Journal of Ovarian Research

RESEARCH

Open Access

Long-read sequencing analysis of non-classical congenital adrenal hyperplasia prevalence and carrier frequency in Chinese polycystic ovarian syndrome patients

Ying Huang[1,2,3,4†], Huahua Jiang[1,2,3,4†], Xiaohui Zhu[1,2,3,4], Aiping Mao[5], Di Cui[5], Yue Zhao[1,2,3,4], Ying Wang[1,2,3,4*], Xiaoyu Long[1,2,3,4*] and Jie Qiao[1,2,3,4]

Abstract

Background Adrenocortical hyperplasia is an autosomal recessive disorder characterized by congenital metabolic dysfunction, resulting in endocrine disturbances and abnormal sexual development (such as female masculinity), requiring lifelong hormonal therapy. Notably, non-classical congenital adrenal hyperplasia (NCCAH) exhibits clinical manifestations resembling polycystic ovary syndrome (PCOS), yet their therapeutic approaches in fertility treatment differ substantially. Importantly, NCCAH carries the risk of delivering offspring with severe adrenal hyperplasia, which presents far graver consequences than PCOS. Accurate differentiation between these two conditions is therefore crucial for proper clinical management. However, to date, no large-scale screening studies have been conducted to investigate the prevalence and carrier status of NCCAH among PCOS populations.

Methods A total of 1,372 suspected PCOS patients were genotyped by long-read sequencing in this study. To further clarify phenotypic characteristics, detailed clinical evaluation and laboratory analysis were conducted on 947 of these patients.

Results The prevalence of NCCAH in suspected PCOS patients was 0.73‰ (95% CIs: 0.02–4.05‰; 1/1,372). The carrier rate for the CAH variants in PCOS patients was 4.45% (61/1,371, 95% CIs: 3.42–5.68%), of which 78.69% were CYP21A2 variants. The carrier frequencies for classic CAH and NCCAH in PCOS patients were 1.46% (95% CIs: 0.89–2.24%) and 2.04% (95% CIs: 1.36–2.94%), respectively. PCOS patients with hirsutism exhibited a significantly higher NCCAH carrier rate compared to those without hirsutism (3.90% vs. 0.37%, $p<0.001$). However, other clinical features such as irregular menstrual cycles, insulin resistance, and obesity showed no correlation with CAH carrier status. No significant differences in clinical or paraclinical characteristics were found among CAH carriers.

†Ying Huang and Huahua Jiang contributed equally to this work.

*Correspondence:
Ying Wang
wangying02114@bjmu.edu.cn
Xiaoyu Long
long_x_y@163.com

Full list of author information is available at the end of the article

Conclusions PCOS patients had similar CAH carrier rates with the general population in China, and carriers of CAH variants are generally asymptomatic.

Keywords Non-classical congenital adrenal hyperplasia, PCOS, Long-read sequencing, Prevalence, Carrier

Introduction

Congenital Adrenal Hyperplasia (CAH) refers to a group of autosomal recessive disorders caused by defects in various catalytic enzymes at different stages of the adrenal cortex steroid synthesis pathway, leading to impaired steroid synthesis [1]. The most common form of CAH, 21-hydroxylase deficiency (21-OHD), is caused by pathogenic variants in the *CYP21A2* gene and accounts for 90–95% of CAH cases [2]. Other genes implicated in CAH include *CYP11B1*, *HSD3B2*, *CYP17A1*, *POR*, *STAR*, and *CYP11A1* [3]. Based on clinical phenotypes and residual 21-hydroxylase activity, 21-OHD is classified into classical and non-classical CAH (NCCAH). Classical CAH includes salt-wasting (SW) and simple-virilizing (SV) forms, which are typically diagnosed at birth or early in life and can result in life-threatening consequences if untreated. In contrast, NCCAH generally presents with mild phenotypes and may be identified in female patients with hirsutism, acne, menstrual disorders, or impaired fertility [4, 5].

Polycystic ovary syndrome (PCOS), characterized by hyperandrogenism, ovulatory dysfunction, and polycystic ovaries, is the most common endocrinopathy affecting 5–15% women of reproductive age [6–8].Notably, reproductive-aged women with NCCAH often exhibit PCOS-like clinical features, including oligo-ovulation and elevated androgen levels [9–11]. However, due to the insidious clinical presentation and limited screening methods, NCCAH is often overlooked, thus delaying opportunities for treatment. Differential diagnosis of NCCAH in women suspected of PCOS can improve female fertility and health. Traditional CAH screening via blood 17α-hydroxyprogesterone (17α-OHP) measurement is prone to false-negative and false-positive results [12]. Genetic testing has now become the gold standard for diagnosing CAH. NCCAH results from low 21-hydroxylase activity due to variants in a key disease-causing gene, *CYP21A2* [13]. The *CYP21A1P* pseudogene shares 99.8% homology with the functional *CYP21A2* gene, which complicates the identification of pathogenic variants. Additionally, the two genes can form complex rearrangements through multiple recombination events. Most laboratories detect pathogenic variants for CAH using multiplex ligation-dependent probe amplification (MLPA) and next-generation sequencing or Sanger sequencing. However, combining these methods is cumbersome and technically challenging, limiting their large-scale clinical application [14, 15]. Long-read sequencing (LRS) has numerous technical advantages, such as the ability to sequence long reads and accurately sequence complex structures (i.e., high GC content regions and highly repetitive regions), and base-free preference, making it particularly useful for accurately distinguishing between *CYP21A2* and *CYP21A1P*. Our team has previously developed an LRS-based CAH diagnostic approach which can effectively distinguish between functional gene and pseudogene, thus accurately identifying point variants, indels, deletions, duplications, and large fragment gene rearrangements [15].

This study aims to use long-read CAH sequencing to screen for NCCAH in women suspected of PCOS in clinic, assess the prevalence and carrier rates, and analyze associated genotypes and phenotypes. These findings will contribute to the accurate identification of NCCAH patients, CAH carriers, and support genetic counseling for Chinese PCOS women.

Materials and methods

Study population and design

From July 2023 to July 2024, we recruited female patients from the Reproductive Center of Peking University Third Hospital for infertility evaluation with suspected PCOS. The diagnosis of suspected PCOS was based on the standard 2003 Rotterdam criteria, confirmed by the presence of at least two of the following three features: hyperandrogenism (clinical or biochemical), irregular menstrual cycles (oligo- or amenorrhea), and polycystic ovary morphology (defined as ≥ 12 antral follicles, 2 to 9 mm in diameter, in unilateral or bilateral ovaries, or an ovarian volume >10 cm^3 on ultrasonography) [6]. Patients with Cushing syndrome, hyperprolactinemia, adrenal or ovarian virilizing tumors, or thyroid dysfunction were excluded from the study. Additionally, none of the patients had received hormonal therapy for at least 12 weeks prior to testing. A total of 1,372 patients with clinically suspected PCOS were enrolled.

