CONGENITAL ADRENAL HYPERPLASIA, UNRESOLVED ISSUES AND IMPLICATIONS ON CLINICAL MANAGEMENT

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CONGENITAL ADRENAL HYPERPLASIA, UNRESOLVED ISSUES AND IMPLICATIONS ON CLINICAL MANAGEMENT

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Editorial: Congenital Adrenal Hyperplasia, Unresolved Issues and Implications on Clinical Management

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Editorial on the Research Topic

Congenital Adrenal Hyperplasia, Unresolved Issues and Implications on Clinical Management

Congenital Adrenal Hyperplasia (CAH) constitutes a group of autosomal recessive disorder, arising from mutations in the genes regulating steroidogenesis. Mutations in the CYP21A2 gene account for ∼95% of CAH cases and most affected individuals are compound heterozygotes arising from inheritance of different paternal and maternal mutant alleles of this gene. However, there is an increasing incidence of true homozygosity due to consanguineous marriage. The incidence of classical CAH in western populations is about 1:40.000 and with a carrier frequency of 1 in 50. Conversely, the prevalence of non-classical CAH, which remains relatively under-diagnosed, may be as high as 1:100-1:1,000 and even higher (1–20) among Hispanics, Yugoslavs, and Ashkenazi Jews.

The discrimination of CAH to Classical and Non-Classical forms is based on the degree of 21-hydroxylase deficiency. Namely, in classic CAH, inactivating mutations of the gene lead to salt wasting due to the absence of both aldosterone and cortisol production, whereas excess adrenal androgens causing signs of virilisation. On the other hand, in Non-Classical CAH, partial enzyme activity is sufficient for cortisol and aldosterone secretion, but increased androgen production are related to variable phenotypes, which may occur prepubertally, during adolescence or later in adulthood. Nevertheless, CAH is better to be considered as a continuum of diseases due to phenotypic variability through time.

Although the Synacthen test is a prerequisite in differential diagnosis, the genotyping of the affected patient is required for confirmation of the diagnosis. Furthermore, in difficult cases, accurate molecular diagnosis essential for optimal genetic counseling and during preparation for pregnancy. On the other hand, genotyping is a challenging process due to the occurrence of both a gene and a highly homologous pseudogene. Nevertheless, as is meticulously explained in their article "*The Complexities in Genotyping of Congenital Adrenal Hyperplasia*" by Pignatelli et al., novel tools are improving the chances of a correct diagnosis and better understanding of the underlying mechanisms of the disease. The new opportunities now provided by genotyping, however, also entail new complexities (Pignatelli et al.). Beyond the 10 classic pathogenic variants usually examined in most laboratories, achievement of an in-depth analysis of 21OH-deficiency cases will today involve complete sequencing of the entire gene and identification of

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gene duplications, which occur frequently and may lead to false positive cases. Moreover, since gene conversions can include several pathogenic variants, it cannot be established with absolute certainty whether both alleles are affected without studying parental DNA samples. The treating clinician should therefore be aware of the challenges, but also of the new possibilities available, and of both the precision of molecular techniques and their difficulties. Most importantly, a suspected diagnosis of NCCAH should certainly not be ruled out until and unless full sequencing of the CYP21A2 gene and the abovementioned procedures have been applied by an experienced and up-to-date laboratory.

Another issue in the diagnosis of CAH is the lack of significant genotype-phenotype association (∼80%), an unusual phenomenon in monogenic diseases. Indeed, in the case of thalassaemia or cystic fibrosis, the phenotype is closely related to a genetic defect. In line with these data, in their article "*Genotype Is Associated to the Degree of Virilization in Patients With Classic Congenital Adrenal Hyperplasia*", Neocleous et al. analyzed the impact of different molecular defects on the degree of virilisation in their cohort of CAH patients. Interestingly, by applying state-of-the-art techniques, they observed disorders ranging from complete male virilisation to clitoromegaly in female newborns carrying CYP21A2 mutations, thus emphasizing the need for full genotyping of patients with the disease regardless of the clinical presentation, especially in cases requiring genetic counseling or involving future fecundity (Neocleous et al.).

Another very important but undervalued issue is the fact that patients with NCCAH are at increased risk of metabolic derangements. Indeed, in females, there is substantial evidence showing a direct link between hyperandrogenaemia and insulin resistance. Furthermore, a significant percentage of patients are on chronic treatment with glucocorticoids, oral contraceptive pills, and/or antiandrogens, therapeutic regimens which induce an unfavorable metabolic profile. Consequently, increased incidence of dysglycaemia, dyslipidaemia, hypertension, fatty liver, and metabolic syndrome is anticipated in this hyperandrogenic population. However, given that the available data evaluating this association are to date limited, the recent publication of three articles aiming at elucidating this relationship is of major importance. First, de Vries et al., in their article "*Obesity and Cardiometabolic Risk Factors in Children and Young Adults With Non-classical 21-Hydroxylase Deficiency*", assessed the metabolic profile of a large number of adolescents with the disease. Of note, 66% of them were on treatment with glucocorticoids. Also of special interest is the authors' observation that patients with NCCAH do not differ from their normal peers as regards obesity status and metabolic profile, irrespective of glucocorticoid treatment.

Regarding cardiovascular risk in patients with CAH, a detailed review entitled "*Cardiovascular Health in Children and Adolescents With Congenital Adrenal Hyperplasia Due to 21-Hydroxilase Deficiency*" was carried out by Improda et al.. Their working hypothesis was that, based on the fact that a cluster of cardiovascular (CV) risk factors have been documented in adults with CAH, and it is highly likely that children and adolescents are prone to develop a similar sequela. As concerns obesity, though few data are as yet available on NCCAH patients, no significant differences were found between classic and non-classic forms. Furthermore, concerning insulin resistance, the available data indicate that both treated and untreated NCCAH patients display reduced insulin sensitivity. Of note, in one study it was observed that patients with NCCAH displayed more pronounced alterations in glucose metabolism compared to patients with classic forms. Taking the above as a whole, it could be suggested that prolonged exposure to androgen excess may contribute to an adverse metabolic profile. Due to the shortage of data, assessment of CV risk factors, such as adipokines, inflammatory markers, and homocysteine, as well as of heart function, was necessary in the NCCAH patients of the above study. Generally speaking, however, young patients with the classic form of the disease exhibit an unbalanced profile of several CV markers associated with vascular dysfunction and increased intramedial thickness of the carotid arteries, as well as with impaired exercise performance. The authors conclude that CAH may be associated with early markers of CV morbidity. Thus, lifestyle counseling and periodic assessment of blood pressure at all ages should be recommended in the management of CAH children, with regular monitoring of CV markers tailored to the individual patient's needs.

Adopting a different approach, Macut et al. analyzed the incidence of growth, obesity, insulin resistance, and bone mineral density in patients with NCCAH at different ages and compared them with subjects with the classic form of the disease. In their article entitled "*Metabolic Perspectives for Non-classical Congenital Adrenal Hyperplasia With Relation to the Classical Form of the Disease*", the authors discuss the metabolic consequences in these patients and describe the therapeutic effects of the different drug regimens, while pointing to the lack of longitudinal follow-up data (Macut et al.). With respect to growth, no difference was found between patients with NCCAH and the normal population, since almost normal final height was observed. It was however noted that adult NCCAH patients are prone to develop metabolic consequences, since many of them presented higher rates of obesity, insulin resistance, and CV risk factors. Finally, decreased bone mineral density and osteoporosis were found in some cases, this possibly being partially attributable to gender as well as to the type and dose of glucocorticosteroids applied.

NCCAH has been detected in women with androgen excess, with varying prevalence (1–10%), while both clinical and hormonal findings in females with NCCAH overlap with those of other hyperandrogenic entities, such as polycystic ovary syndrome (PCOS), hence causing diagnostic difficulties. This important issue has been extensively reviewed by Papadakis et al. in their article entitled "*Polycystic Ovary Syndrome and NC-CAH: Distinct Characteristics and Common Findings. A Systematic Review*". Specifically, the article discusses the differences in the genetics and pathophysiology that regulate these disorders. Meanwhile, however, such common findings as hirsutism, hyperandrogenaemia, polycystic morphology, and pregnancy complications, as well as metabolic disturbances and mood disturbances, are also reviewed, thereby emphasizing that the two entities are also characterized by small differences. Furthermore, the need for Synacthen testing in the differential

diagnosis is presented, while there is additionally a thorough examination of the various therapeutic approaches adopted in accordance with the underlying disorder.

In the same line of thought, Livadas and Bothou, in their article "*Management of the Female With Non-classical Congenital Adrenal Hyperplasia (NCCAH): A Patient-Oriented Approach*", present the best possible approach to the female patient with NCCAH based on the patient's prevailing symptoms at the specific age of presentation (Livadas and Bothou). First, they describe the diverse phenotype of the disease in childhood, during adolescence, or later in adult life, this followed by their recommendations for management. Specifically concerning the treatment decision, they emphasize that treatment (1) should be individualized, (2) is not always indicated, and (3) has to be modified according to the patient's evolving needs. Also described are the therapeutic options for female patients treated from childhood, those who presented the first signs and symptoms during adolescence, and those with hyperandrogenic symptoms post-treatment discontinuation. A detailed discussion on the current therapeutic regimens of, inter alia, glucocorticoids, oral contraceptive pills, and antiandrogens, is provided. Furthermore, the major issue of subfertility, usually encountered in females with NCCAH, is systematically analyzed. Advice on molecular testing of the prospective father is based on the potential mother's genotype. The authors propose a tailor-made approach, incorporating a smooth transition of the patient's management once she is referred from the pediatric to the adult endocrinologist, while underlining that she will require symptom-oriented treatment throughout her life.

Another hot topic concerning the pathophysiology and management of women with hyperandrogenic disorders is the impact of vitamin D deficiency. Indeed, dysregulation of this pleiotropic hormone has been implicated in subclinical inflammation, insulin resistance, and hyperandrogenaemia, all factors that characterize the PCOS phenotype. Yu-Ming et al., in their article "*Association Between Vitamin D Receptor Gene Polymorphisms and Polycystic Ovary Syndrome Risk: A Meta-Analysis*" provide some of the current data on the effect of vitamin D and its receptor polymorphisms on PCOS pathophysiology and, based on their meta-analysis, they suggest that vitamin D receptor gene polymorphisms contribute to PCOS development (Yu-Ming et al.).

Finally, subfertility is a common problem in women suffering from CAH, with many of those suffering from NCCAH often being diagnosed for the first time in a fertility clinic. It is well known that the increased progesterone concentrations usually encountered in this population alter endometrial receptivity and tubal motility and lead to ovulation disorders. In the review "*Assisted Reproduction in Congenital Adrenal Hyperplasia*" by Chatziaggelou et al., these issues are very well-analyzed and an optimal approach is suggested. The authors suggest that administration of an adequate substitution dose of glucocorticoids leads not only to successful assisted reproduction treatment but also, in many cases, to spontaneous pregnancy. Hydrocortisone is today the gold standard treatment, since it restores ovarian function, ovulation, and endometrial receptivity. They recommend that in the event of a pregnancy involving a fetus suspected of having CAH, delivery should ideally be managed by an expert, multidisciplinary team, including a gynecologist, an endocrinologist, and a pediatrician, in a tertiary hospital.

In conclusion in this Editorial illuminating the articles of this series, we have sought to provide the clinician with the current knowledge regarding several aspects of the management of patients with NCCAH. The latter disorder represents a particular form of CAH that is characterized by specificities in clinical presentation, diagnosis, therapeutic approach, and metabolic outcomes. Though concerning a less severe form of CAH, therapeutic management of NCCAH patients remains a challenge, and current treatment regimens do not always allow optimal biochemical control, while overexposure to glucocorticoids as well as to androgen excess may contribute to the development of metabolic and cardiovascular abnormalities. Therefore, we strongly recommend a patient-oriented approach, based on each patient's individual needs, and long-term follow-up.

AUTHOR CONTRIBUTIONS

SL wrote the editorial. CS and DM edited the manuscript.

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ORIGINAL RESEARCH
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Genotype Is Associated to the Degree of Virilization in Patients With Classic Congenital Adrenal Hyperplasia

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Background: Molecular defects of *CYP21A2* consistently decrease 21-hydroxylase activity and result in a variable expression of disease severity in patients with congenital adrenal hyperplasia (CAH).

Aim: The genotype and biochemical findings were examined in an attempt to reveal any association to the degree of virilization in classic CAH patients.

Methods: The study included 18 CAH patients with complete characterization of *CYP21A2* mutations and were sorted based on the severity of the inherited mutations and the expected percentage of 21-hydroxylase enzyme activity.

Results: Eleven out of the 18 patients manifested the SW form with the remaining seven exhibiting the SV form. The most frequent genetic defect in the classic salt-wasting (SW) and simple virilising (SV) forms was the IVS2-13A/C>G (36.1%) mutation, followed by delEX1-3 (19.4%) and p.Ile172Asn (19.4%). Four patients, who shared a combination of two mutations belonging to the most severe type, manifested only the SW form. Four out of five patients who shared homozygosity in the IVS2-13A/C>G mutation, demonstrated the SW form and only one demonstrated the SV form. All four patients who shared the p.Ile172Asn mutation, either in the homozygous or compound heterozygous state, manifested the SV form. Interestingly, a female neonate with SW, bearing the IVS2-13A/C>G/Large del, exhibited complete male virilisation (Prader 5). The remaining four affected female new-borns also exhibited the SW form, with two of them virilised as Prader 3 and the other two as Prader 4. Virilisation with clitoromegaly was also observed in one female, who presented premature adrenarche and carried the least severe p.Pro30Leu mutation.

Conclusion: The frequency of the underlying mutations in our patients, with the classic form of CAH, varies but were quite similar to the ones reported in the Mediterranean region. Therefore, the identification of severe *CYP21A2* defects in Cypriot patients and their comparison with the incidence and severity in different populations, will create a valuable diagnostic tool for genetic counseling in the classic form of CAH.

