L and 9.27 (7.32-15.9) nmol/L in symptomatic heterozygous carriers and symptomatic mutation-free controls, respectively. Stimulated 17-OHP mean levels were 19.51 ± 7.63 nmol/L and 10.77 ± 3.48 nmol/L in symptomatic heterozygous carriers and symptomatic mutation-free controls, respectively. Basal 17-OHP median levels were 3.21 (2.64-4.78) nmol/L and 3.12 (1.82-3.6) nmol/L in asymptomatic heterozygous carriers and asymptomatic mutation-free healthy controls, respectively. Basal 17-OHP mean levels were 3.33 ± 0.84 nmol/L and 2.84 ± 0.71 nmol/L in asymptomatic heterozygous carriers and asymptomatic mutation-free healthy controls, respectively. Stimulated 17-OHP median levels were 14.16 (12.73-16.37) nmol/L and 6.26 (4.9-8.23) nmol/L in asymptomatic heterozygous carriers and asymptomatic mutation-free healthy controls, respectively (Figure 5). Stimulated 17-OHP mean levels were 14.16 ± 1.37 nmol/L and 6.48 ± 1.2 nmol/L in asymptomatic heterozygous carriers and asymptomatic mutation-free healthy controls, respectively.

Heterogeneity and Publication Bias

Funnel plots were drawn in both fixed and random-effect models to determine whether there was a publication

Table 2. The cut-off value for predicting heterozygous individuals with standard dose synacthene stimulation test

SDSST	AUC	95% CI for AUC	p	Cut-off value*	Sensitivity	Specificity
Basal 17-OHP symptomatic	0.67	0.39-0.87	0.279	-	-	-
Stimulated 17-OHP symptomatic	0.80	0.57-1.0	0.034	13.41	100	66.7
Basal 17-OHP asymptomatic	0.68	0.33-1.0	0.347	-	-	-
Stimulated 17-OHP asymptomatic	1.0	1.0-1.0	0.009	10.48	100	100

*ROC curve analysis from study-level data.
SDSST: standard dose synacthen stimulation test, AUC: area under ROC curve, CI: confidence interval, 17-OHP: 17-hydroxyprogesterone, ROC: receiver operating characteristic