Long-read sequencing analysis

Peripheral blood samples were collected from all enrolled subjects and sent to Berry Genomics Corporation, which used Comprehensive Analysis of CAH (CACAH) for detection of CAH-associated variants using long-read sequencing (LRS) based on single-molecule real-time (SMRT) technology [15]. LRS can be demonstrated as a highly efficient tool for the precision genetic diagnosis of CAH, with high analytical sensitivity/specificity in prior studies [15–18]. Briefly, genomic DNA was isolated and amplified using multiplexed PCR primers that covered

the full-length sequences of the *CYP21A2*, *CYP21A1P*, *CYP11B1*, *CYP17A1*, *HSD3B2*, and *STAR* genes. The PCR products were then purified, end-repaired, and ligated to barcode adapters at both ends. Libraries were prepared using the Sequel Binding and Internal Ctrl Kit 3.0 (Pacific Biosciences, Menlo Park, CA) and sequenced on the PacBio Sequel II platform. Raw sub-reads were processed and analyzed using the CCS software application to generate circular consensus sequence (CCS) reads, which were then aligned to the GRCh38 reference genome using BLASR [19]. The FreeBayes software (Biomatters, Inc., San Diego, CA) was used to call single nucleotide variants (SNV) and small insertions and deletions (indels). Structural variants were identified based on reads containing *CYP21A2*, *CYP21A1P*, *TNXA* and *TNXB* primer pairs. For quality control, CCS reads were considered valid if the target sequences were completely detected at least three times. After barcode demultiplexing, the analysis criteria were considered met if ≥ 200 valid CCS reads were obtained for *CYP21A2* + *CYP21A1P*, ≥ 60 for *CYP11B1*, and ≥ 30 for *CYP17A1*, *HSD3B2*, and *STAR*. Pathogenic variants in the *CYP21A2* gene lead to varying degrees of 21-hydroxylase deficiency. Variants that completely abolish enzyme activity are typically associated with the SW phenotype. Variants that reduce activity to approximately 2% are linked to the SV phenotype, while those that retain 10% to 75% of activity are generally associated with the milder NC phenotype [1, 20]. In this study, classification (SW/SV/NC) of the identified variants was categorized using European Molecular Genetic Quality Network (EMQN), and the pathogenicity was categorized according to the American College of Medical Genetics and Genomics (ACMG) criteria [21, 22]. NCCAH was determined when both pathogenic variants were present, with at least one allele being an NCCAH variant. CAH carriers were identified when a pathogenic variant was present on one allele, which included the classic CAH carrier, NCCAH carrier, and other carriers (*CYP11B1*, *CYP17A1*, *STAR*, and *HSD3B2*). A normal genotype was assigned when no pathogenic variants were detected in the *CYP21A2*, *CYP11B1*, *CYP17A1*, *STAR*, and *HSD3B2 genes*. Individuals carrying benign Q319* variant with duplication were considered non-carriers and were not included in the carrier-associated analyses of this study.

Clinical evaluation and laboratory analysis

Among these 1,372 patients, 947 subjects underwent further evaluation, including a complete clinical history and physical assessment. Both reproductive and general medical histories were obtained, with self-reports on the duration of infertility, menstrual cycle and history of spontaneous abortion. A regular menstrual cycle was defined as intermenstrual intervals between 21 and 35 days [23].

Additionally, body mass index (BMI, weight/height^2), waist-to-hip ratio, and hair distribution were assessed. The Modified Ferriman–Gallwey (mFG) score was used to evaluate the degree of hirsutism. Hair growth in nine areas of the body and face (upper lip, chin, arms, chest, upper and lower abdomen, thighs, and upper and lower back) was scored on a scale of 0–4, and the total mFG score was the sum of all individual scores. Given that Asian populations generally exhibit lower mFG score distributions, the Chinese Expert Consensus on the Diagnosis and Management of PCOS recommended mFG score ≥ 4 to define hirsutism, we likewise adopted this cutoff value in this study [23]. All evaluations were performed by a single investigator to minimize intersubject variability. The presence of acne was also recorded according to the Global Acne Grading System (GAGS).

In line with the 2023 International Evidence-based Guideline for the Assessment and Management of Polycystic Ovary Syndrome, tandem mass spectrometry (LC-MS/MS) was utilized for biochemical evaluation of hyperandrogenism [23]. Peripheral blood serum from all subjects was collected, and the androgen levels including total testosterone (T), dihydrotestosterone, androstenedione, dehydroepiandrosterone (DHEA), and dehydroepiandrosterone-sulfate (DHEAS) were measured. Biochemical hyperandrogenism was defined as a total T level >0.60 ng/mL. Reference values were determined by the endocrine laboratory based on supplier information and controls derived from local population testing.

Statistical analysis

The primary outcome of this study was the prevalence of NCCAH patients and CAH carriers in the suspected PCOS population. The 95% confidence intervals (CIs) for prevalence and carrier rates were calculated using the Exact Clopper-Pearson method. Data analysis was performed using SPSS 26.0 (IBM, Armonk, NY, USA). Continuous variables are presented as means ± standard deviations. Student's t-test was used for normally distributed continuous variables, while the Mann-Whitney test was applied for continuous data that did not follow a normal distribution. Categorical variables are expressed as counts and percentages. Comparisons between groups were performed using the $\chi 2$ test (Pearson chi-square when no expected cell count was less than 5; Fisher's exact test when one more cells had expected counts less than 5). $P < 0.05$ was considered statistically significant.