Keywords: Classic CAH, virilisation, CYP21A2, 21-hyrdroxylase deficiency, salt-wasting

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INTRODUCTION

Congenital adrenal Hyperplasia (CAH) is a group of autosomal recessive disorders caused by mutations in gene encoding enzymes, involved in cortisol biosynthesis and defective steroidogenesis (1). The most prevalent form of CAH is 21-hydroxylase (21-OH) (90–95% of cases) followed by the next most frequent type of 11β-hydroxylase (11β-OH) (∼5% of cases) and other rarer types such as 17α-hydroxylase (17α-OH or 17,20-lyase), 3β-hydroxysteroid dehydrogenase type 2 (HSD3B2), steroidogenic acute regulatory protein (StAR), P450 cholesterol side-chain cleavage enzyme (SCC), P450 oxidoreductase (POR) and cytochrome b deficiency (CYB5A) (2, 3). 21-OH deficiency accounts for more than 95% of all CAH cases and is due to the molecular defects in the *CYP21A2* gene (4). The disorder has a broad spectrum of clinical phenotypes and severity depends on the patients' underlying *CYP21A2* genotype (5, 6). The deficiency is present in the course of fetal development and leads to varying degrees of prenatal virilisation of the external genitalia in affected girls. The clarification of the genetic background of CAH has been influential in the diagnosis and the classification of the disease (6, 7). Currently, the disorder is classified into the classic or non-classic (NC late onset) CAH form, respectively (8, 9). The classic form is further divided into the simple virilising (SV) form (∼25% of individuals) and the salt-wasting (SW) form, in which aldosterone production is inadequate (≥75% of individuals). The patients are also divided into the SV and SW groups based on the presence of a milder allele. In the SV patients, excess androgen of the adrenals in the utero, result in genital virilization at birth in 46, XX females. In affected females, the excess androgens result in various degrees of enlargement of the clitoris, fusion of the labioscrotal folds, and formation of a urogenital sinus. Because the anti-müllerian hormone (AMH) is not secreted, the müllerian ducts develop normally into a uterus and fallopian tubes in affected females (10). Patients with the most severe SW classic form, are characterized by salt-wasting and the extremely low enzymatic activity of 21-OH. This leads to the deficiency of both aldosterone and cortisol usually accompanied by vomiting, dehydration, hypoglycaemia and hypotension as well as marked hyperkalaemia and hyponatraemia in the first weeks of after birth (11). Worldwide the estimated incidence of the classic form is 1:10,000 to 1:15,000, while the NC-CAH occurs in a frequency of 1:500 to 1:100 of live births and is estimated to be one of the most common autosomal recessive disorders (12–15).

Data from several new-born screenings and carrier analyses of the general population have estimated that the carrier incidence in the general population is 1:25–1:10 (16–18). Currently, more than 200 mutations in the *CYP21A2* gene, differing in prevalence and severity, have been reported and only 10 of them account for about 95% of the disease-causing alleles (6, 19).

Numerous studies have established a strong correlation between the genotype and the phenotype and over the last few decades mutation detection rates led to the identification of a large number of *CYP21A2* defects (20, 21). In this study, we present the molecular genetic features of the disease in patients with the classic form who are of Cypriot descent, over the last decade. Thus, the aim of this study is to describe a comprehensive *CYP21A2* mutation analysis in a cohort of classic CAH patients and to create a useful tool for clinicians and geneticists, necessary for the genetic diagnosis and management of not only Cypriot patients but also for international patients with 21-hydroxylase deficiency.

METHODS AND RESULTS

Patients and Bioethics Approval

From 2007 to 2018 18 patients of Greek Cypriot origin, with classic CAH, were phenotypically classified by one pediatric endocrinologist (N.S) based on clinical and hormonal criteria. Written and oral informed consent was obtained from the parents or guardians of the minors and all relatives screened for mutations in the *CYP21A2* gene. The project was approved by the Cyprus National Ethics Committee and all methods were performed in accordance with the relevant guidelines and regulations.

Clinical, Biochemical and Genetic Screening at Diagnosis

All patients and their parents were genotyped and were categorized into the most severe SW form and the most severe SV form (**Table 1**). More specifically, patients with the SW form were initially allocated to this form based on clinical and biochemical findings of renal salt wasting (females with virilization at birth and males with vomiting, failure to thrive, hyponatremia, hyperkalemia, high plasma renin activity (PRA), and significantly high 17-OHP>75 nmol/L) in the first 2 weeks of life. The second group of patients categorized as having the SV form, also exhibited clinical symptoms of CAH without electrolyte imbalance (females with virilization at birth or later without any clinical evidence of salt loss at birth and males with clinical signs of sexual precocity with acceleration of growth and bone age, high 17-OHP > 75 nmol/L, and normal or slightly elevated PRA). The *CYP21A2* genes of the total number of patients who participated in the study were analyzed by Sanger DNA sequencing. The genetic investigation was done based on a cascade strategy as formerly described (18, 22). For the amplification of the 5′ untranslated region which is located in the first 167 nucleotides upstream of the ATG codon of the *CYP21A2* gene, the primers P1-P48 (23) were used to amplify a fragment of 370 bp. The 3' untranslated region that is 536 nucleotides downstream of the TGA stop codon of the *CYP21A2* gene was amplified using the primers: 5′AGATGCAGCCTTTCCAAGTG3′ and 5′AGCACAGTGGACCATCAGGT3′ (24). Multiplex ligation-dependent probe amplification (MLPA) technique (MRC Holland, Amsterdam, Netherlands) was used to detect any possible large gene deletions, duplications and large gene conversions in the *CYP21A2* gene of the patients under investigation, as previously described (22).

The type of molecular defects as well as the clinical and biochemical data of patients with classic CAH, are shown in **Table 1**. Eighteen patients with classic CAH were categorized

TABLE 1 | The type of the molecular defects with clinical and biochemical data in the patients with Classic CAH.

	Genotype	Form	Sex	Age of diagnosis	Clinical phenotype	17-OHP nmol/l basal	ACTH <60 pg/ml	Renin PRA* ng/ml/hr (0.2–2.8)
1	IVS2-13A/C>G/IVS2-13A/C>G	SW	F	neonate	Ambiguous genitalia - Prader 3	>75.7	1450	10.3
2	IVS2-13A/C>G/IVS2-13A/C>G	SW	F	neonate	Ambiguous genitalia - Prader 3	>75.7	1355	9.4
3	IVS2-13A/C>G/Large del	SW	F	neonate	Ambiguous genitalia - Prader 5	>75.7	103	3.1
4	IVS2-13A/C>G/p.Gln318stop	SW	F	neonate	Ambiguous genitalia - Prader 4	>75.7	N/A	32.3
5	p.Phe306insT+p.Val281Leu/ p.Phe306insT+p.Val281Leu	SW	F	neonate	Ambiguous genitalia - Prader 4	>75.7	>2100	12
6	IVS2-13A/C>G/IVS2-13A/C>G	SW	M	neonate	Adrenal crisis	>75.7	>2100	11.4
7	IVS2-13A/C>G/IVS2-13A/C>G	SW	M	neonate	Adrenal crisis	>75.7	> 2100	10.7
8	IVS2-13A/C>G/del Exons 1_3	SW	M	neonate	Adrenal crisis	>75.7	2352	9.8
9	del Exons 1_3/del Exons 1_3	SW	M	neonate	Adrenal crisis	>75.7	>2100	8.5
10	del Exons 1_3/del Exons 1_3	SW	M	neonate	Adrenal crisis	>75.7	>2100	10.5
11	del Exons 1_3/p.Gln318stop	SW	M	neonate	Adrenal crisis	>75.7	1680	11.3
12	p.Pro30Leu/p.Pro30Leu	SV	F	6.5 years	Exaggerated premature clitoromegaly	>75.7	76.4	0.4
13	p.Ile172Asn/p.Ile172Asn	SV	F	neonate	Ambiguous genitalia at birth	>75.7	392	8.2
14	p.Ile172Asn/del of *CYP21A2*	SV	M	3 years	GnRH independent precocious puberty	>75.7	569	4.7
15	p.Ile172Asn/p.Ile172Asn	SV	M	5.0 years	GnRH independent precocious puberty	>75.7	38	4.7
16	p.Ile172Asn/p.Ile172Asn	SV	M	3.2 years	GnRH independent Precocious Puberty	>75.7	122	7.5
17	IVS2-13A/C>G/IVS2-13A/C>G	SV	M	5.5 years	GnRH independent precocious puberty	43.7	282	1.23
18	Partial conv with CYP21P:-4C>T, 92C>T, 118T>C, 138A>C/delEx 1_3	SV	M	6.5 years	GnRH independent precocious puberty	>75.7	N/A	N/A

PRA, Plasma Renin Activity.*

in two groups (SW and SV) based on genotype/phenotype correlations (**Table 1**). More specifically, mutations allocated in the SW group resulted in no or minimal residual enzymatic activity (25–28). Mutations allocated to the SV group usually exhibited residual enzymatic activity of about 2% (29–31). The most severe form of CAH, the classic SW, was identified in 11 neonates (**Table 1**). Seven children with the SV form were also identified, at a median presentation age of 5 years (interquartile range (IQR) 3.2–6.5). The clinical presentation at diagnosis was considerably different between the SW and SV group (**Table 1**). All five females with SW CAH exhibited an expected electrolyte imbalance (hyponatremia, hypekalemia) and a variable degree of virilization of the external genitalia in accordance with the severity of mutations that they carried (**Table 1**).

All males with the SW CAH presented clinical signs of adrenal crisis, hyponatremia, hyperkalemia, dehydration, and hypovolemic shock. The children belonging to the SV group had no electrolyte imbalance. The girls with SV CAH presented ambiguous genitalia at birth and the boys manifested GnRH independent precocious puberty (**Table 1**).

The splice site mutation IVS2-13A/C>G in homozygosity was the most frequently detected genotype. Five out of 18 patients with the classic SW form of CAH were found in the homozygosity of the severe causing IVS2-13A/C>G splice mutant. The remaining 13 patients had a combination of compound heterozygote genotypes belonging to the most severe *null group* and the second most severe *group A* mutations as described in a previous study by our group (22). One patient affected with the SW form, was associated with the rare genotype p.Phe306insT+p.Val281Leu/p.Phe306insT+p.Val281Leu. The same genotype was detected both on the paternal and the maternal alleles.

Using the MLPA analysis, several deletions (DelEx1-3, del *CYP21A2*, Large del, 30 kb del) and a partial conversion (Partial conv with CYP21P:-4C>T, 92C>T, 118T>C, 138A>C) were identified. The DelEx1-3 was identified as the second most severe frequent defect and was detected in homozygosity or in the compound heterozygosity state in thirteen patients with various degrees of severity (**Table 1**).

In total, nine different variants were identified in the cohort of 18 patients with classic CAH and consisted of (a) two (22.22%) missense mutations, (b) one (11.11%) nonsense mutation, (c) one (11.11%) splicing mutation, (d) one (11.11%) frameshift mutation, (e) one (11.11%) partial conversion, and finally (f) three (33.33%) large deletions. The overall frequency of the identified molecular defects detected in our patients is also depicted in **Figure 1**. In the 36 non-related alleles, the most frequent mutation was IVS2-13A/C>G (36.11%) followed by DelEx1-3 (19.44%). A series of seven other less frequent and mostly severe mutations were identified and are also depicted in **Figure 1**.

DISCUSSION

Our data represent a comprehensive portrayal of the classic clinical forms of CAH over a period of time in Cyprus. From 2007 to 2017, 18 patients with either the SW or SV form of CAH were categorized and genotyped at the Molecular Genetics, Function and Therapy (MGFT) department of the Cyprus Institute of Neurology and Genetics. With an estimated population of 701,000 Greek Cypriots (Cyprus statistical service 2016_ http://www.mof.gov.cy/mof/cystat/statistics.nsf/populationcondition_21main_puparchive_en/populationcondition_21main_puparchive_en?OpenForm&yr=2016) and the recent report by our group that the true carrier frequency of *CYP21A2* in Greek-Cypriots is 1:10 (18), the CAH prevalence is therefore predicted to be around ∼1750 (701,000 × ½ × ½ × $^{1}/10$ × $^{1}/10$ = 1752.5). Subsequently, the current 18 classic CAH patients identified by our group make up only a 1.03% of the total of CAH cases projected (∼1,750) to exist in the Greek Cypriot population. Prompt screening in combination with *CYP21A2* genetic analyses, enables clinicians to manage severe cases in the neonatal period promptly, even before the appearance of any electrolyte imbalance and/or urgent adrenal crisis.

As expected, the clinical presentation of our classic CAH patients showed a spectrum of phenotypes and as demonstrated from the current and previous studies, the clinical presentation was substantially different between the SW and SV groups (22, 32). The female neonates with SW presented a variable degree of virilization in accordance with the severity of the genetic defect accompanied by hyponatremia and hyperkalemia, whereas all males exhibited signs of adrenal crisis (electrolyte imbalance and hypovolemic shock). Interestingly, one of the five female neonates with SW had an external genital appearance of Prader 5, with the remaining four classified as Prader 3 or 4. The neonate of our study with SW and the external genital appearance of Prader 5, were carried in the compound heterozygote state, the IVS2-13A/C>G and a large deletion. Several recent and older reports have shown that complete deletion of *CYP21A2* in Caucasians changes the genomic organization in the RCCX module to the status of C4A-CYP21A1P-TNXA/TNXB (21, 33). To date, at least nine kinds of chimeric TNXA/TNXB genes have been identified and associated with Ehlers-Danlos syndrome as well as with CAH (33). This combination of the IVS2-13A/C>G with a large deletion has been associated with the most severe SW phenotype (33–36). This phenotype is part of a group of chimeras and is common among CAH patients of Caucasian origin and has been referred to as a classic or common type of chimera (37). None of the children in the SV group had any electrolyte imbalance as expected. All males belonging to the SV group exhibited GnRH independent precocious puberty (pubic hair, penile increase, pre pubertal testes) at different ages.

Currently, more than 200 mutations in the *CYP21A2* gene have been described in several studies and there is a good correlation between the clinical phenotype and the patient genotypic findings (1, 21, 38–43). In general, our genotype-phenotype correlation was in accordance with previous studies and showed a positive predictive value for patients carrying mutations belonging to the null group (44–46). Patients carrying the supposedly milder mutation p.Pro30Leu, have previously been reported to demonstrate poor genotype-phenotype correlation and showed a divergence between the

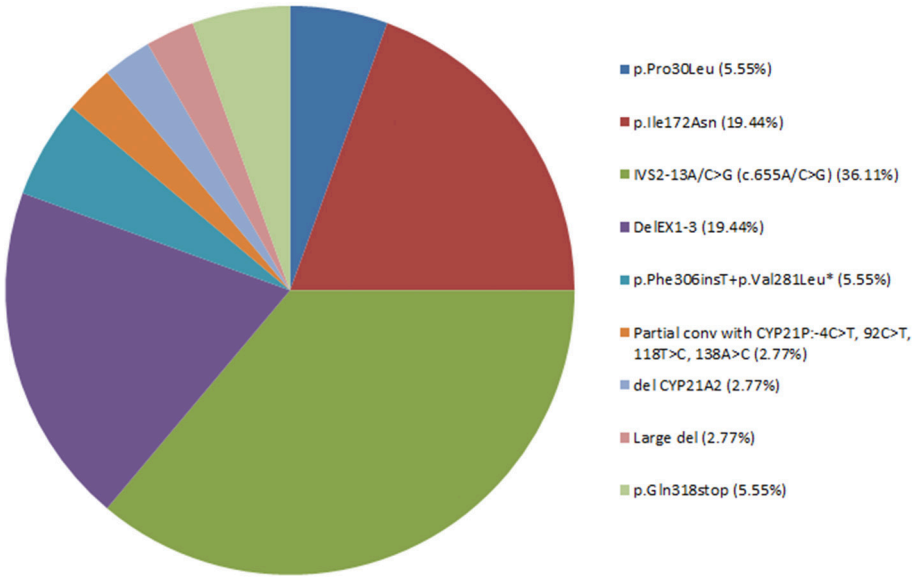

FIGURE 1 | Pie-chart showing the percentage of mutations across the 18 classic CAH patients. *Mutation p.Phe306insT+p.Val281Leu fall under the category of multiple mutations because they are found *in cis* on the same allele.

observed and predicted phenotype (6, 46). In a similar fashion a female patient from our cohort, homozygous for p.Pro30Leu, was clinically and biochemically identified with the SV form, with a premature pubarche clitoromegaly at 6.5 yrs. It is possible that other genetic variation(s) might also exist in other genes known to be implicated in the salt balance of CAH, for this specific female, that carried a homozygosity p.Pro30Leu. Such candidate genes where variations have been reported to exist are the *CYP2C19* and *CYP34A3* (47). Therefore, the infrequent phenomenon of digenic inheritance (DI), where the patients co-inherit biallelic or even triallelic mutations in two distinct genes (48, 49), *in cis* or *in trans*, and are sufficient to cause pathology with a usually defined and severe diagnosis, could also be the case with the female patient of our cohort carrying the homozygosity p.Pro30Leu.