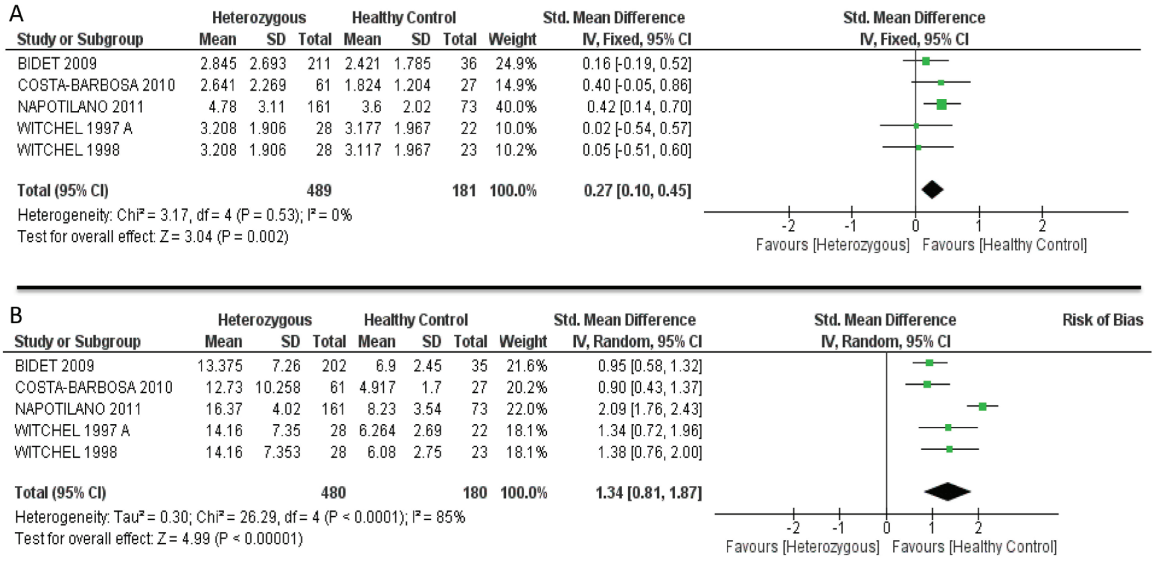

Figure 4. Forest plot of 17-hydroxyprogesterone response to standard dose synacthen stimulation test in asymptomatic heterozygous vs. asymptomatic mutation-free healthy controls

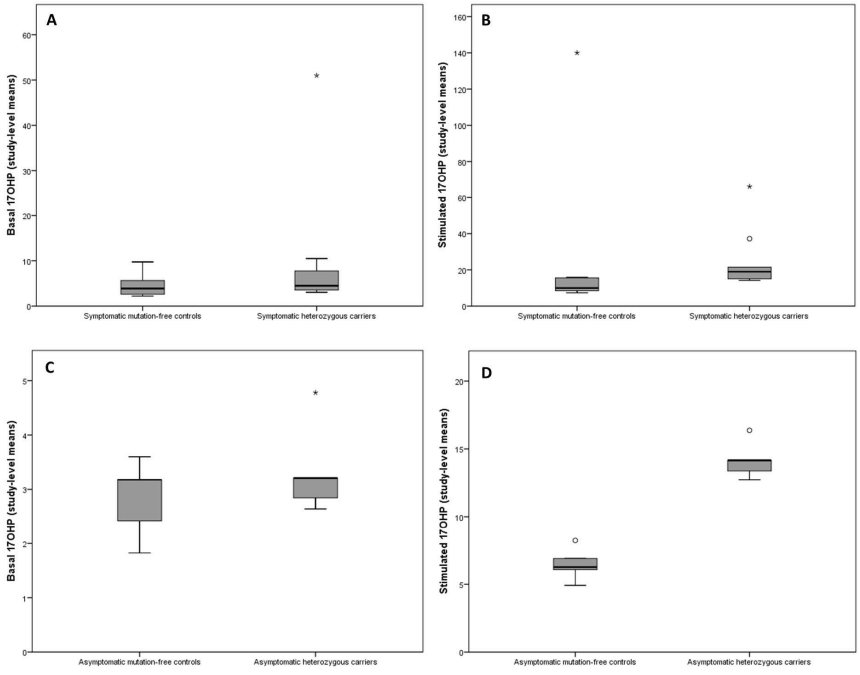

Figure 5. Box plot for median of basal and stimulated 17-hydroxyprogesterone levels in symptomatic and asymptomatic groups

bias in the included papers. In both models, CI were also presented in the funnel plot. As funnel plots seemed almost symmetrical in all meta-analyses, it was concluded that publication bias was weak (Figure 6).

Discussion

Measurement of serum 17-OHP was introduced in 1968 (42), and it is now used most widely for the diagnosis of adrenal enzymatic defects (43) in combination with SDSST, a gold standard and commonly used biochemical test in the evaluation of adrenal gland function. The SDSST for evaluation of adrenal gland function has been investigated in various clinical conditions, such as pre-clinical Addison's disease (44), immediately after pituitary surgery (45), patients with primary hypothyroidism (46), and in patients with primary fibromyalgia syndrome (47), in women with PCOS (48) and hirsutism and/or oligomenorrhea (49),