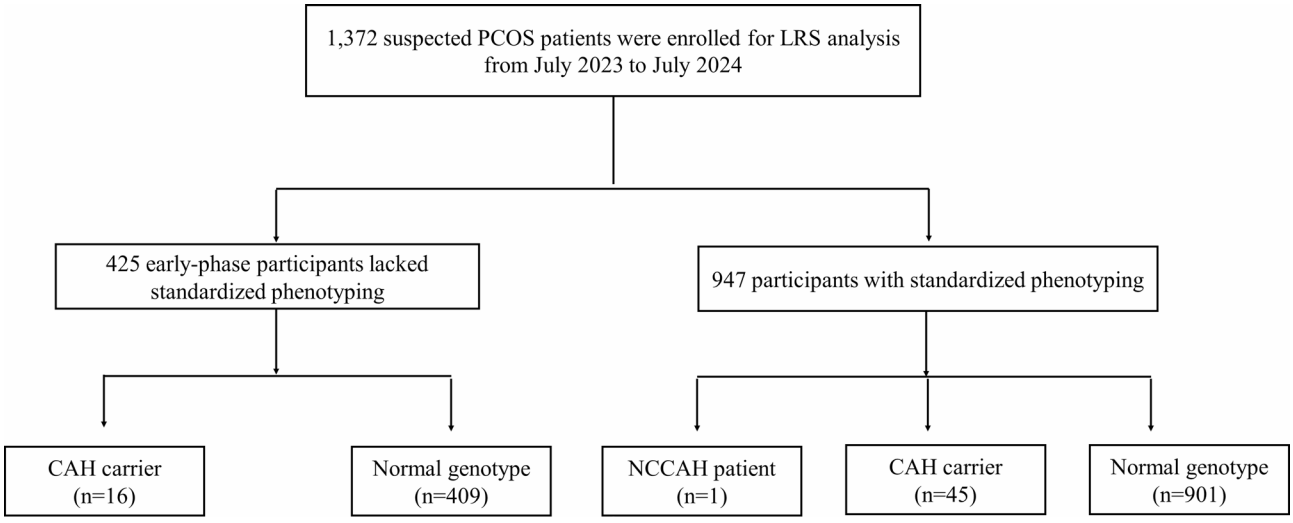

Fig. 1 Flow diagram of the participants in this study. Abbreviations: *PCOS* polycystic ovarian syndrome, *LRS* Long-read sequencing, *CAH* congenital adrenal hyperplasia, *NCCAH* non-classical congenital adrenal hyperplasia

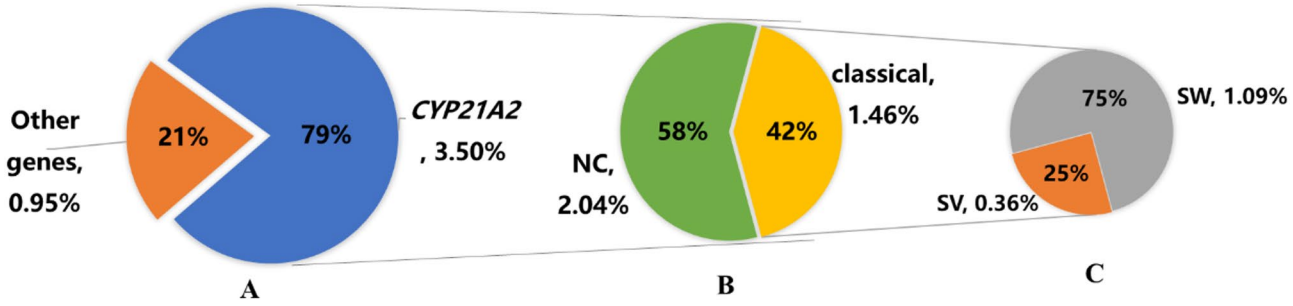

Fig. 2 Prevalence of CAH carriers in PCOS patients. Note: The outer ring values in each pie chart display the carrier frequencies of respective variants (carriers/total screened, n=1,371). The inner sectors show: (**A**) Variant distribution among all carriers (n=61), Other genes included *CYP11B1*, *CYP17A1*, *STAR*, and *HSD3B2*; (**B**) Distribution of carrier types in *CYP21A2* variant carriers (n=48); (**C**) Subtype distribution among classical CAH carriers (n=20). Abbreviations: *NC* non-classical, *SW* salt-wasting, *SV* simple virilizing

Result

Prevalence of NCCAH patient and CAH carrier in PCOS patients

The flow diagram of the participants in this study was summarized in Fig. 1. A total of 1,372 suspected PCOS patients underwent LRS analysis in the study. Among them, only one patient was genetically diagnosed with NCCAH, resulting in a prevalence rate of 0.73‰ (1/1,372; 95% CIs: 0.02–4.05‰). The identified NCCAH patient was a normal-sized woman with oligo-ovulation and mild hyperandrogenemia (mFG score of 4). Her genotype was *CYP21A2*: c.518T > A/c.844G > T, compound heterozygous (verified by a confirmational test performed on the parents). After the genetic diagnosis of NCCAH, the patient was referred for endocrine evaluation where subsequent 17-hydroxyprogesterone testing demonstrated elevated levels (basal: 7.2 ng/mL; 60-minute post-cosyntropin stimulation: 59.76 ng/mL). Following initiation of oral glucocorticoid therapy by the endocrinology team, the patient achieved spontaneous conception and delivered a healthy female infant at term.

61 patients were identified as CAH carriers in this study, yielding a carrier rate of 4.45% (61/1,371; 95% CIs: 3.42–5.68%). The details of the variants were presented in supplemental Table 1. As shown in Fig. 2, of the 61 carriers, 48 patients (48/61, 78.69%) were *CYP21A2* variant carriers, and 13 patients carried non-CYP21A2 gene variants. The carrier frequency of *CYP21A2* was 3.50% (48/1,371; 95% CIs: 2.59–4.62%) in PCOS population. Among them, 58.33% carried NCCAH variants (2.04% in total, 28/1,371; 95% CIs: 1.36–2.94%), and 41.67% carried classic CAH variants. (1.46% in total, 20/1371; 95% CIs: 0.89–2.24%).

Among the classic CAH carriers, 75% carried SW CAH variants (15 cases) and 25% carried SV CAH variants (5 cases). For patients identified as carriers of classic CAH variants, we recommend subsequent LRS analysis for their spouses to assess the risk of transmitting the condition to their offspring. Notably, in one case of a SW

CAH carrier (genotype c.1451_1452delinsC), the spouse was also found to be a carrier of SW CAH (genotype CYP21A2: c.293–13 C > G/+). In this case, their offspring would have a 25% risk of developing classic CAH. After reproductive genetic counseling, this couple opted for in vitro fertilization with preimplantation genetic testing (IVF-PGT) for assisted reproduction. Following transfer of a single PGT-M-screened blastocyst free of pathogenic variants, she is currently at 22 weeks' gestation.

Comparison of CAH carrier frequency among different clinical features of PCOS

A detailed medical history, physical examination and androgen determination were performed on 947 patients. After excluding one patient diagnosed with NCCAH, a total of 946 PCOS patients were included in analysis. The patients were divided into subgroups according to menstrual cycle characteristics, presence of hirsutism, biochemical hyperandrogenism, insulin resistance, and obesity. The differences in the total CAH carrier rate, classical CAH carrier rate, NCCAH carrier rate, and other CAH carrier rate were further analyzed across these subgroups. Irregular menstrual cycle was defined as the occurrence of eight or fewer menstrual cycles per year or menstrual cycles > 35 days. Biochemical hyperandrogenism was defined as total T level > 0.60 ng/ml. Insulin resistance was assessed using the HOMA-IR index, calculated as fasting blood glucose (mmol/L) × fasting insulin (μU/mL)/22.5, with a value of >2.69 considered indicative of insulin resistance. Hirsutism was defined by mFG score ≥ 4 and obesity was defined by BMI ≥ 28.