According to genetic findings from previous studies as well as our present study, 17 different variants have been identified in the Greek-Cypriot population and are scattered throughout the entire coding sequence of the *CYP21A2* gene (22, 32, 40, 42, 43). In the present study we identified nine different variants and the most frequent defect among the 36 tested alleles was the IVS2-13A/C>G (36.11%) followed by DelEx1-3 (19.44%) and a series of seven other less frequent mutations.

In conclusion, the pathogenesis and the clinical presentation of the classic CAH depend on the severity of the underlying *CYP21A2* gene defects. Our study describes the complexities encountered in patients with classic CAH. The genotypic analysis of our patients with classic CAH confirmed their diagnosis in one of the two main forms of the disease, with an exceptional genotype-phenotype correlation. Knowing about the genetic defects will be valuable in the antenatal diagnosis, management and genetic counseling of existing and future families affected by these gene defects.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Association Between Vitamin D Receptor Gene Polymorphisms and Polycystic Ovary Syndrome Risk: A Meta-Analysis

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Objective: Published studies have demonstrated a closer association between vitamin D receptor (VDR) gene polymorphisms and polycystic ovary syndrome (PCOS) risk, but the results were inconsistent. We therefore performed this meta-analysis to explore the precise associations between VDR gene polymorphisms and PCOS risk.

Methods: Five online electronic databases (PubMed, Embase, SCI index, CNKI and Wanfang) were searched. Odds ratios (ORs) with 95% confidence interval (CIs) were calculated to assess the association between VDR Fok I C/T (rs10735810), BsmI A/G (rs1544410), ApaI A/C (rs7975232), and TaqI T/C (rs731236) polymorphisms and PCOS risk. In addition, heterogeneity, accumulative/sensitivity analysis and publication bias were conducted to check the statistical power.

Results: Overall, 10 publications (31 independent case-control studies) involving 1,531 patients and 1,174 controls were identified. We found that the C mutation of ApaI A/C was a risk factor for PCOS (C vs. A: OR = 1.20, 95%CI = 1.06–1.35, $P < 0.01$, $I^2 = 29.7\%$; CC vs. AA: OR = 1.49, 95%CI = 1.17–1.91, $P < 0.01$, $I^2 = 0\%$; CC vs. AA+AC: OR = 1.36, 95%CI = 1.09–1.69, $P = 0.01$, $I^2 = 12.8\%$). Moreover, the BsmI A/G polymorphism also showed a dangerous risk for PCOS in Asian population (G vs. A: OR = 1.62, 95%CI = 1.24–2.11, $P < 0.01$, $I^2 = 0\%$; AG vs. AA: OR = 2.08, 95%CI = 1.26–3.20, $P < 0.01$, $I^2 = 0\%$; GG vs. AA: OR = 2.21, 95%CI = 1.29–3.77, $P < 0.01$, $I^2 = 0\%$; AG+GG vs. AA: OR = 2.12, 95%CI = 1.42–3.16, $P < 0.01$, $I^2 = 0\%$). In addition, no significant association of Fok I C/T, and TaqI T/C polymorphisms was observed.

Conclusions: In summary, our meta-analysis suggested that VDR gene polymorphisms contribute to PCOS development, especially in Asian populations.

Keywords: vitamin D receptor, polycystic ovary syndrome, polymorphism, meta-analysis, risk

INTRODUCTION

Polycystic ovary syndrome (PCOS), characterized by clinical features including menstrual disorder, persistent anovulation, and polycystic ovaries, is one of the most common reproductive, endocrine, and metabolic disorder syndromes among women of reproductive age (Sirmans and Pate, 2013). Polycystic ovary syndrome (PCOS) has become a highly prevalent disorder that affects women in their reproductive age and contributes to multiple complications. According to the NIH 1990 criteria and/or Rotterdam 2003 criteria, the cumulative prevalence of PCOS was ∼4–21% worldwide (Knochenhauer et al., 1998; Asuncion et al., 2000; Azziz et al., 2004). High prevalence and elevated risk of the development of type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD) were reported in women with PCOS (Repaci et al., 2011; Ollila et al., 2017). Moreover, long-term complications including the mental dysfunctions, such as mood and sleeping disorders, are also found. However, the precise etiology and underlying pathological mechanism of PCOS remain unclear.

Vitamin D, a steroid hormone, plays an important role in maintaining calcium homeostasis and promoting bone mineralization (Shen et al., 2013). Beyond these fundamental relationships, accumulating evidence indicates a close association of vitamin D status with the pathogenesis, signs and symptoms of PCOS (Wehr et al., 2009; Krul-Poel et al., 2013). A recent meta-analysis found significant differences in serum 25-hydroxyvitamin D, serum insulin, total cholesterol, triglycerides, and low-density lipoprotein cholesterol in patients with PCOS compared with that in healthy controls (Bacopoulou et al., 2017).

Vitamin D receptor (VDR) is widely distributed in several tissues of the female reproductive system (Kato, 2000). Vitamin D receptor (VDR) could mediate the biological responses of the $1\alpha,25(OH)_2D_3$ hormone, through generating a signal transduction complex with a heterodimer of $1\alpha,25(OH)_2D_3$-liganded VDR and unoccupied retinoid X receptor (RXR). Then, this transcriptional unit combines with the vitamin D response element (VDRE) in the promoter region of genes and regulates its actions through altering the transcriptional expression of target genes (Haussler et al., 2011). Single nucleotide polymorphisms (SNPs) are the most frequent nucleotide variations in the human genome. The VDR gene is located on chromosome 12q13.11, includes eight protein coding exons and one untranslated exon, and encodes a 427-amino-acid protein (Baker et al., 1988). To date, four most common VDR polymorphisms of FokI (rs10735810 C>T), BsmI (rs1544410 G>A), ApaI (rs7975232 G>T), and TaqI (rs731236 T>C) have been investigated to explore the association between VDR and PCOS susceptibility. However, the results were conflicting and inconclusive owing to the small sample size and limited statistical power. We therefore conducted this comprehensive meta-analysis to evaluate the association between the above polymorphisms and PCOS susceptibility precisely.

MATERIALS AND METHODS

This meta-analysis was conducted according to the guideline of Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (Moher et al., 2009). All included data were based on published studies, and no ethical issues were involved.

Search Strategy

Five online electronic databases (PubMed, Embase, SCI index, CNKI, and Wanfang) were searched with the following terms from their inception up to March 20, 2018: "vitamin D receptor," "VDR," "rs10735810," "rs1544410," "rs7975232," "rs731236," "polymorphism," "variant," "mutation" "polycystic ovary syndrome," and "PCOS." Some relevant references cited within retrieved articles were reviewed with manual searched.

The following search strategy was used:
#1 vitamin D receptor
#2 VDR
#3 rs10735810
#4 rs1544410
#5 rs7975232
#6 rs731236
#7 #1 OR #2 OR #3 OR #4 OR #5 OR #6
#8 polymorphism
#9 variant
#10 mutation
#11 #8 OR #9 OR #10
#12 polycystic ovary syndrome
#13 PCOS
#14 #12 OR #13
15 #7 AND #11 AND #14

Eligibility Criteria

Studies were selected when they met the following criteria by two independent investigators (NYM and HYY): (1) the study followed a case-control design; (2) at least one polymorphisms of VDR gene was reported; (3) sufficient information about the distribution frequency of different polymorphism loci could be extracted to calculate the odds ratios (ORs) and 95% confidence intervals (CIs); (4) the most recent or largest sample sizes were selected when multiple publications were repeatedly reported with same team; and (5) the articles were written in English and Chinese.

Data Extraction

All included studies were reviewed and extracted by two independent investigators (NYM and WYD). Disagreements and compared results were settled through discussion. The following information and data were extracted from included studies: the first author of each study, published year, study country or region where the study was conducted, ethnicity of research population, the source of the controls, the sample sizes of patients with PCOS and healthy controls, data of the frequency genotype of distribution, and the genotyping method.

Risk Assessment of Bias Within Studies

All included studies in this meta-analysis were subject to make risk assessment of bias by two independent authors (JGB and BG) via the modified Newcastle-Ottawa Quality Assessment Scale (Niu et al., 2015). The score was based on five parameters (representativeness of cases, source of controls, Hardy-Weinberg

equilibrium (HWE) in controls, genotyping examination and association assessment), with a maximum score of 11 points. Studies of at least a score of 8 were identified with a high quality (**Table 1**).

Statistical Analysis

Crude ORs with 95% CIs were calculated to examine the statistical power of the association between the VDR gene polymorphisms and PCOS risk. For example, four genetic models of Fok I C/T polymorphisms were calculated: allele contrast (T vs. C), co-dominant models (heterozygote model: CT vs. CC, homozygote model: TT vs. CC), dominant model (CT+TT vs. CC), and recessive model (TT vs. CC+CT) (Minelli et al., 2005; Lewis and Knight, 2012). Similar genetic models were also calculated with the others [BsmI A/G (rs1544410), ApaI A/C (rs7975232), TaqI T/C (rs731236)]. Subgroup analysis based on HWE status, ethnicity difference, control design and genotyping methods were performed to clarify the potential risk. Heterogeneity was investigated by I^2 index which describes the percentage of variation among the included studies in the pooled analysis (Huedo-Medina et al., 2006). The fixed-effect model (Mantel-Haenszel method) was used when the I^2 value was <40% (Mantel and Haenszel, 1959). Otherwise, a random-effects model (DerSimonian and Laird method) was adopted (DerSimonian, 1996). Cumulative analyses were conducted to explore the tendency of the results whit the published studies were added. Sensitivity analyses were performed to investigate the stability of the results when each study was removed one at a time. Publication bias was assessed with the Egger's bias test and Begg's funnel plots (Begg and Mazumdar, 1994; Egger et al., 1997). Data analysis was conducted using STATA version 14.0 (Stata Corporation, College Station, TX, USA). $P < 0.05$ indicated a statistically significant difference.

TABLE 1 | Scale for quality evaluation.

Criteria	Score
Representativeness of cases	
Consecutive/randomly selected cases with clearly defined sampling frame	2
Not consecutive/randomly selected case or without clearly defined sampling frame	1
Not described	0
Source of controls	
Population-based	2
Hospital-bases or healthy-bases	1
Not described	0
Hardy-weinberg equilibrium in controls	
Hardy-weinberg equilibrium	2
Hardy-weinberg disequilibrium	1
Not available	0
Genotyping examination	
Genotyping done under "blinded" condition and repeated again	2
Genotyping done under "blinded" condition or repeated again	1
Unblinded done or not mentioned and unrepeated	0
Subjects	
Number ≥300	1
Number <300	0
Association assessment	
Assess association between genotypes and PCOS with appropriate statistics and adjustment for confounders	2
Assess association between genotypes and PCOS with appropriate statistics and without adjustment for confounders	1
Inappropriate statistics used	0

RESULTS

Study Characteristics

At first, 120 publications were identified through the systematic literature search. Three important steps according to the eligibility criteria were conducted to screen the selected studies were as follows: duplicate check, title and abstract check and text review. The selection of screening is presented in **Figure 1**. Finally, 10 studies were included in the meta-analysis with 1,531 patients with PCOS and 1,174 control individuals (Mahmoudi, 2009; Wehr et al., 2011; Bagheri et al., 2012, 2013; El-Shal et al., 2013; Dasgupta et al., 2015; Jedrzejuk et al., 2015; Mahmoudi et al., 2015; Cao and Tu, 2016; Siddamalla et al., 2018). The studies comprised seven case-control studies on FokI C/T (Mahmoudi, 2009; Wehr et al., 2011; Bagheri et al., 2012; Dasgupta et al., 2015; Jedrzejuk et al., 2015; Mahmoudi et al., 2015; Cao and Tu, 2016), seven case-control studies on BsmI A/G (Mahmoudi, 2009; Wehr et al., 2011; Bagheri et al., 2012; Jedrzejuk et al., 2015; Mahmoudi et al., 2015; Siddamalla et al., 2018), eight case-control studies on ApaI A/C (Mahmoudi, 2009; Wehr et al., 2011; El-Shal et al., 2013; Dasgupta et al., 2015; Jedrzejuk et al., 2015; Mahmoudi et al., 2015; Cao and Tu, 2016; Siddamalla et al., 2018), and nine case-control studies on TaqI T/C (Mahmoudi, 2009; Wehr et al., 2011; Bagheri et al., 2013; El-Shal et al., 2013; Dasgupta et al., 2015; Jedrzejuk et al., 2015; Mahmoudi et al., 2015; Cao and Tu, 2016; Siddamalla et al., 2018), respectively. Furthermore, three publications involved the Asians (Dasgupta et al., 2015; Cao and Tu, 2016; Siddamalla et al., 2018), and seven studies involved Caucasians (Mahmoudi, 2009; Wehr et al., 2011; Bagheri et al., 2012, 2013; El-Shal et al., 2013; Jedrzejuk et al., 2015; Mahmoudi et al., 2015). In the control groups, there are two case-control studies in BsmI A/G (Mahmoudi, 2009; Siddamalla et al., 2018), three case-control studies in ApaI A/C (Wehr et al., 2011; Dasgupta et al., 2015; Siddamalla et al., 2018) and two case-control studies in TaqI T/C (Cao and Tu, 2016; Siddamalla et al., 2018) polymorphisms deviated from the HWE. The main characteristics of the selected studies are shown in **Table 2**.