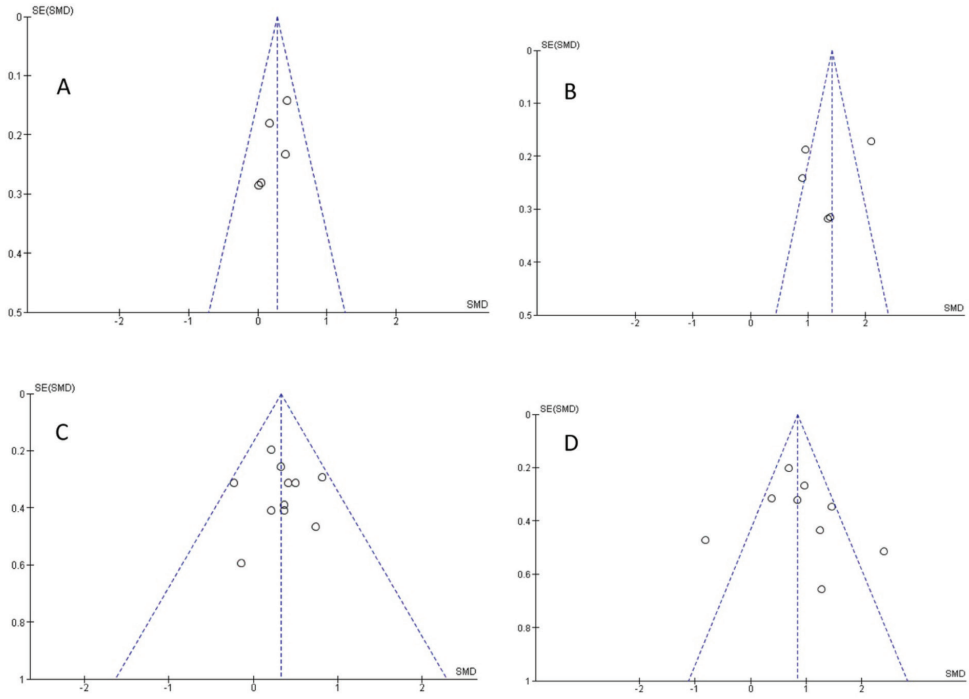

Figure 6. Funnel plots to detect publication bias of the meta-analysis. A: Basal 17-hydroxyprogesterone (17-OHP) levels of asymptomatic heterozygous vs. asymptomatic mutation-free healthy volunteers B: Stimulated 17-OHP levels of asymptomatic heterozygous vs. asymptomatic mutation-free healthy volunteers C: Basal 17-OHP levels of symptomatic heterozygous vs. symptomatic mutation-free volunteers D: Stimulated 17-OHP of symptomatic heterozygous vs. symptomatic mutation-free volunteers. The standardized mean difference (SMD) on the x-axis is plotted against the standard error of the SMD on the y-axis. Symmetrical distribution of studies indicates the absence of publication bias

in adolescents with PP (15), and in patients with CAH or NCAH (50). Compared to normal female individuals, female carriers of 21-OHD frequently demonstrate an exaggerated secretion of the 21-OH precursor 17-OHP after ACTH administration (51). It has been reported that 50-80% of carriers exhibit 17-OHP levels above the 95th percentile of the control level after ACTH stimulation (51,52,53).

To our knowledge, this is the first meta-analysis of differences in 17-OHP responses to SDSST in heterozygous and mutation-free symptomatic and asymptomatic volunteers, considering only high-quality studies with the same SDSST criteria and *CYP21A2* mutation analyses at a molecular level excluding HLA typing. In the literature, SDSST was recommended when the basal 17-OHP ≥6 nmol/L. So 21OHD-NCAH was unlikely in cases with lower basal 17-OHP has become widely accepted. Possible heterozygote carrier status was considered for the patients with baseline 17-OHP >6 nmol/L or those with baseline 17-OHP <6 nmol/L and ACTH stimulated 17-OHP <30 nmol/L (54,55). Stimulated 17-OHP >30 nmol/L was considered as the criterion for 21OH deficiency-related NCAH (56). In another study, patients were considered to be heterozygote carriers of 21OHD with ACTH-stimulated 17-OHP concentrations between 12.1 nmol/L and 30.2 nmol/L (57). In yet another study, stimulated 17-OHP levels >45 nmol/L after ACTH stimulation were suggested to have NCAH while levels between 15 and 45 nmol/L were suggested to be probable mutation carriers and levels below 15 nmol/L were interpreted as normal (41).