As shown in Table 1, the carrier rate of CAH was higher in PCOS patients with hirsutism than patients without hirsutism (6.34% vs. 3.54%, $p = 0.045$). Further subdividing by the types of variants carried showed that the NCCAH carrying rate was remarkably higher in patients with hirsutism (3.90% vs. 0.37%, $p<0.001$), while the carrier rates of classical CAH and other CAH variants were similar between the two groups. However, when patients were grouped by the presence of biochemical hyperandrogenism, no significant difference was found in either total CAH nor NCCAH carrier rates (1.35% vs. 2.15%, $p = 0.402$). Additionally, the NCCAH carrier rate showed no significant correlation with menstrual regularity, insulin resistance, or obesity. Specifically, the NCCAH carrier rates in the irregular menstruation group and the regular menstruation group were 1.88% and 2.04%, respectively ($p = 0.751$); the NCCAH carrier rates in the insulin resistance group and the non-insulin resistance group were 2.41% and 1.08%, respectively ($p = 0.213$); and the NCCAH carrier rates in the obese group and the non-obese group were 2.38% and 1.58%, respectively ($P = 0.380$). These results suggest that the NCCAH carrier status may not be significantly correlated with the metabolic characteristics of PCOS patients. The 95% CIs for the carrier rate across different clinical characteristics of the PCOS patients were provided in Supplemental Table 2.

Table 1 CAH carrier rates across different clinical characteristics in PCOS patients

Clinical characteristics		Total CAH		Classic CAH		NCCAH		Other types	
		***n* (rate)**	***P***	***n* (rate)**	***P***	***n* (rate)**	***P***	***n* (rate)**	***P***
Irregular menstrual cycle	Yes	40/799 (5.01%)	0.401	14/799 (1.75%)	0.489	15/799 (1.88%)	0.751	11/799 (1.38%)	0.704
	No	5/147 (3.40%)		1/147 (0.68%)		3/147 (2.04%)		1/147 (0.68%)	
Biochemical hyperandrogenemia	Yes	11/296 (3.72%)	0.310	5/296 (1.69%)	0.863	4/296 (1.35%)	0.402	2/296 (0.68%)	0.359
	No	34/650 (5.23%)		10/650 (1.54%)		14/650 (2.15%)		10/650 (1.54%)	
Hirsutism	Yes	**26/410 (6.34%)**	**0.045**	5/410 (1.22%)	0.430	**16/410 (3.90%)**	**<0.001**	5/410 (1.22%)	0.906
	No	**19/536 (3.54%)**		10/536 (1.87%)		**2/536 (0.37%)**		7/536 (1.31%)	
Insulin resistance	Yes	13/249 (5.22%)	0.395	4/249 (1.61%)	0.798	6/249 (2.41%)	0.213	3/249 (1.20%)	1.000
	No	14/369 (3.79%)		5/369 (1.36%)		4/369 (1.08%)		5/369 (1.36%)	
Obesity	Yes	18/378 (4.76%)	0.995	5/378 (1.32%)	0.598	9/378 (2.38%)	0.380	4/378 (1.06%)	0.772
	No	27/568 (4.75%)		10/568 (1.76%)		9/568 (1.58%)		8/568 (1.41%)	

Chi square test was used in the categorical data (Pearson chi-square when no expected cell count was less than 5; Fisher's exact test when one more cells had expected counts less than 5). P < 0.05 was considered statistically significant

CAH Congenital adrenal hyperplasia, *NCCAH* Non-classical congenital adrenal hyperplasia

Comparison of CAH carrier rates among four PCOS phenotypes

PCOS is a heterogeneous disorder that presents with different phenotypes. According to the Rotterdam criteria, PCOS is classified into four phenotypes: Phenotype A (olig-anovulation + hyperandrogenism + polycystic ovary morphology), Phenotype B (olig-anovulation + hyperandrogenism), Phenotype C (hyperandrogenism + polycystic ovary morphology), and Phenotype D (olig-anovulation + polycystic ovary morphology) [6]. In this study, the most common phenotype among the included PCOS patients was Phenotype A (56.13%, n = 531), while Phenotype B represented the smallest proportion (4.5%, n = 43). Additionally, 119 patients had Phenotype C (12.58%), and 253 patients had Phenotype D (26.74%).

The carrier rates of total CAH, classical CAH, and NCCAH were evaluated across the four PCOS phenotypes. As shown in Table 2, although the carrier rates of classical CAH and NCCAH were higher in Phenotype B compared to the other phenotypes, the differences were not statistically significant. Overall, no significant differences were observed in either the total CAH or NCCAH carrier rates across the four PCOS phenotypes. Corresponding 95% CIs for these rates were provided in Supplemental Table 3.

Demographic and clinical characteristics of the PCOS participants

The 946 PCOS patients were divided into two groups based on the presence of CAH pathogenic variants. The demographic and clinical characteristics of the two groups were compared. The analysis revealed that CAH carriers had a significantly higher proportion of hirsutism (57.8% vs. 42.6%, $p = 0.045$) and a lower fasting blood glucose (FBG) level (4.83 ± 0.55 vs. 5.09 ± 0.83, $p = 0.032$). However, other parameters, including waist-to-hip ratio, years of infertility, history of spontaneous abortion, blood lipid levels, baseline hormone levels, and AMH were comparable between the two groups (Table 3). To eliminate the influence of confounding factors on the results, multivariate analyses (generalized linear models for FBG levels and logistic regression analysis for hirsutism) were performed. The variables included in the analysis comprised maternal age, BMI, waist-to-hip ratio, AMH, HOMA-IR, menstural cycle, presence of PCOM, carrier status for total CAH and NCCAH variants. The result demonstrated no significant association between CAH variant carrier status and either hirsutism ($p = 0.186$) or FBG ($p = 0.224$) (Supplemental Tables 4 and 5). While NCCAH mutation carrier significantly increased hirsutism risk (OR = 24.63, 95% CI 1.899–319.4, $p = 0.014$) (Supplemental Table 4). Given the low prevalence of NCCAH carriers in non-hirsute patients, the resulting OR had an excessively wide confidence interval, limiting the statistical power of the analysis.

Discussion

Our study represents the first large-scale investigation utilizing LRS technology to screen for CAH-related genes in a PCOS cohort. The genetic analysis covers nearly all types of CAH-related variant sites, therefore providing reliable data on the prevalence of NCCAH patients and carriers in the PCOS population.