Quantitative Analysis

Fok I C/T Locus and PCOS Risk

Seven case-control studies with 1,241 PCOS cases and 846 control individuals were identified with regard to the association between Fok I C/T locus and PCOS risk. Overall, the pool analysis did not find any significant association between this locus on PCOS risk in five genetic models (T vs. C: OR = 1.04, 95%CI = 0.83–1.30, $P = 0.77$, $I^2 = 53.2\%$; CT vs. CC: OR = 1.08, 95%CI = 0.89–1.32, $P = 0.40$, $I^2 = 7.0\%$; TT vs. CC: OR = 0.89, 95%CI = 0.64–1.25, $P = 0.50$, $I^2 = 35.6\%$; CT+TT vs. CC: OR = 1.06, 95%CI = 0.88–1.27, $P = 0.56$,

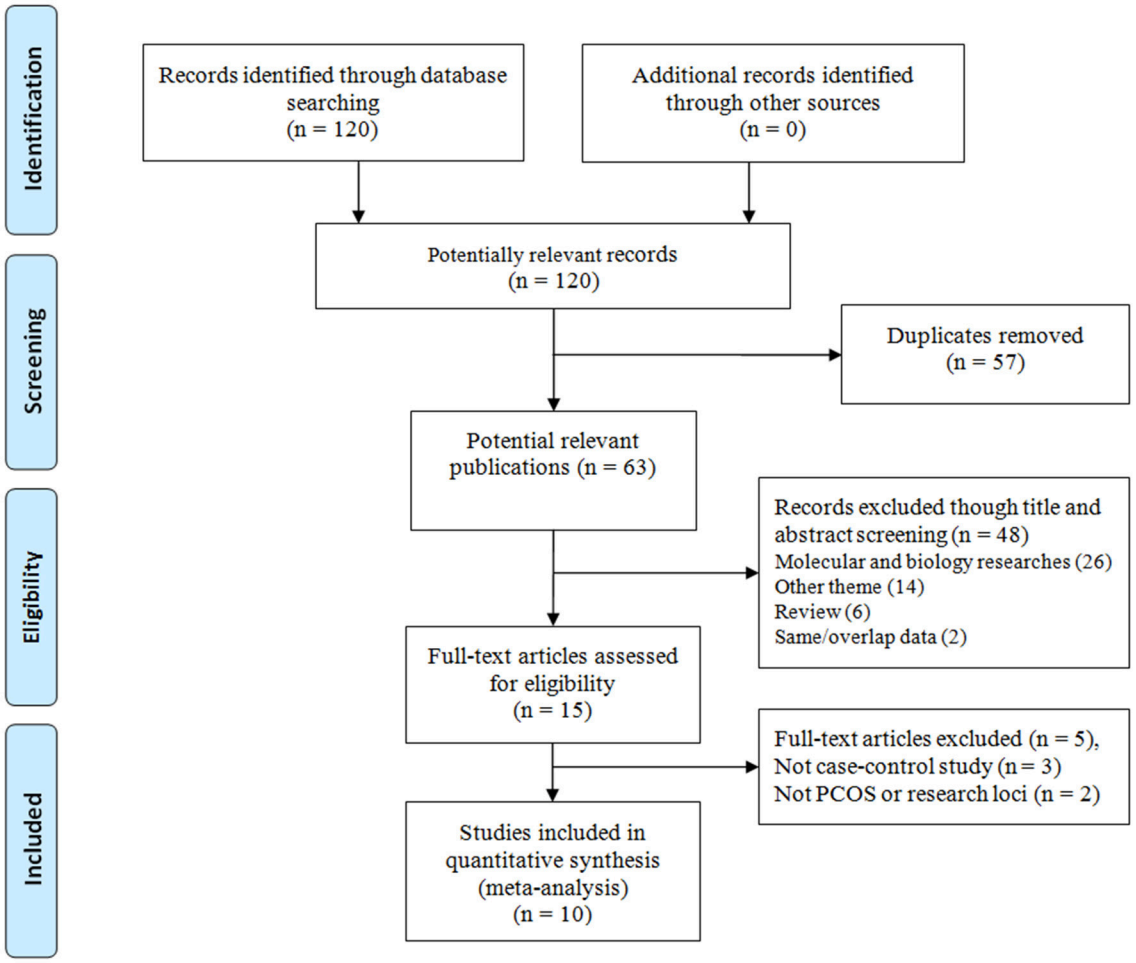

FIGURE 1 | Flow diagram of the study selection process.

I^2 = 36.4%; TT vs. CC+CT: OR = 0.86, 95%CI = 0.63–1.18, $P = 0.34$, $I^2 = 23.6\%$) (**Table 3**, **Figure 2A** for TT vs. CC model). Heterogeneity was only indentified in allele contrast and the meta-regression analyses did not find any distinct factors that contributed to the heterogeneity. Furthermore, no significant association was identified in stratified analysis of HWE status, ethnicity difference, and control design and genotyping methods (**Table 3**). Cumulative analyses by publication date showing the negative results according to the new studies were added (**Figure 2B** for TT vs. CC model). Sensitivity analysis presented a consistent tendency of negative results without any apparent changes (**Figure 2C** for TT vs. CC model). Publication bias was assessed using the Egger's bias test and Begg's funnel plot tests, and no significant asymmetrical evidence was found (T vs. C: $P = 0.23$; CT vs. CC: $P = 0.20$; TT vs. CC: $P = 0.33$; CT+TT vs. CC: $P = 0.27$; TT vs. CC+CT: $P = 0.37$) (**Figure 2D** for TT vs. CC model).

BsmI A/G Locus and PCOS Risk

Seven case-control studies with 1,085 PCOS cases and 728 control individuals were identified on the association between BsmI A/G locus and PCOS risk. Overall, the pool analysis did not find any significant association between this locus on PCOS risk in five genetic models (G vs. A: OR = 1.17, 95%CI = 0.95–1.45, $P = 0.14$, $I^2 = 49.6\%$; AG vs. AA: OR = 1.15, 95%CI = 0.75–1.78, $P = 0.52$, $I^2 = 59.9\%$; GG vs. AA: OR = 1.28, 95%CI = 0.95–1.74, $P = 0.11$, $I^2 = 35.7\%$; AG+GG vs. AA: OR = 1.22, 95%CI = 0.82–1.81, $P = 0.34$, $I^2 = 57.1\%$; GG vs. AA+AG: OR = 1.16, 95%CI = 0.93–1.45, $P = 0.18$, $I^2 = 19.8\%$) (**Table 3**, **Figure 3A** for GG vs. AA model). Heterogeneity was observed in allele contrast, heterozygote model and dominant model. Meta-regression analyses were conducted, and the results indicated that the ethnicity diversity maybe the critical factors contributing to the existed heterogeneity (G vs. A: $P_{ethnicity} = 0.04$; AG vs. AA: $P_{ethnicity} = 0.04$; AG+GG vs. AA: $P_{ethnicity} = 0.03$). In addition, the subgroup of ethnicity proved that the heterogeneity was alleviated in the Asian and Caucasian subgroups apparently. Furthermore, the subgroup analyses based on ethnicity presented an increased risk in Asian populations in some genetic models (G vs. A: OR = 1.62, 95%CI = 1.24–2.11, $P < 0.01$, $I^2 = 0\%$; AG vs. AA: OR = 2.08, 95%CI = 1.26–3.20, $P < 0.01$, $I^2 = 0\%$; GG vs. AA: OR = 2.21, 95%CI = 1.29–3.77, $P < 0.01$,

TABLE 2 | Characteristics of included studies on vitamin D receptor gene polymorphisms and polycystic ovary syndrome risk.

References	Country/region	Racial	Source of controls	Case	Control	Genotype distribution						Genotyping methods	*P* for HWE	MAF in control	NOS
						Case			Control						
						Fok I C/T (rs10735810)									
						CC	**CT**	**TT**	**CC**	**CT**	**TT**				
Mahmoudi, 2009	Iran	Caucasian	Hospital-B	162	162	83	67	12	96	59	7	PCR-RFLP	0.58	0.23	8
Wehr et al., 2011	Austria	Caucasian	Hospital-B	538	135	215	241	82	53	60	22	Genotyping assay	0.47	0.39	8
Bagheri et al., 2012	Iran	Caucasian	Healthy-B	46	46	22	20	4	29	15	2	PCR-RFLP	0.97	0.21	7
Jedrzejuk et al., 2015	Poland	Caucasian	Healthy-B	90	98	28	51	11	23	50	25	PCR- SNaPshot	0.84	0.51	7
Dasgupta et al., 2015	India	Asian	Healthy-B	250	250	155	85	10	153	82	15	PCR-RFLP	0.37	0.22	8
Mahmoudi et al., 2015	Iran	Caucasian	Healthy-B	35	35	16	17	2	24	10	1	PCR-RFLP	0.97	0.17	8
Cao and Tu, 2016	China	Asian	Hospital-B	120	120	70	40	10	65	45	10	PCR-RFLP	0.60	0.27	7
						BsmI A/G (rs1544410)									
						AA	**AG**	**GG**	**AA**	**AG**	**GG**				
Mahmoudi, 2009	Iran	Caucasian	Hospital-B	162	162	24	85	53	18	91	53	PCR-RFLP	0.02	0.61	8
Wehr et al., 2011	Austria	Caucasian	Hospital-B	537	137	77	244	216	22	66	49	Genotyping assay	0.98	0.60	8
Bagheri et al., 2012	Iran	Caucasian	Healthy-B	46	46	15	27	4	20	24	2	PCR-RFLP	0.12	0.30	7
Jedrzejuk et al., 2015	Poland	Caucasian	Healthy-B	90	98	14	45	31	13	42	43	PCR- SNaPshot	0.59	0.65	7
Mahmoudi et al., 2015	Iran	Caucasian	Healthy-B	35	35	10	12	13	5	23	7	PCR-RFLP	0.06	0.53	8
Cao and Tu, 2016	China	Asian	Hospital-B	120	120	23	60	37	40	55	25	PCR-RFLP	0.45	0.44	7
Siddamalla et al., 2018	India	Asian	Hospital-B	95	130	35	45	15	72	41	17	PCR-RFLP	0.01	0.29	7
						ApaI A/C (rs7975232)									
						AA	**AC**	**CC**	**AA**	**AC**	**CC**				
Mahmoudi, 2009	Iran	Caucasian	Hospital-B	162	162	58	68	36	49	90	23	PCR-RFLP	0.07	0.42	8
Wehr et al., 2011	Austria	Caucasian	Hospital-B	543	145	142	274	127	48	60	37	Genotyping assay	0.04	0.46	7
El-Shal et al., 2013	Egypt	Caucasian	Healthy-B	150	150	63	65	22	68	64	18	PCR-RFLP	0.62	0.33	8
Jedrzejuk et al., 2015	Poland	Caucasian	Healthy-B	90	98	19	52	19	32	49	17	PCR- SNaPshot	0.81	0.42	7
Dasgupta et al., 2015	India	Asian	Healthy-B	250	250	117	120	13	122	116	12	PCR-RFLP	0.02	0.28	7
Mahmoudi et al., 2015	Iran	Caucasian	Healthy-B	35	35	15	11	9	8	21	6	PCR-RFLP	0.23	0.47	8
Cao and Tu, 2016	China	Asian	Hospital-B	120	120	22	58	40	39	55	26	PCR-RFLP	0.43	0.45	7
Siddamalla et al., 2018	India	Asian	Hospital-B	95	130	42	21	32	70	35	25	PCR-RFLP	<0.01	0.33	7
						TaqI T/C (rs731236)									
						TT	**TC**	**CC**	**TT**	**TC**	**CC**				
Mahmoudi, 2009	Iran	Caucasian	Hospital-B	162	162	71	71	20	72	76	14	PCR-RFLP	0.33	0.32	8
Wehr et al., 2011	Austria	Caucasian	Hospital-B	536	137	226	238	72	49	65	23	Genotyping assay	0.85	0.41	8
Bagheri et al., 2013	Iran	Caucasian	Healthy-B	38	38	16	14	8	17	19	2	PCR-RFLP	0.26	0.30	7
El-Shal et al., 2013	Egypt	Caucasian	Healthy-B	150	150	40	74	36	69	61	20	PCR-RFLP	0.27	0.34	8
Jedrzejuk et al., 2015	Poland	Caucasian	Healthy-B	90	98	37	45	8	49	37	12	PCR- SNaPshot	0.24	0.31	7
Dasgupta et al., 2015	India	Asian	Healthy-B	250	250	112	91	47	109	104	37	PCR-RFLP	0.14	0.36	8
Mahmoudi et al., 2015	Iran	Caucasian	Healthy-B	35	35	15	14	6	15	16	4	PCR-RFLP	0.93	0.34	8
Cao and Tu, 2016	China	Asian	Hospital-B	120	120	57	52	11	40	72	8	PCR-RFLP	<0.01	0.37	6
Siddamalla et al., 2018	India	Asian	Hospital-B	95	130	40	31	24	71	42	17	PCR-RFLP	0.01	0.29	7

HWE in control. MAF, Minor allele frequency in control group; Hospital-B, Hospital-based control; Healthy-B, Healthy-based control.

TABLE 3 | Summary ORs and 95% CI of vitamin D receptor gene polymorphisms and polycystic ovary syndrome risk.