In our study, MD and SMD of the basal 17-OHP were 0.7 nmol/L and 0.33 nmol/L in the symptomatic group while MD and SMD in the asymptomatic group were 0.62 nmol/L and 0.27 nmol/L compared to the control group, respectively. SMD levels in both groups were slightly higher than the small effect size limit of <0.2. In our meta-analyses, in the asymptomatic group, both the basal median 17-OHP level and the SMD level were found to be lower than in the symptomatic group. The authors of some of the included studies had attempted to identify a cut-off level for basal 17-OHP that will exclude the diagnosis of NCAH and avoid unnecessary SDSST, especially for countries where synthetic ACTH is not widely available (58). Temeck et al (59) found that 13-14% of patients with NCAH would be missed if a basal 17-OHP level of 6 nmol/L

was used. Escobar-Morreale et al (60) proposed a cut-off level of 5.1 nmol/L with 100% sensitivity and 88.6% specificity in a cohort of women with hyperandrogenism. Leite et al (11) showed that basal level of 17-OHP >3 nmol/L was sufficient for the diagnosis of NCAH. Gönç et al (61) determined that only one of the NCAH cases would be missed when 4.69 nmol/L was used as the basal 17-OHP cut-off level in patients with PA and these authors also suggested that including the patient's clinical phenotype in the evaluation of the basal 17-OHP level can increase the accuracy of the SDSST. On the other hand, there is no consensus for identification of heterozygosity, and so 17-OHP levels between those of NCAH and normal are accepted as indicating heterozygousity (41).

In these meta-analyses, MD and SMD of the stimulated 17-OHP were 7.2 nmol/L and 0.9 nmol/L in the symptomatic group, and 7.57 nmol/L and 1.34 nmol/L in the asymptomatic group, respectively. SMD levels in both groups were higher than the large effect size limit of >0.8. The stimulated 17-OHP median levels were determined to be 17.29 nmol/L (14.22-37.2) and 9.27 nmol/L (7.32-15.9) in symptomatic heterozygous carriers and symptomatic mutation-free controls, respectively, and median stimulated 17-OHP levels were 14.16 nmol/L (12.73-16.37) and 6.26 nmol/L (4.9-8.23) in asymptomatic heterozygous carriers and asymptomatic mutation-free healthy controls, respectively. ROC analysis showed that the basal 17-OHP level was not discriminative in both symptomatic and asymptomatic groups, but the stimulated 17-OHP level was informative. The cut-off level for the asymptomatic heterozygous individuals was 10.48 nmol/L, while the cut-off level for the symptomatic group was 13.41 nmol/L. Both the cut-off levels were lower than 15 nmol/L, which were interpreted as normal. Besides, the stimulated 17-OHP median level of symptomatic mutation-free controls was higher than that of asymptomatic mutation-free healthy controls (9.27 vs. 6.26 nmol/L). Similarly, the stimulated 17-OHP median level of symptomatic heterozygous carriers was higher than that of asymptomatic heterozygous carriers (17.29 vs. 14.16 nmol/L). The stimulated 17-OHP level of the symptomatic group was in the heterozygous range (>15 nmol/L and <45 nmol/L), while it was below the level considered heterozygous (<15 nmol/L) in the asymptomatic group. This result supports the hypothesis that clinical phenotype effects the test. Similarly, Admoni et al (41) made a comparison between symptomatic heterozygous and family member carriers, which revealed that the symptomatic carriers had a significantly higher ACTH-stimulated 17-OHP than family member carriers.