NCCAH is a common autosomal recessive disease, with its frequency varying across different ethnicities. To date, population-based data on the prevalence of NCCAH is still scarce, as neonatal screening is relatively insensitive in identifying children affected by NCCAH [10]. As the exclusion of NCCAH is mandatory for diagnosing PCOS [24, 25], a significant body of data on the prevalence of NCCAH among hyperandrogenic women is available. However, the prevalence reported in the literatures varies greatly, ranging from 1% to 10%, depending on the ethnicity of the study population [26–35]. Given that hyperandrogenism affects approximately 10% of the female population [24, 25], it can be estimated that NCCAH affects between 1:1000/2000 and 1:100 women in the general population. A meta-analysis of published studies achieved a more accurate estimation, suggesting that the worldwide prevalence of NCCAH among hyperandrogenic women was 4.2% [10].

In contrast to the high prevalence of NCCAH reported in previous studies, the prevalence of NCCAH among Chinese PCOS patients in our study is less than 1 in 1000,

Table 2 Comparison of CAH carrier rates across four PCOS phenotypes

Carrier rate	Phenotype A *n* (rate)	Phenotype B *n* (rate)	Phenotype C *n* (rate)	Phenotype D *n* (rate)	*P*
Total CAH	28/531(5.27%)	4/43(9.30%)	4/119(3.36%)	9/253(3.56%)	0.296
Classical CAH	8/531(1.51%)	2/43(4.65%)	1/119(0.84%)	4/253(1.58%)	0.349
NCCAH	13/531(2.45%)	2/43(4.65%)	2/119(1.68%)	1/253(0.40%)	0.072
Other types	7/531(1.32%)	0/43(0.00%)	1/119(0.84%)	4/253(1.58%)	0.953

Chi square test was used in the categorical data (Pearson chi-square when no expected cell count was less than 5; Fisher's exact test when one more cells had expected counts less than 5). P < 0.05 was considered statistically significant. Phenotype A was defined as olig-anovulation + hyperandrogenism + polycystic ovary morphology, Phenotype B was defined as olig-anovulation + hyperandrogenism; Phenotype C was defined as hyperandrogenism + polycystic ovary morphology; Phenotype D was defined as olig-anovulation + polycystic ovary morphology

CAH Congenital adrenal hyperplasia, *NCCAH* Non-classical congenital adrenal hyperplasia

Table 3 Demographic and biochemical characteristics of CAH carriers in PCOS patients

Clinical characteristics		Normal genotype (*n* = 901)	CAH carrier (*n* = 45)	*P*
Demographic characteristics				
Maternal age (year)		30.80 ± 3.79	31.02 ± 4.10	0.631
Duration of infertility (year)		3.00 ± 2.68	2.50 ± 2.79	0.065
BMI (Kg/m^2)		26.85 ± 5.27	26.71 ± 5.20	0.907
Waist (cm)		83.39 ± 11.54	84.13 ± 12.17	0.786
Hip (cm)		100.34 ± 10.52	100.42 ± 10.52	0.735
Waist–to-hip ratio		0.83 ± 0.06	0.84 ± 0.08	0.878
mFG score		3.43 ± 3.88	4.36 ± 4.70	0.174
Hirsutism	mFG score ≥ 4	384 (42.6%)	26 (57.8%)	**0.045**
	mFG score <4	517 (57.4%)	19 (42.2%)	
Acne	Yes	306 (34.0%)	20 (44.4%)	0.150
	No	594 (66.0%)	25 (55.6%)	
History of spontaneous abortion	Yes	163 (18.1%)	4 (8.9%)	0.114
	No	738 (81.9%)	41 (91.1%)	
Menstrual cycle	≤ 35days	142 (15.8%)	5 (11.1%)	0.696
	36–45 days	139 (15.4%)	7 (15.6%)	
	>45 days	620 (68.8%)	33 (73.3%)	
Biochemical characteristics				
Basal PRL(ng/ml)		14.95 ± 10.14	13.47 ± 8.27	0.236
Basal LH (mIU/ml)		8.49 ± 6.44	7.48 ± 6.02	0.202
Basal P (nmol/L)		1.54 ± 2.59	1.36 ± 1.03	0.636
Basal FSH (mIU/ml)		5.52 ± 1.62	5.59 ± 1.49	0.808
Basal E2 (pmol/L)		150.4 ± 111.9	166.6 ± 202	0.497
AMH (ng/ml)		9.13 ± 5.29	10.98 ± 6.37	0.127
Fasting blood glucose level (mmol/L)		**5.09 ± 0.83**	**4.83 ± 0.55**	**0.032**
HOMA-IR		2.96 ± 3.44	2.86 ± 1.61	0.450
ALT (U/L)		24.06 ± 19.46	26.44 ± 19.08	0.299
AST (U/L)		22.99 ± 15.51	24.14 ± 9.45	0.136
Total cholesterol (mmol/L)		5.41 ± 16.13	4.99 ± 0.75	0.147
Triglyceride (TG, mmol/L)		1.85 ± 5.56	1.62 ± 0.85	0.573
High-density lipoprotein cholesterol (HDL-C, mmol/L)		1.24 ± 0.47	1.25 ± 0.51	0.592
Low-density lipoprotein cholesterol (LDL-C, mmol/L)		2.96 ± 1.03	3.06 ± 0.79	0.446
25OH-vitamin D (ng/mL)		23.84 ± 11.95	23.53 ± 11.63	0.982
Total testosterone (ng/mL)		0.54 ± 0.41	0.49 ± 0.35	0.338
Androstenedione (ng/mL)		2.30 ± 1.32	2.07 ± 1.13	0.181
Dihydrotestosterone (pg/mL)		121.17 ± 66.85	131.60 ± 74.86	0.561
DHEA (ng/mL)		6.17 ± 3.22	5.73 ± 2.95	0.325
DHEAS (ng/mL)		2482.8 ± 1131	2306.1 ± 994	0.314

Categorical data: n (rate). Continuous data: mean±SD. Student's t test or Mann-Whitney test were used in the continuous data. Chi square test was used in the categorical data (Pearson chi-square when no expected cell count was less than 5; Fisher's exact test when one more cells had expected counts less than 5). P < 0.05 was considered statistically significant

CAH Congenital adrenal hyperplasia, *BMI* Body mass index, *AMH* Anti-Mullerian hormone, *E2* Estradiol, *P* Progesterone, *PRL* Prolactin, *LH* Luteinizing Hormone, *FSH* Follicle-Stimulating Hormone, *mFG* Modified Ferriman–Gallwey, *HOMA-IR* Homeostatic Model Assessment of Insulin Resistance, *ALT* Alanine Aminotransferase, *AST* Aspartate Aminotransferase, *DHEA* Dehydroepiandrosterone, *DHEAS* Dehydroepiandrosterone Sulfate

which is consistent with the general population. This discrepancy may be attributed to several factors, including ethnic diversity, diagnostic methodology variability (e.g., genetic confirmation versus biochemical criteria) and differences in inclusion criteria. The participants in our study were infertility patients who sought treatment at the reproductive center and were clinically diagnosed with PCOS based on the Rotterdam criteria. In contrast, the majority of the previous studies included individuals who sought treatment at endocrinology or gynecology departments due to concerns over hirsutism or other symptoms of hyperandrogenism, which put them at a higher risk for NCCAH. Therefore, our results provide a more accurate reflection of the true prevalence rate of NCCAH in suspected PCOS patients and suggest that the PCOS phenotype does not show a higher prevalence of NCCAH in the population.