Locus	N*	OR	95% CI	*P*	I^2	OR	95% CI	*P*	I^2	OR	95% CI	*P*	I^2	OR	95% CI	*P*	I^2	OR	95% CI	*P*	I^2
Fok I C/T (rs10735810)			**T vs. C**				**CT vs. CC**				**TT vs. CC**				**CT+TT vs. CC**				**TT vs. CC+CT**		
Total	7	1.04	0.83–1.30	0.77	53.2	1.08	0.89–1.32	0.42	7.0	0.89	0.64–1.25	0.50	35.6	1.06	0.88–1.27	0.56	36.4	0.86	0.63–1.18	0.34	23.6
ETHNICITY																					
Caucasian	5	1.14	0.80–1.60	0.47	66.6	1.18	0.92–1.53	0.20	19.3	1.07	0.52–2.19	0.86	54.1	1.22	0.84–1.77	0.31	49.5	0.96	0.53–1.76	0.91	45.0
Asian	2	0.91	0.72–1.16	0.46	0	0.95	0.70–1.30	0.77	0	0.77	0.41–1.43	0.40	0	0.92	0.69–1.24	0.60	0	0.79	0.43–1.45	0.44	0
DESIGN																					
Hospital-B	3	1.04	0.86–1.27	0.65	28.1	1.04	0.80–1.36	0.75	0.0	1.07	0.70–1.56	0.76	0	1.05	0.82–1.36	0.68	15.9	1.06	0.71–1.59	0.78	0
Healthy-B	4	1.08	.0.69–1.69	0.72	68.7	1.14	0.85–1.52	0.39	33.8	0.78	0.33–1.83	0.56	45.1	1.19	0.72–1.97	0.50	57.5	0.62	0.37–1.03	0.07	23.7
GENOTYPING																					
PCR-RFLP	5	1.17	0.89–1.54	0.26	45.9	1.15	0.91–1.46	0.27	26.6	1.14	0.70–1.84	0.59	11.6	1.22	0.88–1.68	0.24	42.3	1.09	0.68–1.75	0.72	0
Other	2	0.82	0.56–1.19	0.30	57.7	0.95	0.67–1.35	0.76	0	0.62	0.25–1.53	0.30	66.6	0.88	0.63–1.23	0.47	0	0.65	0.29–1.44	0.28	66.5
BsmI A/G (rs1544410)			**G vs. A**				**AG vs. AA**				**GG vs. AA**				**AG+GG vs. AA**				**GG vs. AA+AG**		
Total	7	1.17	0.95–1.45	0.14	49.6	1.15	0.75–1.78	0.52	59.9	1.28	0.95–1.74	0.11	35.7	1.22	0.82–1.81	0.34	57.1	1.16	0.93–1.45	0.18	19.8
HWE-yes	5	1.17	0.90–1.51	0.24	45.2	1.10	0.67–1.82	0.70	50.8	1.38	0.96–2.00	0.09	36.9	1.19	0.76–1.89	0.48	47.5	1.26	0.84–1.89	0.27	42.2
HWE-no	2	1.20	0.70–2.07	0.50	78.2	1.27	0.40–4.01	0.68	84.7	1.15	0.48–2.72	0.76	61.2	1.26	0.43–3.64	0.67	84.1	1.06	0.72–1.58	0.16	0
ETHNICITY																					
Caucasian	5	1.02	0.86–1.21	0.84	0.0	0.90	0.64–1.26	0.54	31.4	0.99	0.68–1.44	0.95	0.0	0.94	0.68–1.30	0.72	11.7	1.08	0.84–1.39	0.57	27.2
Asian	2	1.62	1.24–2.11	<0.01	0.0	2.08	1.26–3.20	<0.01	0	2.21	1.29–3.77	<0.01	0.0	2.12	1.42–3.16	<0.01	0	1.51	0.95–2.39	0.08	0
DESIGN																					
Hospital-B	4	1.26	0.97–1.64	0.08	60.1	1.35	0.81–2.24	0.25	64.5	1.43	0.87–2.35	0.16	51.3	1.41	0.86–2.29	0.17	65.5	1.22	0.95–1.57	0.12	0
Healthy-B	3	0.96	0.71–1.31	0.82	20.8	0.82	0.33–2.00	0.66	59.8	0.90	0.45–1.78	0.76	0	0.90	0.45–1.80	0.76	40.0	1.28	0.49–3.30	0.62	58.9
GENOTYPING																					
PCR-RFLP	5	1.29	0.99–1.99	0.06	46.3	1.17	0.62–2.20	0.63	71.6	1.49	0.85–2.60	0.16	40.7	1.29	0.74–2.25	0.37	66.9	1.30	0.97–1.80	0.07	0
Other	2	0.98	0.68–1.41	0.89	55.3	1.04	0.65–1.65	0.87	0	1.04	0.65–1.69	0.86	28.1	1.04	0.67–1.61	0.85	0	0.94	0.53–1.66	0.84	62.2
ApaI A/C (rs7975232)			**C vs. A**				**AC vs. AA**				**CC vs. AA**				**AC+CC vs. AA**				**CC vs. AA+AC**		
Total	8	1.20	1.06–1.35	<0.01	29.7	1.10	0.80–1.49	0.56	59.7	1.49	1.17–1.91	<0.01	0	1.21	0.94–1.57	0.15	51.3	1.36	1.09–1.69	0.01	12.8
HWE-yes	5	1.22	1.03–1.45	0.02	32.6	1.00	0.58–1.72	0.99	72.6	1.61	1.13–2.27	0.01	0	1.14	0.70–1.83	0.60	68.8	1.55	1.15–2.10	<0.01	0
HWE-no	3	1.21	0.95–1.54	0.13	49.0	1.21	0.94–1.56	0.14	0	1.38	0.97–1.97	0.07	18.1	1.26	1.00–1.59	0.06	0	1.25	0.71–2.20	0.44	62.7
ETHNICITY																					
Caucasian	5	1.11	0.95–1.30	0.18	0	0.98	0.60–1.62	0.95	72.6	1.28	0.94–1.74	0.12	0	1.07	0.74–1.57	0.71	58.0	1.20	0.91–1.57	0.20	0
Asian	3	1.41	1.02–1.95	0.04	63.9	1.19	0.90–1.57	0.23	18.9	1.97	1.30–2.97	<0.01	22.8	1.42	0.96–2.10	0.08	48.6	1.72	1.19–2.50	<0.01	0

(Continued)

TABLE 3 | Continued

Locus	N*	OR	95% CI	P	I^2 (%)[a]	OR	95% CI	P	I^2 (%)[a]	OR	95% CI	P	I^2 (%)[a]	OR	95% CI	P	I^2 (%)[a]	OR	95% CI	P	I^2 (%)[a]
DESIGN																					
Hospital-B	4	1.31	1.02–1.67	0.03	55.8	1.16	0.71–1.89	0.56	68.9	1.59	1.17–2.15	<0.01	38.1	1.33	0.90–1.97	0.16	61.1	1.50	0.98–2.30	0.06	59.9
Healthy-B	4	1.11	0.93–1.34	0.24	0	1.03	0.64–1.64	0.91	60.5	1.31	0.85–2.02	0.22	0	1.10	0.74–1.62	0.64	49.9	1.26	0.85–1.87	0.25	0
GENOTYPING																					
PCR-RFLP	5	1.22	1.00–1.49	0.05	45.4	0.95	0.66–1.36	0.78	58.5	1.60	1.18–2.16	<0.01	3.8	1.10	0.80–1.54	0.52	57.8	1.63	1.24–2.15	<0.01	0
Other	2	1.17	0.94–1.46	0.16	0	1.61	1.12–2.32	0.01	0	1.30	0.85–2.00	0.22	0	1.50	1.07–2.10	0.02	0	0.98	0.68–1.41	0.89	0
TaqI T/C (rs731236)			**C vs. T**				**TC vs. TT**				**CC vs. TT**				**TC+CC vs. TT**				**CC vs. TT+TC**		
Total	9	1.16	0.94–1.44	0.18	66.5	1.00	0.75–1.34	0.99	58.5	1.44	0.97–2.15	0.07	54.2	1.10	0.82–1.48	0.52	65.4	1.37	1.09–1.74	0.01	38.3
HWE-yes	7	1.16	0.93–1.46	0.19	61.3	1.07	0.79–1.45	0.66	51.2	1.39	0.87–2.22	0.17	57.8	1.15	0.85–1.56	0.38	58.1	1.31	0.90–1.90	0.16	43.4
HWE-no	2	1.15	0.52–2.53	0.73	88.0	0.81	0.32–2.05	0.65	81.1	1.65	0.65–4.18	0.29	56.3	0.95	0.33–2.80	0.93	87.9	1.91	1.09–3.33	0.02	0
ETHNICITY																					
Caucasian	6	1.20	0.89–1.60	0.23	67.2	1.13	0.79–1.63	0.50	54.2	1.46	0.79–2.71	0.23	64.8	1.20	0.82–1.76	0.34	62.8	1.33	0.81–2.17	0.26	52.5
Asian	3	1.11	0.74–1.67	0.60	76.4	0.82	0.51–1.32	0.41	63.0	1.45	0.99–2.12	0.06	36.9	0.95	0.55–1.65	0.86	75.9	1.55	1.08–2.22	0.02	0
DESIGN																					
Hospital-B	4	1.03	0.74–1.44	0.85	74.5	0.83	0.59–1.17	0.29	48.0	1.23	0.66–2.27	0.52	63.5	0.91	0.60–1.38	0.66	68.0	1.33	0.79–2.22	0.28	54.0
Healthy-B	5	1.31	1.00–1.70	0.05	48.0	1.21	0.78–1.87	0.40	57.9	1.70	0.98–2.96	0.06	46.0	1.33	0.89–1.98	0.16	56.4	1.51	1.09–2.08	0.01	30.8
GENOTYPING																					
PCR-RFLP	7	1.25	0.97–1.60	0.08	64.5	0.98	0.69–1.40	0.91	61.8	1.74	1.30–2.33	<0.01	27.3	1.14	0.79–1.63	0.50	68.5	1.68	1.28–2.22	<0.01	0
Other	2	0.89	0.71–1.13	0.34	38.9	1.09	0.55–2.17	0.81	71.8	0.73	0.44–1.19	0.20	0	1.01	0.55–1.86	0.98	68.1	0.75	0.48–1.18	0.22	0

**Numbers of comparisons.*

Hospital-B, Hospital-based control; Healthy-B, Healthy-based control; RT-PCR, Real-time PCR.

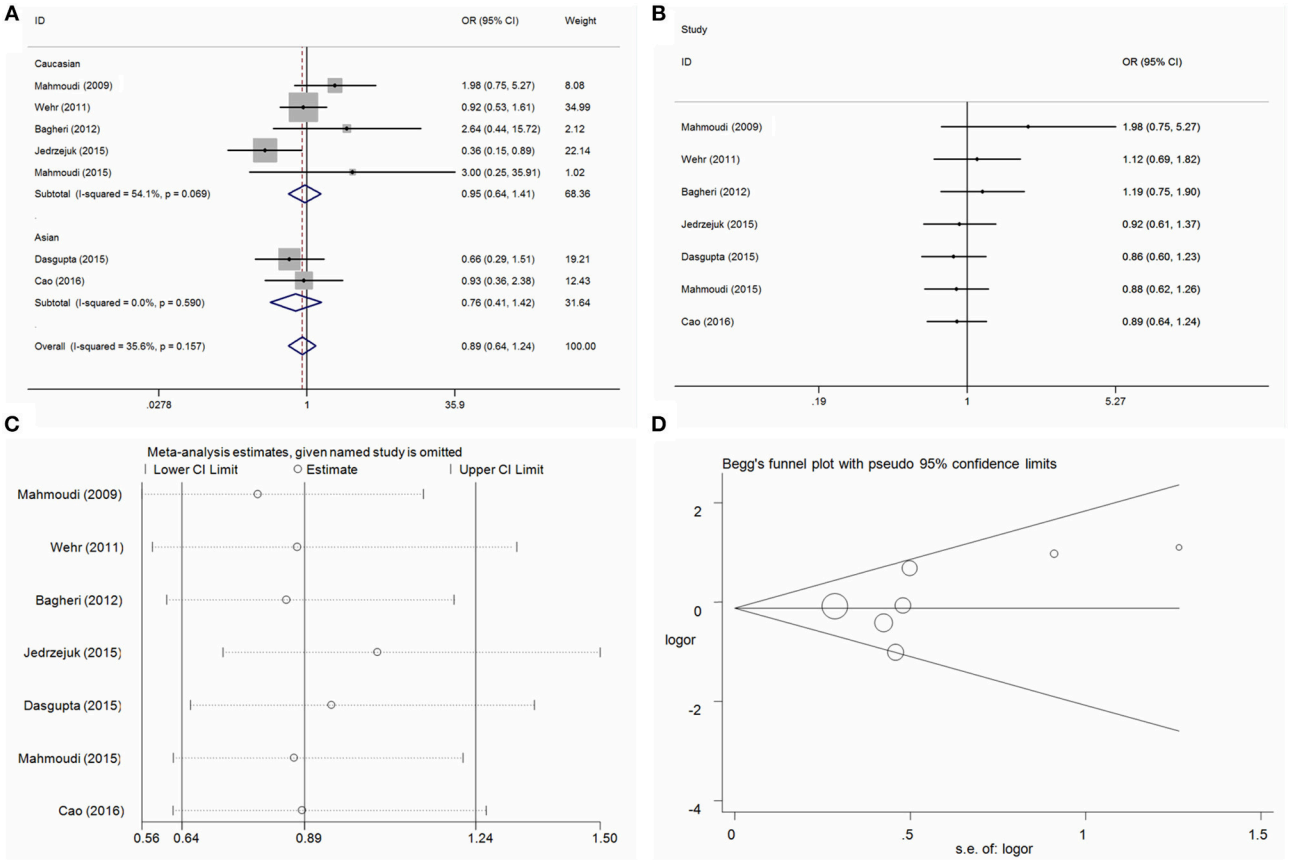

FIGURE 2 | Statistical analysis of the association between VDR Fok I C/T polymorphism and PCOS risk in the TT vs. CC model. **(A)** ORs and 95% CIs; **(B)** cumulative analysis; **(C)** sensitivity analysis; **(D)** publication bias.

I^2 = 0%; AG+GG vs. AA: OR = 2.12, 95%CI = 1.42–3.16, $P < 0.01$, I^2 = 0%). Cumulative analyses by publication date were conducted and indicated apparent consistence and stability of pool results (**Figure 3B** for GG vs. AA model). Sensitivity analysis was conducted and indicated some changes of results in allele contrast, homozygote, and recessive models without the publication by Jedrzejuk et al. (2015) (**Figure 3C** for GG vs. AA model). Publication bias was assessed using the Egger bias test and a Begg funnel plot test, and no significant asymmetrical evidence was found (T vs. C: $P = 0.82$; CT vs. CC: $P = 0.17$; TT vs. CC: $P = 0.94$; CT+TT vs. CC: $P = 0.19$; TT vs. CC+CT: $P = 0.36$) (**Figure 3D** for GG vs. AA model).

ApaI A/C Locus and PCOS Risk

Eight case-control studies with 1,445 cases and 1,090 controls individuals were identified on the association between ApaI A/C locus and PCOS risk. Overall, significant association of increased risk were observed in three genetic models (C vs. A: OR = 1.20, 95%CI = 1.06–1.35, $P = 0.01$, I^2 = 29.7%; CC vs. AA: OR = 1.49, 95%CI = 1.17–1.91, $P < 0.01$, I^2 = 0%; CC vs. AA+AC: OR = 1.36, 95%CI = 1.09–1.69, $P = 0.01$, I^2 = 12.8%) (**Table 3**, **Figure 4A** for CC vs. AA model). Heterogeneity was observed in heterozygote model (AC vs. AA) and dominant model (AC+CC vs. AA), and the meta-regression analyses did not find any distinct factors that contributed to the heterogeneity. Subgroup analyses by ethnicity presented an increased risk in Asian populations in the genetic models mentioned (C vs. A: OR = 1.22, 95%CI = 1.03–1.45, $P = 0.02$, I^2 = 32.6%; CC vs. AA: OR = 1.61, 95%CI = 1.13–2.27, $P < 0.01$, I^2 = 0%; CC vs. AA+AC: OR = 1.55, 95%CI = 1.15–2.10, $P = 0.01$, I^2 = 0%). Moreover, the same significant PCOS risk was observed in some genetic models in these subgroups of HWE-yes, hospital control and genotyping groups (**Table 3**). Cumulative analyses demonstrated a significant alteration when the study of Cao and Tu (2016) was added in 2016 (**Table 2**, **Figure 4B** for CC vs. AA model). Sensitivity analysis was conducted in every genetic model and did not indicate some apparent changes except for the dominant modes (**Figure 4C** for CC vs. AA model). Publication bias was assessed using the Egger bias test and a Begg's funnel plot test, and no significant asymmetrical evidence was found (C vs. A: $P = 0.74$; AC vs. AA: $P = 0.55$; CC vs. AA: $P = 0.97$; AC+CC vs. AA: $P = 0.86$; CC vs. AA+AC: $P = 0.37$) (**Figure 4D** for CC vs. AA model).