CYP21A2 gene mutation is not the only factor causing androgen excess symptoms. Other hormones or genes may also play a role, resulting in a similar clinical phenotype. Additionally, both adrenal and gonadal steroid hormone biosynthesis is a complex phenomenon, regulated by the feedback mechanism between different tissues and dozens of genes belonging to different gene families. The biosynthetic pathways have not been fully elucidated yet and there is still much to be understood (62).

The *CYP21A2* heterozygous mutation carrier should be considered in the differential diagnosis of hyperandrogenic symptoms (63). Determination of carrier status is also compulsory for genetic counselling of the parents affected by CAH/NCAH and in families that includes a parent with confirmed heterozygous mutation, since genetic counselling plays an important role in the control of genetic diseases. The heterozygous individuals may be diagnosed with NCAH due to false-positivity of the SDSST, especially in V281L heterozygous mutation (21). Therefore, there is a need for up-to-date studies on the specificity and sensitivity of the SDSST to distinguish *CYP21A2* carriers and 21OHD-NCAH.

The V281L mutation is compatible with the NCAH allele and the resulting protein exhibits 30-50% residual enzymatic activity (64). It has been shown that the ACTH test result gave a level close to the cut-off level used for NCAH in heterozygous V281L mutation (21,41). Escobar-Morreale et al (23) hypothesized one possible explanation for the abnormal 21-hydroxylase function in subjects with one normal allele and a "half functioning" allele was a dominant-negative mutation. This is postulated to happen because the product of a mutation adversely affects the wild-type gene product within the same cell, as seen in different diseases such as familial hypertrophic cardiomyopathy (65) and alfa-mannosidosis (66).

Considering the developing technology, more precise basal and stimulated 17-OHP cut-off levels can be identified because it will be possible to combine genetic analysis, now more widely available and less expensive, with more precise hormone measurements obtained through LC/MS-MS. This kind of work can be valuable under conditions in which hormone determinations are possible but access to genetic testing is limited due to financial restrictions of health care systems or health insurance. Moreover, the SDSST is faster and cheaper than genetic analyses. We suggest that adding the clinical phenotype and the type of mutation to basal and stimulated 17-OHP evaluation may increase the accuracy of the test and yield better results.

Study Limitations

Our study had a few limitations. Firstly, the included articles did not report data separately by gender. Secondly, the ages of the subjects in the included publications varied widely. Thirdly, copy number variation of *CYP21A2* was not investigated in all studies included. Fourthly, study assays, number of individuals and 17-OHP units differed between included studies. Our study also had some strengths. Firstly, a genetic analysis was performed in all the subjects. Secondly, almost all 17-OHP measurements were performed by using the same hormone measurement method (RIA), with the exception of two studies (37,40).

Conclusion

In conclusion, this meta-analysis supports the idea that stimulated 17-OHP level has the potential to identify *CYP21A2* carriers by SDSST, although basal 17-OHP level may not be informative. ROC curve analysis from study-level data may produce some bias, and so the cut-off values from our results should be used with caution. In addition, the SDSST needs further investigation to increase specificity and sensitivity to determine heterozygosity. A greater increase in stimulated 17-OHP levels was found in mutation-free symptomatic individuals than those who were asymptomatic. Therefore, we suggest that individuals might be better evaluated by SDSST by considering the clinical phenotype and type of mutation to increase the accuracy of the test.

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Ethics

Ethics Committee Approval: This study is a meta-analysis study.

Informed Consent: This study is a meta-analysis study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Seher Polat, Concept: Seher Polat, Yusuf Kemal Arslan, Design: Seher Polat, Yusuf Kemal Arslan, Data Collection or Processing: Seher Polat, Yusuf Kemal Arslan, Analysis or Interpretation: Seher Polat, Yusuf Kemal Arslan, Literature Search: Seher Polat, Yusuf Kemal Arslan, Writing: Seher Polat.

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