The prevalence of CAH carrier rates in the general population varies largely by ethnicity. Additionally, genetic testing methods play a key role in these differences, as level of comprehensiveness in tests carried out in different studies vary widely due to the complexity of the CYP21A2 gene locus. Through neonatal screening programs, the prevalence of classical CAH is more clearly defined. Data from roughly 6.5 million newborn infants screened in 13 countries showed an overall prevalence of one in 15,000 livebirths for the classical CAH [36]. Thus, the *CYP21A2* carrier frequency of classical CAH is estimated to be about one in 60 individuals, with salt-wasting CAH accounting for 67% of cases and simple virilizing CAH accounting for 33% [37]. Consistent with the estimate, a carrier screening study in China reported a classical CAH carrier frequency of 1.61% (47/2928) in the general Chinese population [38]. A study from Bulgaria also demonstrated an allele frequency of 1.67% (1/60) for the classical CAH carrier in Eastern European PCOS patients [39]. Our result revealed a similar finding, with a classical CAH carrier rate of 1.46%, consistent with the carrier rate in the general population. Currently, specific

data on the carrier rate of NCCAH in the general Chinese population remain unavailable. However, existing carrier screening studies for monogenic diseases suggest a combined (classical and NCCAH) *CYP21A2* carrier frequency of 2.81–2.98% in this population [40, 41]. Our study identified a similar carrier frequency (3.50%, 48/1,371) in PCOS patients, similar with these population estimates. However, some small-scale screening studies have reported higher carrier frequencies in other ethnic populations. Baumgartner-Parzer et al. demonstrated a carrier frequency of 9.5% in an unselected middle European (Austrian) population, with 5.5% of individuals carrying the so-called "classic" variant and 4% carrying the "non-classical" *CYP21A2*-gene aberrations [42]. In New Zealand, the carrier frequency of CYP21A2 was 2.8% for classical CAH, and 2.0% for non-classical CAH in neonates [43]. A recent screening study in Turkey revealed a surprisingly high prevalence of NCCAH (7 of 270, 2.2%) and high variant-carrier frequency in randomly-selected Turkish women. The carrier frequency for CAH due to 21-OHD was found to be 12.6%, with 9.3% associated with classical CAH, and 3.3% associated with non-classical CAH in the study group [44].

The significance of being a CAH carrier in relation to clinical PCOS symptoms remains unclear. Admoni et al. found that carriers of *CYP21A2* variants are at risk of clinically significant hyperandrogenism [45]. Similarly, a study in a Sicilina cohort revealed that heterozygous carriers of *CYP21A2* variants are at increased risk of developing hyperandrogenism and related comorbidities, and they frequently show hirsutism, oligomenorrhoea, overweight and a PCOS-like phenotype [46]. However, other researchers hold the opposite view that carriers of *CYP21A2* variants exhibit only subtle abnormalities in the functioning of the HPA axis. Carriers are mostly asymptomatic, without experiencing adrenal crises, hyperandrogenic symptoms, or growth and puberty disorders [37, 47]. Besides, no significant differences in the frequency of *CYP21A2* variant carriers (not specifically classified as classical or non-classical) were found between hirsute/hyperandrogenic patients and healthy controls [44, 48–50]. As for PCOS patients, a cross-sectional study in adolescents demonstrated that the frequencies of heterozygous carriers of NCCAH variations were similar between PCOS patients (3/55, 5.45%) and healthy controls (3/49,6.12%) [51]. S. Polat et al. also found that the PCOS group (16.6%) had a similar *CYP12A2* carrier frequency to the control group (13.2%) [44]. These findings collectively suggest that carrying the *CYP21A2* variant is often asymptomatic and does not appear to increase the risk of hyperandrogenism, hirsutism, or PCOS in the general population. Contrary to the above data, our results showed that PCOS patients who are NCCAH carriers exhibited a higher proportion of hirsutism.

It is noteworthy that our result demonstrated a relatively low incidence of CAH carrier status in PCOS patients, with 1.46% carrying classical CAH and 2.04% carrying NCCAH, which is comparable to the general population [38, 40, 41]. This finding is important, as it suggests that having PCOS does not increase the risk of CAH in offspring. Therefore, carrier screening in PCOS patients can be approached in the same manner as in the general population of women of reproduction age. Notably, during the screening process, a couple was unexpectedly identified as both being carriers of classical CAH. Through genetic counseling and PGT, the risk of their offspring inheriting classical CAH was mitigated. While this case shows the potential benefits of genetic counseling, it does not alter population-level screening recommendations. Routine CAH genetic testing remains unnecessary for PCOS patients.

Although the PCOS population as a whole does not exhibit an increased prevalence or carrier rate of NCCAH, the carrier rate of NCCAH variants is significantly higher among PCOS patients with hirsutism compared to those without. This suggests that carrying NCCAH variants may play a role in the development of hirsutism in Chinese women, though the specific mechanisms remain unclear and require further study. Additionally, although we did not find a statistical difference in carrier rates among different PCOS phenotypes, this may be due to small sample size. The limited number of patients with certain phenotypes, particularly Phenotype B, may have impacted statistical analysis. While our data provides novel insights into NCCAH epidemiology in PCOS patients, the small number of carriers in certain subgroups limit the clinical interpretability of these results. Larger sample sizes are required to validate these findings and improve the statistical power for rare variant analyses.

Conclusion

PCOS patients had similar CAH carrier rates with the general population in China, and carriers of CAH variants are generally asymptomatic.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13048-025-01824-x.

Supplementary Material 1.

Acknowledgements

The authors thank Rui Wang for her work in data collecting in this study.

Authors' contributions
Xiaoyu Long and Jie Qiao supervised the entire study, Xiaoyu Long, Ying Wang participated in the interpretation of the study data and revisions to the article. Ying Huang and Huahua Jiang collected the data and performed the statistical analysis. Ying Huang drafted the manuscript. Xiaohui Zhu, Aiping Mao and Di Cui performed the sequencing detection and genetic data analysis. Yue Zhao involved in designing the study. All authors reviewed the manuscript.