TaqI T/C Locus and PCOS Risk

Nine case-control studies with 1,476 cases and 1,120 controls individuals were identified on the association between TaqI T/C

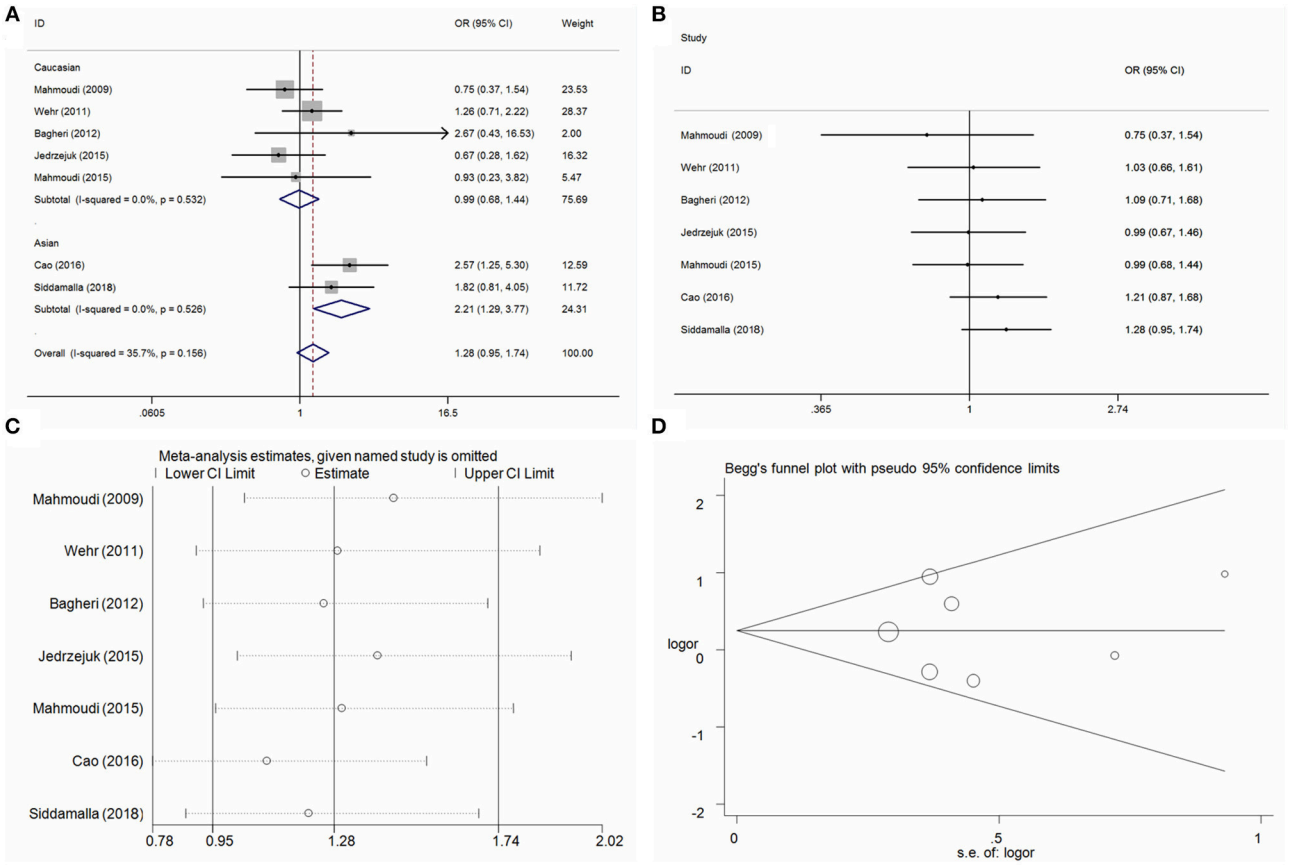

FIGURE 3 | Statistical analysis of the association between VDR BsmI A/G polymorphism and PCOS risk in the GG vs. AA model. **(A)** ORs and 95% CIs; **(B)** cumulative analysis; **(C)** sensitivity analysis; **(D)** publication bias.

locus and PCOS risk. Overall, the increased risk was observed only in the recessive model (CC vs. TT+TC: OR = 1.37, 95%CI = 1.09-1.74, $P = 0.01$, I^2 =38.3%) (**Table 3**, **Figure 5A** for CC vs. TT model). Heterogeneities were identified in the allele contrast (C vs. T), heterozygote model (TC vs. TT), homozygote (CC vs. TT), and dominant model (TC+CC vs. TT). Meta-regression analyses only found that the genotyping methods contributed to the existing heterogeneity in the dominant model, but not in other models. Subgroup analyses revealed an increased PCOS risks in Asian populations (CC vs. TT+TC: OR = 1.55, 95%CI = 1.08-2.22, $P = 0.02$, I^2 =0%) and other subgroups in the recessive model (**Table 3**). Cumulative analyses by publication date demonstrated a negative association except for the recessive model (**Figure 5B** for CC vs. TT model). Sensitivity analysis indicated some slight alterations when the studies of Wehr et al. (2011) and El-Shal et al. (2013) were deleted in the homozygote and recessive models, respectively (**Figure 5C** for CC vs. TT model). Publication bias was assessed using the Egger bias test and a Begg funnel plot test, and no significant asymmetrical evidence was found (C vs. T: $P = 0.48$; TC vs. TT: $P = 0.74$; CC vs. TT: $P = 0.49$; TC+CC vs. TT: $P = 0.62$; CC vs. TT+TC: $P = 0.38$) (**Figure 5D** for CC vs. TT model).

DISCUSSION

To date, the pathogenesis and etiology of PCOS have remained unknown. The complex gene-gene and gene-environment interactions have been reported to be an important risk for PCOS development. Consistent epidemiologic evidence demonstrated that PCOS always suffered a series of complications, comprising hyperandrogenism, oligo-anovulation, insulin resistance and associated metabolic abnormalities.

Many studies proved that a dysregulated vitamin D serum level is closely related to PCOS occurrence. In addition, the vitamin D supplementation therapy would decreases fasting plasma glucose, serum insulin concentrations, and homeostasis model assessment-estimated insulin resistance (HOMA-IR) (Asemi et al., 2015; Foroozanfard et al., 2017). All these evidences suggested that vitamin D disorder is associated with multiple metabolic risks in women with PCOS.

Noteworthily, the $1\alpha,25(OH)_2D_3$ is an important active form of vitamin D, it is mediated by the vitamin D receptor [$1\alpha,25(OH)_2D_3$ receptor, VDR] (Yoshizawa et al., 1997). Vitamin D receptor (VDR) is a DNA-binding transcription factor, combined with a heterodimer of the $1a,25(OH)_2D_3$-ligand VDR and unoccupied RXR to generate an active signal transduction

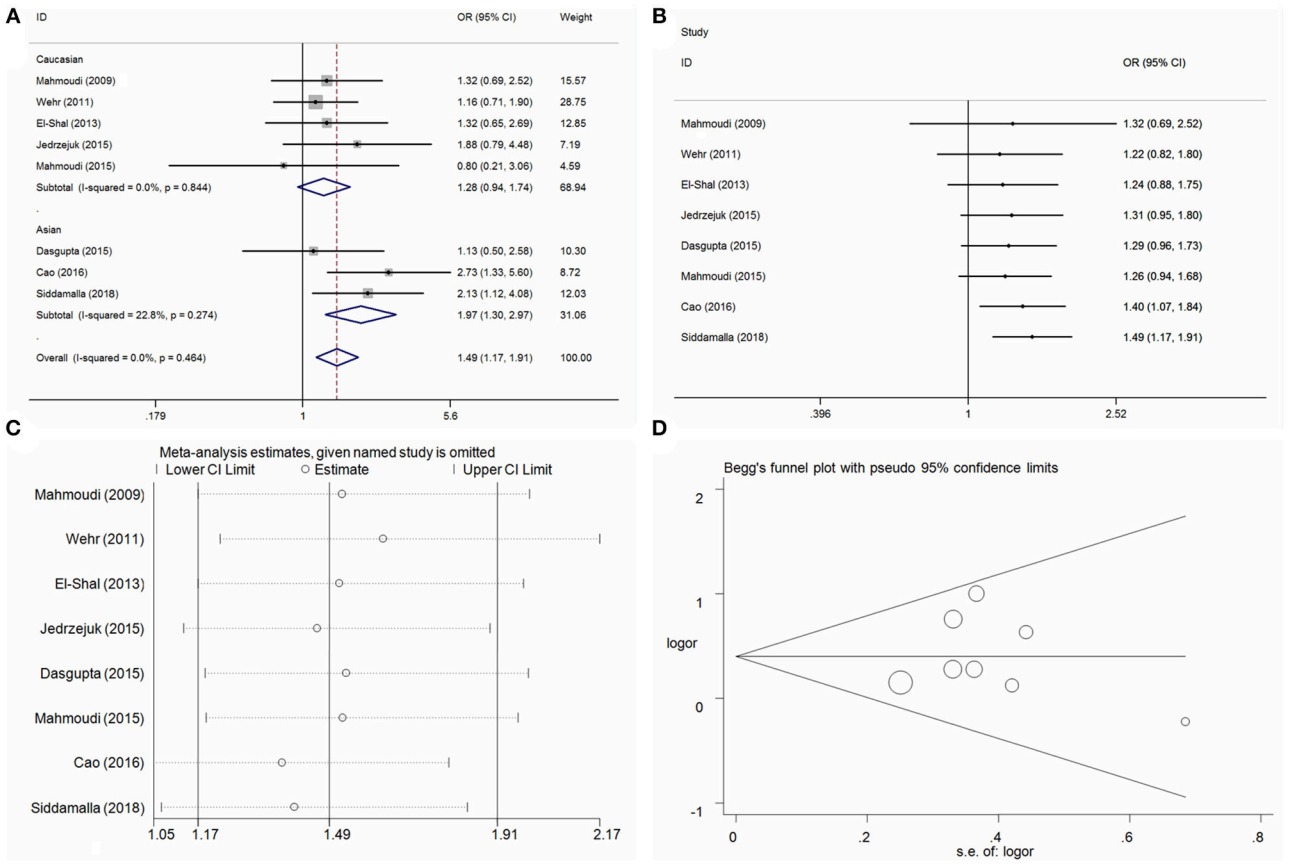

FIGURE 4 | Statistical analysis of the association between VDR ApaI A/C polymorphism and PCOS risk in the CC vs. AA model. **(A)** ORs and 95% CIs; **(B)** cumulative analysis; **(C)** sensitivity analysis; **(D)** publication bias.

complex (Haussler et al., 2011). To date, several functional SNP loci reported in these polymorphisms presented an increased susceptibility of various diseases (Valdivielso and Fernandez, 2006), such as multiple cancers (Vidigal et al., 2017), diabetes mellitus (Yu et al., 2017), rheumatoid arthritis (Tizaoui et al., 2014), and cardiocerebrovascular disease (Moradi et al., 2017).

In 2009, Mahmoudi et al. published the first case-control study to explore the association between the above four polymorphisms and PCOS susceptibility in the Iranian population, and the results suggested that these individuals with an CC genotype have an increased risk for PCOS compared with the AA genotype. Since then, a series of case-control studies was conducted to evaluate the association between the vitamin D polymorphisms and PCOS susceptibility, but some controversies arose and bewildered us completely. In 2017, Reis et al. published a system review on vitamin D polymorphisms and PCOS with most literature on this theme (Reis et al., 2017). However, the synthesis and calculation of all selected data were not conducted. We also made a comprehensive understanding of all the studies but failed to draw a clear conclusion. Thus, we conducted the meta-analysis to investigate the precise relationships between VDR Fok I C/T, BsmI A/G, ApaI A/C, and TaqI T/C polymorphisms and PCOS risk based on 10 published case-control studies.

In the current meta-analysis, the pooled results indicated some significant association between ApaI A/C polymorphism and PCOS susceptibility in allele contrast, homozygote genotype and recessive models, presenting 1.20-, 1.49- and 1.36-fold high risk for PCOS. Furthermore, an increased PCOS risk was observed in the subgroup analysis of the HWE-yes group and hospital based group, especially in the Asian group. In BsmI A/G polymorphism, only some increased PCOS risks were observed in the Asians based on ethnic diversity. These pieces of evidence demonstrated that the ethnicity differences may play an important role, contributing to the varying PCOS susceptibility among the Asian and Caucasian races. In addition, no signification association was observed in TaqI T/C and Fok I C/T polymorphisms for PCOS risk, except for a few scattered cases of increased PCOS risk in the former in the recessive models.

The restriction fragment length polymorphism sties of BsmI and ApaI are located in the intron (between exons 8 and 9), and the TaqI polymorphism was located in exon 9 (Zmuda et al., 2000). They are all located near the 3'-untranslated region of the VDR gene, which was suggested to be involved in the regulation of gene expression by modulating mRNA stability (Zmuda et al., 2000; Ogunkolade et al., 2002). In

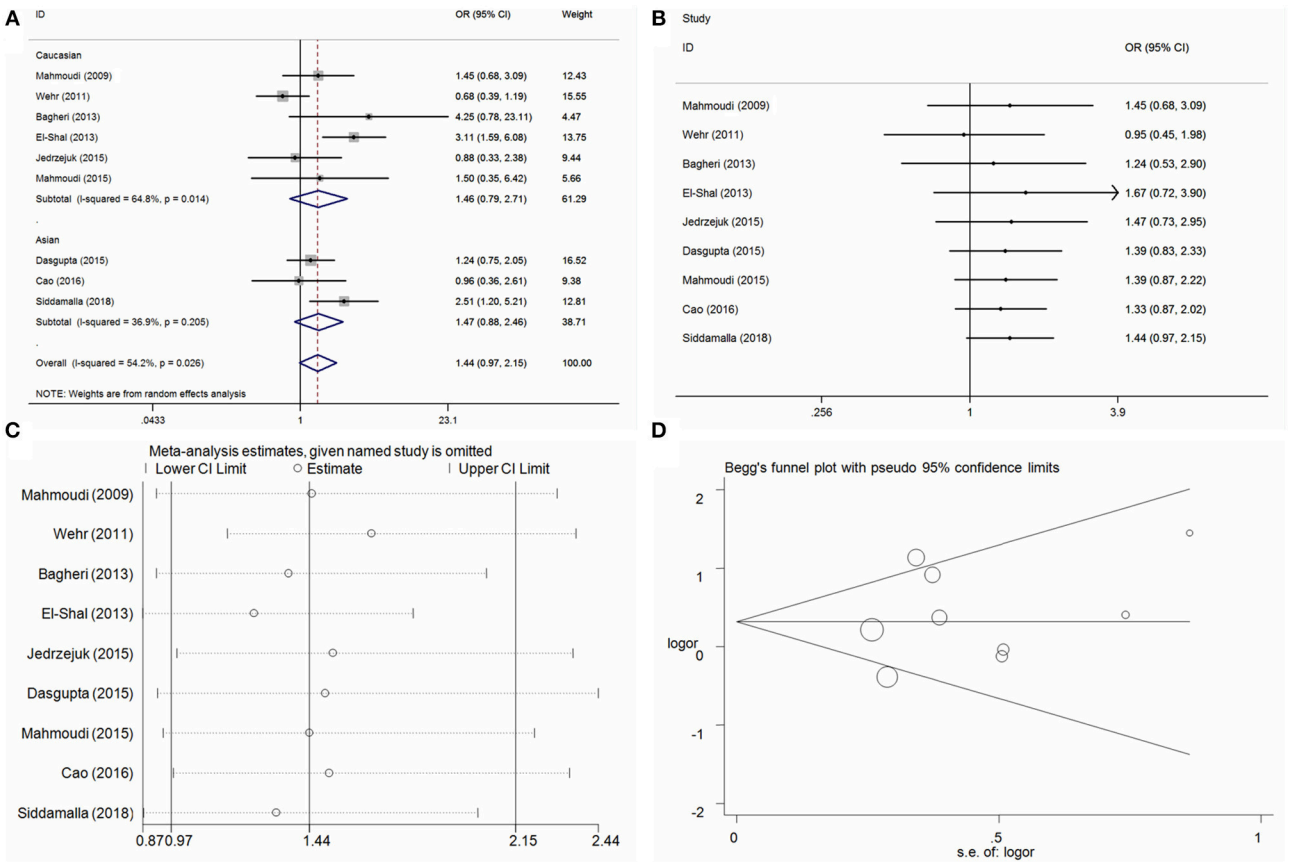

FIGURE 5 | Statistical analysis of the association between VDR TaqI T/C polymorphism and PCOS risk in the CC vs. TT model. **(A)** ORs and 95% CIs; **(B)** cumulative analysis; **(C)** sensitivity analysis; **(D)** publication bias.

this meta-analysis, some increased and significant risks were observed in the above three polymorphism, indicating that the potential synergism among these polymorphisms would play an important role for PCOS occurrence. Regrettably, this hypothesis could not be verified without valid haplotype data to assess interaction between the adjacent polymorphism loci with all included studies. FokI polymorphism located in exon 2, resulting in a incorporation VDR protein production in the NH2 terminal, which was suggested to influence the transcriptional activity of VDR gene combined with the modulation of transcription factor IIB (Jurutka et al., 2000; Whitfield et al., 2001). Some publications indicated that this polymorphism would regulate the expression of mRNA and contribute to susceptibility to various diseases (Arai et al., 1997; Colombini et al., 2014), but no significant association between FokI polymorphism and PCOS was found based on the current meta-analysis. So, all these evidences indicated that there were a causation between the mutation of the above SNPs located in VDR gene and PCOS occurrence (Hill, 1965).

To our knowledge, this is the first meta-analysis to assess the association between VDR polymorphisms and PCOS risk. Some advantages were presented in this meta-analysis compared with the published case-control studies: First, all case-control studies published on the four polymorphisms were considered, and the risk assessment of bias within studies would enhance the statistical power and help understand the association between VDR polymorphisms and PCOS risk. Second, a stratified analysis based on ethnic diversity, control design, and genotyping methods was conducted to explore the potential relationships that were modulated under these subgroup biologic factors. Third, a scientific retrieval strategy and rigorous methodology were used, including cumulative analyses and sensitivity analyses. Publication bias was also used to guarantee the stability and credibility of the conclusions of the analysis.

However, there were some limitations of this study, which should be pointed out. First, only 10 publications were included in this present meta-analysis. The studies and sample size of each polymorphic locus were limited, and the pooled results and subgroup analysis could not reveal the reality association between VDR polymorphisms and PCOS susceptibility. Second, interactive risk factors, such as living habits, diet, age, and family history were not adjusted in this meta-analysis due to data deficiency. Third, included studies were written only in English and Chinese, and the included subjects were mostly Asian and Caucasian populations. Therefore, the results of this meta-analysis cannot represent all ethnic populations, and the application of the conclusions was restricted. Fourth, all examined polymorphisms were assessed separately, and the

gene-gene interactions especially the haplotype analyses were not assessed due to the insufficient data.

In conclusion, this meta-analysis suggests that VDR gene polymorphisms play an important role in PCOS development, especially on the ApaI A/C and BsmI A/G among the Asian populations. Further case-control studies on various ethnic populations with a larger sample size are need to verify the current conclusions in the future.

AUTHOR CONTRIBUTIONS

Y-MN, Y-DW, and Y-YH conceived the study. Y-MN and Y-DW searched the databases and extracted the data. X-FL, G-BJ, and GB analyzed the data. Y-MN, Y-YH, and H-BC wrote the draft of the paper. Y-DW and MS reviewed the manuscript. All the authors approved the final manuscript.

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REVIEW
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Cardiovascular Health in Children and Adolescents With Congenital Adrenal Hyperplasia Due to 21-Hydroxilase Deficiency

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Increasing evidence indicates that adults with Congenital Adrenal Hyperplasia (CAH) may have a cluster of cardiovascular (CV) risk factors. In addition, ongoing research has highlighted that children and adolescents with CAH are also prone to developing unfavorable metabolic changes, such as obesity, hypertension, insulin resistance, and increased intima-media thickness, which places them at a higher risk of developing CV disease in adulthood. Moreover, CAH adolescents may exhibit subclinical left ventricular diastolic dysfunction and impaired exercise performance, with possible negative consequences on their quality of life. The therapeutic management of patients with CAH remains a challenge and current treatment regimens do not always allow optimal biochemical control. Indeed, overexposure to glucocorticoids and mineralocorticoids, as well as to androgen excess, may contribute to the development of unfavorable metabolic and CV abnormalities. Long-term prospective studies on large cohorts of patients will help to clarify the pathophysiology of metabolic alterations associated with CAH. Meanwhile, further efforts should be made to optimize treatment and identify new therapeutic approaches to prevent metabolic derangement and improve long-term health outcomes of CAH patients.

Keywords: Congenital Adrenal Hyperplasia, cardiovascular risk factors, 21-hydroxilase deficiency, excess androgens, obesity, cardiovascular disease

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INTRODUCTION

Deficiency of 21-hydroxylase (21-OH) is an autosomal recessive disease accounting for about 95% of the cases of Congenital Adrenal Hyperplasia (CAH). It has an incidence estimated at 1/10000-1/20000 live newborns (1). Deletions or mutations of the *CYP21A2* gene, encoding the 21-OH enzyme, impair the enzyme activity at a variable extent, resulting in glucocorticoid and/or mineralocorticoid deficiency, which lead to increased secretion of ACTH, adrenal hyperplasia, and excess production of androgens and steroid precursors before the enzymatic block (1). Based on the residual enzyme activity, CAH shows a continuum of phenotypes, ranging from a severe classic form, usually presenting in infancy, to a non-classic (NC) form, which may be diagnosed from childhood to adult life, because of signs of excess androgens production. The classic form is sub-classified as either salt-wasting (SW) or simple virilising (SV) form, depending on the degree of residual enzyme activity, which influences the amount of mineralocorticoids produced by the adrenal cortex.

Current treatment consists of glucocorticoid and, when necessary, mineralocorticoid substitution to prevent adrenal crises and to suppress the excess androgen production (2). Following the introduction of glucocorticoid treatment in the 1950s, survival of CAH patients has dramatically improved, so that CAH is now recognized as a lifelong chronic disease. However, despite recent advances in its management (i.e., prenatal diagnosis, neonatal screening, improved knowledge on adrenal medulla dysfunction) CAH still has relevant morbidity and mortality (1–4).

The therapeutic spectrum of glucocorticoids is narrow, and patients need an accurate and tailored follow-up to prevent both under- and over-treatment with glucocorticoids and/or mineralocorticoids, which may increase the cardiovascular (CV) risk of CAH patients.

Increasing evidences suggest that adult patients with CAH have a higher risk to develop long-term health problems including cardiovascular diseases, impaired fertility and bone health (3–5). However, signs and symptoms of forerunner conditions of adult disease are already detectable in childhood (6, 7).

The aim of this review is to summarize current data available on traditional and non-traditional CV risk in children and adolescents with CAH.

METHODS

We searched the PubMed database from the National Library of Medicine using the keywords "obesity," "body composition," "hypertension," "lipids," "dyslipidemia," "glucose intolerance," "insulin," "insulin resistance," "endothelial function," "Intima-Media Thickness," "adipokines" "inflammatory cytokines," "blood pressure," "cardiac morphology," "cardiac function" associated with "Congenital Adrenal Hyperplasia," with the limits set to only English-language articles and those involving human subjects. We included all case-control studies, case series and meta-analysis that were published in English from 1992 to date. We excluded studies involving <6 patients.

TRADITIONAL CV RISK FACTORS

Obesity and Body Composition

Several studies from large National cohorts (4, 8–10) indicate that obesity is common in adults with CAH, ranging from 22 to 52% of the subjects (9, 10). An increased prevalence of obesity has also been reported in children with CAH in most studies (8, 11–24) (**Table 1**), although these studies are limited by small patient number and heterogeneity of cohorts and study design. So far, only 3 relatively large cross-sectional studies reporting prevalence of obesity in CAH children are available (8, 16, 25).

In a study involving 170 patients, age range 0.6–17 years, about 35% of children were obese, regardless of the clinical CAH form and the glucocorticoids dose (8). Obese children presented significantly greater concentrations of insulin, leptin, and androstenedione compared to normal and overweight children. Only one patient fulfilled the diagnostic criteria for the metabolic syndrome.

Higher prevalence of obesity (16.8%) compared to the reference population (2.27%) was also reported in a cross-sectional retrospective study on 89 classic CAH patients aged 0.2–17.9 years (16). In this cohort, body mass index (BMI) positively correlated with age, hydrocortisone dose and parental BMI.

Finally, Subbarayan et al. (25) documented a prevalence of obesity of 23.6% among a cohort of 107 classic CAH patients aged 0.4 to 20.5 years. Of note, mean BMI was lower than that reported in a previous health survey from the same center almost 10 years before (21), possibly due to the use of lower glucocorticoid doses in recent years.

Whether the increased prevalence of obesity results from an increased lean body mass (as a possible consequence of excess androgens) or total/regional fat mass has been explored through bioimpedance analysis (11, 26, 27), dual x-ray absorptiometry (DEXA) (12, 15, 28–32), and computer tomography (CT) imaging (33) (**Table 1**). Several studies relying on DEXA documented that either males or females with classic CAH exhibit higher total fat mass (12, 15, 28–30) and reduced lean body mass (29) than controls. Moreover, some studies also documented higher indexes of visceral adiposity, such as waist/hip (11, 15) and waist-to-height ratio (WHtR) (30) with respect to controls.

Only a few data are available regarding NC CAH (27, 28, 32), documenting no significant differences between classic and NC forms. Interestingly, one study reported a higher lean body mass in NC CAH in comparison to matched controls, suggesting an effect of longer exposure to excess androgens (28).

A weak correlation between 6-months cumulative glucocorticoid dose and total fat mass was found only in females (12), whereas duration of treatment was positively correlated to the percentage of total fat mass and to fat/lean ratio (29).

In contrast to these results, two recent DEXA studies failed to identify significant differences in body composition between CAH patients and controls (31, 32). One of these studies (32) used DEXA to estimate visceral adipose tissue (VAT), which was also comparable between CAH patients and controls.

To date, only one study performing CT imaging is available, revealing greater amount of both VAT and subcutaneous adipose tissue (SAT) and VAT/SAT ratio in 28 adolescents and young adults with classic CAH, compared with age-, gender-, and BMI-matched controls (33). SAT and VAT were positively correlated to adiposity indexes (BMI, WHtR, trunk, and total body fat mass), homeostasis model assessment (HOMA), and lipid profile (total cholesterol, low density lipoproteins (LDL), very low density lipoproteins (VLDL) and triglycerides), but did not correlate with hydrocortisone dose or markers of hormonal control.

Interestingly, adiposity rebound, consisting in the re-increase of BMI after its nadir, occurs significantly earlier in CAH patients than non-affected children, possibly predicting development of obesity (15, 34). In a recent study (35) on 29 classic CAH patients identified by neonatal screening and followed-up for at least 10 years, multivariate regression analysis identified lower BMI at birth as an independent predictor of early adiposity rebound, thus suggesting that early alterations in fetal life may predispose to the development of metabolic problems.

TABLE 1 | Summary of main studies documenting the prevalence of obesity and/or altered body composition in CAH patients.

Study	Patients and CAH form	Age range (years)	Mean HC dose (mg/m^2/day)	Mean FC dose (μg/m^2/day)	Technique	Outcomes
Finkielstain et al. (8)	-139 Classic (97 SW, 42 SV) -31 Non-classic	0.6–17	15	n.a.	n.a.	↑ Prevalence of obesity (35%) vs. normal pediatric population (17%), with no difference between Classic and NC CAH; No difference between males and females ↑ HOMA-IR ↑ BP (about 40% Classic and 20% NC CAH)
Gonçalves et al. (11)	-28 Classic females (18 SW, 10 SV) -112 Controls	4–23	15–20	n.a.	BIA	↑ BMI vs. controls, ↑ fat mass and waist/hip ratio vs. controls
Stikkelbroeck et al. (12)	-27 Classic (24 SW, 3 SV) -30 Controls	17–25	n.a.	n.a.	DXA	↑ BMI vs. controls, ↑ fat mass vs. controls
Völkl et al. (16)	-89 Classic (78 SW, 11 SV)	0.2–17.9	14.7	63.1	n.a.	↑ Prevalence of obesity (16.8%) vs. normal population (2.27%) No difference between males and females
Roche et al. (21)	-38 Classic (SW)	6.1–18.2	17.5 (prepubertal) 17.3 (pubertal) 17.8 (postpubertal)	120 (prepubertal) 113 (pubertal) 103 (postpubertal)	n.a.	↑ BMI vs. normal population ↑ SPB and DBP vs. normal population ↓ nocturnal dip in BP No difference between males and females
Subbarayan et al. (25)	-107 Classic (79 SW, 28 SV)	0.4-20.5	13.3	102	n.a.	↑ Prevalence of obesity (23.6%) vs. normal population (14.8-17.1 %) ↑ SBP vs. normal population 9.5% high plasma triglycerides No difference between males and females ↓ HOMA-IR vs. controls
Isguven et al. (26)	-16 Classic (SW) -18 Controls	1.4–10.5	15	n.a.	BIA	↑ BMI vs. controls, No difference between males and females ↑ Fat mass in female patients vs. controls
Janus et al. (27)	-61 Classic (51 SW, 10 SV) -9 Non-classic	3–17.9	17.2 (SW) 19.5 (SV) 11.9 (NC)	66.5 (SW) 28.6 (SV)	BIA	Normal BMI, no differences in body composition between classic and NC forms; No difference between males and females No hypertension
Williams et al. (28)	-25 Classic -12 Non -classic -41 Controls	0.5–15.8	13.9 8.2	82 μg/day 18 μg/day	DXA	↑ fat mass in classic CAH vs. controls, ↑ lean mass in NC CAH vs. controls Comparable HOMA-IS between CAH (classic and NC) and controls ↑ stimulated glucose and insulin in NC CAH vs. controls Comparable BP and lipids between classic CAH and controls ↑ SBP in NC CAH vs. controls
Abd El Dayem et al. (29)	-28 Classic (27 SW, 1 SV) -11 Controls	3–18	15.5	50–200 μg/day	DXA	↑ BMI, ↑ fat mass and ↓ lean mass vs. controls No difference between males and females
Marra et al. (30)	-20 Classic (15 SW, 5 SV) -20 Controls	11.1–16.1	15	54.8	DXA	↑ BMI and ↑ fat mass and waist/height ratio vs. controls; ↑ HOMA-IR vs. controls; No difference between males and females Comparable lipids and BP between CAH and controls ↓ Exercise capacity vs. controls LV diastolic dysfunction in CAH male patients
Ariyawatkul et al. (31)	-21 Classic (10 SW, 11 SV) -21 Controls	9–28	21.4	50–150 μg/day	DXA	BMI, fat and lean mass comparable between CAH and controls ↑ Waist/hip and waist/height ratio vs. controls Comparable HOMA, BP, lipids, leptin between CAH and controls
Halper et al. (32)	-32 Classic (19 SW, 13 SV) -10 Non- classic -101 controls	7.6–17.7	11.3	n.a.	DXA	↓ BMI vs. controls, VAT comparable between patients and controls