Funding
This work was supported by the special fund of the National Clinical Key Specialty Construction Program, P. R. China (2023) and Capital's Funds for Health Improvement and Research (2024-2-40910).

Data availability
The data sets used and/or analyzed during the current study are available from the database of Center for Reproductive Medicine in Peking University Third Hospital on reasonable request.

Declarations

Ethics approval and consent to participate
This study was approved by the Ethics Committee of Peking University Third Hospital (No: M2023387). All the recruited subjects provided written informed consent.

Consent for publication
Written informed consent was obtained from the individuals for the publication of any potentially identifiable images or data included in this article.

Competing interests
The authors declare no competing interests.

Author details
[1]Center for Reproductive Medicine, Department of Obstetrics and Gynecology, Peking University Third Hospital, Beijing 100191, China
[2]National Clinical Research Center for Obstetrics and Gynecology, Peking University Third Hospital, Beijing, China
[3]Key Laboratory of Assisted Reproduction (Peking University), Ministry of Education, Beijing, China
[4]Beijing Key Laboratory of Reproductive Endocrinology and Assisted Reproductive Technology, Beijing, China
[5]Department of Research & Development, Berry Genomics Corporation, Beijing 102200, China

Received: 26 June 2025 / Accepted: 23 September 2025
Published online: 11 November 2025

References

1. Auer MK, Nordenström A, Lajic S, Reisch N. Congenital adrenal hyperplasia. Lancet. 2023;401:227–44.
2. Hannah-Shmouni F, Chen W, Merke DP. Genetics of congenital adrenal hyperplasia. Endocrinol Metab Clin North Am. 2017;46:435–58.
3. Gidlöf S, Wedell A, Guthenberg C, von Döbeln U, Nordenström A. Nationwide neonatal screening for congenital adrenal hyperplasia in Sweden: a 26-year longitudinal prospective population-based study. JAMA Pediatr. 2014;168:567–74.
4. Nordenström A, Falhammar H. Management of endocrine disease: diagnosis and management of the patient with non-classic CAH due to 21-hydroxylase deficiency. Eur J Endocrinol. 2019;180:R127–45.
5. Nordenström A, Falhammar H, Lajic S. Current and novel treatment strategies in children with congenital adrenal hyperplasia. Horm Res Paediatr. 2023;96:560–72.
6. Revised. 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). *Hum Reprod* 2004, 19:41–47.
7. Li R, Zhang Q, Yang D, Li S, Lu S, Wu X, et al. Prevalence of polycystic ovary syndrome in women in China: a large community-based study. Hum Reprod. 2013;28:2562–9.
8. Huddleston HG, Dokras A. Diagnosis and treatment of polycystic ovary syndrome. JAMA. 2022;327:274–5.
9. Falhammar H, Nordenström A. Nonclassic congenital adrenal hyperplasia due to 21-hydroxylase deficiency: clinical presentation, diagnosis, treatment, and outcome. Endocrine. 2015;50:32–50.
10. Carmina E, Dewailly D, Escobar-Morreale HF, Kelestimur F, Moran C, Oberfield S, et al. Non-classic congenital adrenal hyperplasia due to 21-hydroxylase deficiency revisited: an update with a special focus on adolescent and adult women. Hum Reprod Update. 2017;23:580–99.
11. Teede HJ, Misso ML, Costello MF, Dokras A, Laven J, Moran L, et al. Recommendations from the international evidence-based guideline for the assessment and management of polycystic ovary syndrome. Fertil Steril. 2018;110:364–79.
12. Speiser PW, Arlt W, Auchus RJ, Baskin LS, Conway GS, Merke DP, et al. Congenital adrenal hyperplasia due to steroid 21-Hydroxylase deficiency: an endocrine society clinical practice guideline. J Clin Endocrinol Metab. 2018;103:4043–88.
13. Merke DP, Auchus RJ. Congenital adrenal hyperplasia due to 21-Hydroxylase deficiency. N Engl J Med. 2020;383:1248–61.
14. Concolino P. Issues with the detection of large genomic rearrangements in molecular diagnosis of 21-hydroxylase deficiency. Mol Diagn Ther. 2019;23:563–7.
15. Liu Y, Chen M, Liu J, Mao A, Teng Y, Yan H, et al. Comprehensive analysis of congenital adrenal hyperplasia using long-read sequencing. Clin Chem. 2022;68:927–39.
16. Wang R, Luo X, Sun Y, Liang L, Mao A, Lu D, et al. Long-read sequencing solves complex structure of CYP21A2 in a large 21-hydroxylase deficiency cohort. J Clin Endocrinol Metab. 2025;110:406–16.
17. Li H, Zhu X, Yang Y, Wang W, Mao A, Li J, et al. Long-read sequencing: an effective method for genetic analysis of CYP21A2 variation in congenital adrenal hyperplasia. Clin Chim Acta. 2023;547:117419.
18. Yuan D, Cai R, Mao A, Tan J, Zhong Q, Zeng D, et al. Improved genetic characterization of congenital adrenal hyperplasia by long-read sequencing compared with multiplex ligation-dependent probe amplification plus Sanger sequencing. J Mol Diagn. 2024;26:770–80.
19. Chaisson MJ, Tesler G. Mapping single molecule sequencing reads using basic local alignment with successive refinement (BLASR): application and theory. BMC Bioinformatics. 2012;13:238.
20. New MI, Abraham M, Gonzalez B, Dumic M, Razzaghy-Azar M, Chitayat D, et al. Genotype-phenotype correlation in 1,507 families with congenital adrenal hyperplasia owing to 21-hydroxylase deficiency. Proc Natl Acad Sci U S A. 2013;110:2611–6.
21. Baumgartner-Parzer S, Witsch-Baumgartner M, Hoeppner W. EMQN best practice guidelines for molecular genetic testing and reporting of 21-hydroxylase deficiency. Eur J Hum Genet. 2020;28:1341–67.
22. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American college of medical genetics and genomics and the association for molecular pathology. Genet Med. 2015;17:405–24.
23. Teede HJ, Tay CT, Laven J, Dokras A, Moran LJ, Piltonen TT, et al. Recommendations from the 2023 international evidence-based guideline for the assessment and management of polycystic ovary syndrome. Fertil Steril. 2023;120:767–93.
24. Azziz R, Carmina E, Dewailly D, Diamanti-Kandarakis E, Escobar-Morreale HF, Futterweit W, et al. Positions statement: criteria for defining polycystic ovary syndrome as a predominantly hyperandrogenic syndrome: an androgen excess society guideline. J Clin Endocrinol Metab. 2006;91:4237–45.
25. Azziz R, Carmina E, Dewailly D, Diamanti-Kandarakis E, Escobar-Morreale HF, Futterweit W, et al. The androgen excess and PCOS society criteria for the polycystic ovary syndrome: the complete task force report. Fertil Steril. 2009;91:456–88.
26. Romaguera J, Moran C, Diaz-Montes TP, Hines GA, Cruz RI, Azziz R. Prevalence of 21-hydroxylase-deficient nonclassic adrenal hyperplasia and insulin resistance among hirsute women from Puerto Rico. Fertil Steril. 2000;74:59–62.
27. Chetkowski RJ, DeFazio J, Shamonki I, Judd HL, Chang RJ. The incidence of late-onset congenital adrenal hyperplasia due to 21-hydroxylase deficiency among hirsute women. J Clin Endocrinol Metab. 1984;58:595–8.
28. Morán C, Tapia MC, Hernández E, Vázquez G, García-Hernández E, Bermúdez JA. Etiological review of hirsutism in 250 patients. Arch Med Res. 1994;25:311–4.
29. Kuttenn F, Couillin P, Girard F, Billaud L, Vincens M, Boucekkine C, et al. Late-onset adrenal hyperplasia in hirsutism. N Engl J Med. 1985;313:224–31.

30. Innanen VT, Vale JM. Assessment of the one hour adrenocorticotrophic hormone test in the diagnosis of attenuated 21-hydroxylase deficiency. J Clin Pathol. 1990;43:493–5.
31. Escobar-Morreale HF, Sanchón R, San Millán JL. A prospective study of the prevalence of nonclassical congenital adrenal hyperplasia among women presenting with hyperandrogenic symptoms and signs. J Clin Endocrinol Metab. 2008;93:527–33.
32. Zreik RS, Nasrallah MP. The prevalence of endocrinopathies among Lebanese women presenting with hirsutism to an endocrine clinic. J Med Liban. 2014;62:27–32.
33. Arnaout MA. Late-onset congenital adrenal hyperplasia in women with hirsutism. Eur J Clin Invest. 1992;22:651–8.
34. Khandekar S, Lata V, Dash RJ. Screening for late onset congenital adrenal hyperplasia due to 21-hydroxylase deficiency. Indian J Med Res. 1990;92:79–82.
35. Moran C, Azziz R, Carmina E, Dewailly D, Fruzzetti F, Ibañez L, Knochenhauer ES, Marcondes JA, Mendonca BB, Pignatelli D, et al. 21-Hydroxylase-deficient nonclassic adrenal hyperplasia is a progressive disorder: a multicenter study. Am J Obstet Gynecol. 2000;183:1468–74.
36. Therrell BL. Newborn screening for congenital adrenal hyperplasia. Endocrinol Metab Clin North Am. 2001;30:15–30.
37. Merke DP, Bornstein SR. Congenital adrenal hyperplasia. Lancet. 2005;365:2125–36.
38. Li S, Hua R, Han X, Xu Y, Li M, Gao L, et al. Targeted long-read sequencing facilitates effective carrier screening for complex monogenic diseases including spinal muscular atrophy, α-/β-thalassemia, 21-hydroxylase deficiency, and fragile-X syndrome. J Transl Med. 2025;23:307.
39. Robeva R, Andonova S, Todorov T, Feyzullova A, Elenkova A, Kirilov G, Savov A, Zacharieva S, Todorova A. CYP21A2 intron 2 genetic variants might be associated with the clinical characteristics of women with PCOS. Biomedicines. 2024;12:1528.
40. Hu P, Tan J, Yu F, Shao B, Zhang F, Zhang J, et al. A capillary electrophoresis-based multiplex PCR assay for expanded carrier screening in the Eastern Han Chinese population. NPJ Genom Med. 2022;7:6.
41. Fang Y, Li J, Zhang M, Cheng Y, Wang C, Zhu J. Clinical application value of expanded carrier screening in the population of childbearing age. Eur J Med Res. 2023;28:151.
42. Baumgartner-Parzer SM, Nowotny P, Heinze G, Waldhäusl W, Vierhapper H. Carrier frequency of congenital adrenal hyperplasia (21-hydroxylase deficiency) in a middle European population. J Clin Endocrinol Metab. 2005;90:775–8.
43. Fitness J, Dixit N, Webster D, Torresani T, Pergolizzi R, Speiser PW, et al. Genotyping of CYP21, linked chromosome 6p markers, and a sex-specific gene in neonatal screening for congenital adrenal hyperplasia. J Clin Endocrinol Metab. 1999;84:960–6.
44. Polat S, Karaburgu S, Unluhizarci K, Dundar M, Ozkul Y, Arslan YK, et al. Unexpectedly high mutation rate of *cyp11b1* compared to *cyp21a2* in randomly-selected Turkish women: a large screening study. J Endocrinol Invest. 2023;46:2367–77.
45. Admoni O, Israel S, Lavi I, Gur M, Tenenbaum-Rakover Y. Hyperandrogenism in carriers of CYP21 mutations: the role of genotype. Clin Endocrinol (Oxf). 2006;64:645–51.
46. Guarnotta V, Niceta M, Bono M, Marchese S, Fabiano C, Indelicato S, et al. Clinical and hormonal characteristics in heterozygote carriers of congenital adrenal hyperplasia. J Steroid Biochem Mol Biol. 2020;198:105554.
47. Knochenhauer ES, Cortet-Rudelli C, Cunnigham RD, Conway-Myers BA, Dewailly D, Azziz R. Carriers of 21-hydroxylase deficiency are not at increased risk for hyperandrogenism. J Clin Endocrinol Metab. 1997;82:479–85.
48. Escobar-Morreale HF, San Millán JL, Smith RR, Sancho J, Witchel SF. The presence of the 21-hydroxylase deficiency carrier status in hirsute women: phenotype-genotype correlations. Fertil Steril. 1999;72:629–38.
49. Witchel SF, Aston CE. The role of heterozygosity for CYP21 in the polycystic ovary syndrome. J Pediatr Endocrinol Metab. 2000;13(Suppl 5):1315–7.
50. Glintborg D, Hermann AP, Brusgaard K, Hangaard J, Hagen C, Andersen M. Significantly higher adrenocorticotropin-stimulated cortisol and 17-hydroxyprogesterone levels in 337 consecutive, premenopausal, Caucasian, hirsute patients compared with healthy controls. J Clin Endocrinol Metab. 2005;90:1347–53.
51. Lidaka L, Bekere L, Lazdane G, Dzivite-Krisane I, Kivite-Urtane A, Gailite L. Non-Classical congenital adrenal Hyperplasia-Causing alleles in adolescent girls with PCOS and in risk group for PCOS development. Diagnostics. 2021;11:980.

Publisher's Note