(Continued)

TABLE 1 | Continued

Study	Patients and CAH form	Age range (years)	Mean HC dose (mg/m^2/day)	Mean FC dose (μg/m^2/day)	Technique	Outcomes
Kim et al. (33)	-28 Classic (20 SW, 8 SV) -28 Controls	12.4–18.8	19.5	210 μg/day	CT	60.7% of patients obese or overweight; ↑ VAT, SAT, and VAT/SAT vs. controls; No difference between males and females Elevated HOMA in 18% of CAH
Mooij et al. (23)	-26 Classic -(24 SW, 2 SV)	8-16	11.2	98.5	n.a.	↑ BMI vs. reference population (25.9% obese and 14.8% overweight) 44.4% of patients HOMA-IR > 75th and 29.6% HOMA-IR >90th percentile ↑ SBP in 18.5%, lower than reference population 48.1% ↓ nocturnal dip
Amr et al. (24)	-32 Classic (24 SW, 8 SV) -32 Controls	3-17	15	50-100	n.a.	↑ BMI in patients (22% obese) vs. controls ↑ HOMA-IR, CIMT, SBP vs. controls ↑ Stimulated glucose levels vs. controls Comparable lipids, stimulated insulin vs. controls

CAH, Congenital Adrenal Hyperplasia; HC, hydrocortisone; FC, fludrocortisones; SW, salt wasting; SV, simple virilizing; NC, non-classic; HOMA, homeostatic model assessment; HOMA-IS, HOMA insulin sensitivity; BP, blood pressure; BIA, bioelectrical impedance analysis; BMI, body mass index; DXA, dual X-ray absorptiometry; SBP, systolic blood pressure; DBP, diastolic blood pressure; HOMA-IR, HOMA insulin resistance; LV, left ventricle; HDL, high density lipoproteins; T-COL, total cholesterol; VAT, visceral adipose tissue; SAT, subcutaneous adipose tissue; CIMT, carotid intima media thickness; CRP, C reactive protein. n.a., not available/not applicable. ↑ increased; ↓ decreased.

In conclusion, current data point toward a tendency to develop increased adiposity in children and adolescents with CAH. Supra-physiological dosages of glucocorticoids have been advocated as a key causative factor, however, data regarding this association are not univocal, and thus other predisposing factors may contribute to an increased risk of obesity.

Even though obesity has potential negative effects in terms of cardiac and metabolic consequences, recent Endocrine Society Guidelines on CAH (2) recommend against routine evaluation for cardiac and metabolic disease in patients with CAH beyond that advised for the general population, and suggest early lifestyle counseling in order to prevent such consequences.

Insulin Resistance

Several studies have demonstrated a tendency toward insulin resistance (IR) in children and adolescents with CAH, as assessed by increased plasma insulin concentrations (30) and HOMA-IR (8, 23, 24, 30, 33, 36–38) (**Table 2**).

In a recent study, 8 out of 27 (29.6%) classic CAH patients presented a HOMA-IR above the 90th percentile, which was significantly correlated to hydrocortisone dose and BMI (23).

On the other hand, other studies have reported normal (31) or even better (25) insulin sensitivity in CAH patients compared to controls. In particular in a large cohort of 107 children and adolescents with classic CAH (25), despite increased BMI, CAH children had lower levels of fasting blood glucose, insulin and HOMA-IR compared to controls.

Glucose tolerance and insulin sensitivity status have also been assessed through oral glucose tolerance test (OGTT) (**Table 2**). In a recent study (24) on 32 patients with classic CAH aged 3–17 years, impaired fasting glucose was found in 34% of the patients, while 19% had impaired glucose tolerance and 2.7% a slight increase in HOMA-IR. Noteworthy, in this study BMI was significantly higher than controls, with 22% of the patients labeled as obese. However, other studies suggest a tendency toward IR even in the absence of obesity (28, 38). Zimmerman et al. (38) performed OGTT in 27 normal weight children and young adults with classic CAH showing higher post-load glucose and insulin values than matched controls. Moreover, insulin resistance index (IRI) and HOMA-insulin secretion B cell (HOMA-B) were found to be higher and insulin sensitivity index (ISI) to be lower in patients compared with controls. IRI was significantly correlated with hydrocortisone dose and duration of treatment (38).

With regard to NC CAH, only a few data on small cohorts are available (28, 39–42). Most of the studies documented that both treated (28, 42) and untreated (39, 41) NC CAH patients have reduced insulin sensitivity, thus suggesting that prolonged exposure to androgen excess before diagnosis may contribute to IR. Williams et al. (28) documented that, despite more favorable body composition, NC CAH had more pronounced alterations in glucose metabolism compared to classic forms.

Finally, the results of a recent meta-analysis of 14 studies, reporting data for 437 children and adolescents with CAH, confirm that CAH patients display reduced insulin sensitivity compared to controls (6).

In summary, several data suggest that children and adolescents with CAH have impaired insulin sensitivity, with preserved glucose tolerance. Obesity and altered body composition are risk factors for IR; however, unfavorable changes in glucose metabolism may also be detected in non-obese CAH subjects. Indeed, glucocorticoid excess and hyperandrogenism may both contribute to the development of IR.

Dyslipidemia

Dyslipidemia may be a possible consequence of increased body fat. However, in the majority of CAH children the lipid profile is comparable to controls (24, 28, 30, 42, 43). Subtle alterations in lipid metabolism have been detected only in a few studies on small cohorts of children with several methodological limitations

TABLE 2 | Summary of main studies addressing insulin-sensitivity in CAH patients.

Study	Patients and CAH form	Age range (years)	Mean HC dose (mg/m^2/day)	Mean FC dose (μg/m^2/day)	Methods	Outcomes
Finkielstain et al. (8)	-139 Classic (97 SW, 42 SV) -31 Non-classic	0.6–17	15	n.a.	n.a.	↑ Prevalence of obesity (35%) vs. normal pediatric population (17%), with no difference between Classic and NC CAH and between males and females ↑ HOMA-IR ↑ BP (about 40% Classic and 20% NC CAH)
Marra et al. (30)	-20 Classic (15 SW, 5 SV) -20 Controls	11.1–16.1	15	54.8	HOMA	↑ BMI and ↑ fat mass and waist/height ratio vs. controls; ↑ HOMA-IR vs. controls; No difference between males and females Comparable lipids and BP between CAH and controls ↓ exercise capacity vs. controls LV diastolic dysfunction in CAH male patients
Kim et al. (33)	-28 Classic (20 SW, 8 SV) -28 Controls	12.4–18.8	19.5	210 μg/day	HOMA	60.7% of patients obese or overweight; ↑ VAT, SAT and VAT/SAT vs. controls; No difference between males and females Elevated HOMA in 18% of CAH
Charmandari et al. (36)	-16 Classic (12 SW, 4 SV) -28 Controls	2–12	14.8	114 μg/day	HOMA	BMI comparable to controls ↑ HOMA-IR vs. controls ↑ Leptin in patients vs. controls; No difference between males and females
Harrington et al. (37)	-14 Classic (11 SW, 3 SV) 53 Controls	11.6–18	13.3	108.3 μg/day	HOMA	BMI comparable to controls, ↑ WHtR vs. controls ↑ HOMA-IR vs. controls ↑ SBP vs. controls ↓ FMD and smooth muscle function vs. controls IMT, CRP, lipids comparable between CAH and controls
Mooij et al. (23)	-26 Classic (24 SW, 2 SV)	8–16	11.2	98.5	HOMA	↑ BMI vs. reference population (25.9% obese and 14.8% overweight) 44.4% of patients HOMA-IR > 75th and 29.6% HOMA-IR >90th percentile ↑ SBP in 18.5%, lower than reference population 48.1% ↓ nocturnal dip
Amr et al. (24)	-32 Classic (24 SW, 8 SV) -32 Controls	3–17	15	50–100	HOMA OGTT	↑ BMI in patients (22% obese) vs. controls ↑ HOMA-IR, CIMT, SBP vs. controls ↑ Stimulated glucose levels vs. controls Comparable lipids, stimulated insulin vs. controls
Zimmermann et al. (38)	-27 Classic (12 SW, 15 SV) -27 Controls	4–31	21.5 (SW) 16.2 (SV)	50–100 μg/day	HOMA IRI ISI OGTT	BMI comparable to controls ↓ HOMA-IS and ↑ HOMA-IR, HOMA-B, IRI, stimulated glucose and insulin levels vs. controls ↑ Small dense LDL subfractions and HDL vs. controls Comparable LDL, T-COL, triglycerides between CAH and controls
Ariyawatkul et al. (31)	-21 Classic (10 SW, 11 SV) -21 Controls	9–28	21.4	50–150 μg/day	HOMA	BMI, fat and lean mass comparable between CAH and controls ↑ Waist/hip and waist/height ratio vs. controls Comparable HOMA, BP, lipids, leptin between CAH and controls
Subbarayan et al. (25)	-107 Classic (79 SW, 28 SV)	0.4–20.5	13.3	102	HOMA	↑ Prevalence of obesity (23.6%) vs. normal population (14.8–17.1%) ↑ SBP vs. normal population 9.5% high plasma triglycerides No difference between males and females ↓ HOMA-IR vs. controls
Williams et al. (28)	-25 Classic -12 Non-classic -41 Controls	0.5–15.8	13.9 8.2	82 μg/day 18 μg/day	HOMA OGTT	↑ Fat mass in classic CAH vs. controls ↑ Lean mass in NC CAH vs. controls Comparable HOMA-IS between CAH (classic and NC) and controls ↑ Stimulated glucose and insulin in NC CAH vs. controls Comparable BP and lipids between classic CAH and controls ↑ SBP in NC CAH vs. controls
Saygili et al. (39)	-18 Non-classic -26 Controls	25.7 ± 8.9 (mean ± SD)	Untreated	Untreated	HOMA	BMI comparable to controls ↑ HOMA-IR vs. controls

(Continued)

TABLE 2 | Continued

Study	Patients and CAH form	Age range (years)	Mean HC dose ($mg/m^2/day$)	Mean FC dose ($\mu g/m^2/day$)	Methods	Outcomes
Bayraktar et al. (40)	-50 Non-classic -25 Controls	22.1 ± 2.91 (mean ± SD)	Untreated	Untreated	HOMA	BMI comparable to controls Comparable HOMA-IR and lipids between CAH and controls
Speiser et al. (41)	-6 Non-classic -12 Controls	16–45	Untreated	Untreated	i.v. GTT	BMI comparable to controls ↓ Insulin sensitivity vs. controls
Wasniewska et al. (42)	-9 Classic SW -9 Non-classic -16 Controls	13.3–20.4	17.1	100 μg/day	HOMA	BMI comparable to controls ↑ HOMA-IR in Classic CAH vs. controls Comparable HOMA-IR between NC CAH and controls ↑ SBP in classic CAH vs. controls ↑ DBP in classic CAH and NC CAH vs. controls Comparable lipids between CAH and controls ↑ IMT in classic CAH and NC CAH vs. controls

CAH, Congenital Adrenal Hyperplasia; HC, hydrocortisone; FC, fludrocortisones; SW, salt wasting; SV, simple virilizing; HOMA, homeostatic model assessment; BMI, body mass index; HOMA-IR, HOMA insulin resistance; BP, blood pressure; LV, left ventricle; VAT, visceral adipose tissue; SAT, subcutaneous adipose tissue; WHtR, waist-to-height ratio; SBP, systolic blood pressure; FMD, flow-mediated dilatation; IMT, intima media thickness; CRP, C reactive protein; OGTT, oral glucose tolerance test; IRI, insulin resistance index; ISI, insulin sensitivity index; LDL, low density lipoproteins; HDL, high density lipoproteins; NC, non-classic; T-COL, total cholesterol; i.v. GTT, intravenous glucose tolerance test; DBP, diastolic blood pressure. ↑ increased; ↓ decreased.

(38, 44). Indeed, higher triglycerides (44), lower HDL cholesterol and higher concentrations of small dense LDL subfractions (sd-LDL) (38) have been reported.

A recent study on a large cohort of 107 children with CAH aged 9.2 years (range 0.4–20.5 years) documented a slight increase in the prevalence of hyperlipidemia (9.5%), even though a proper control group to assess the significance of these findings was lacking (25).

At present, there are not sufficient data documenting dyslipidemia in CAH children, therefore, regular assessment of lipid profile seems to be unnecessary and further studies are needed to better address this topic.

Hypertension

The vast majority of studies investigating blood pressure (BP) in CAH patients, either by ambulatory or 24 h BP measurement, documented increased systolic and/or diastolic BP values (6, 8, 21–23, 25, 43, 45–47), as summarized in **Table 3**. Moreover, even in the absence of overt hypertension (27), CAH children may display a reduction of the physiological nocturnal dipping in BP (21, 23, 46).

Subbarayan et al. (25) reported systolic hypertension in 20.9%, diastolic hypertension in 8.8%, and both systolic and diastolic hypertension in 3% of a large cohort of CAH children, regardless of gender. However, only systolic BP (SBP) was significantly higher compared to the reference population. Interestingly, the prevalence of hypertension was lower compared to previous studies (21, 46), possibly due to the use of lower glucocorticoid and mineralocorticoid doses in recent years. Both SBP and diastolic BP (DBP) were negatively related to age, possibly due to a reduction in the dose of fludrocortisone.

A recent longitudinal multicenter study (22) on a large cohort of classic CAH patients reported a prevalence of hypertension of 12.5%. The increase in SBP was more marked than what was observed for DBP. SW patients exhibited higher BP values than SV patients. BP values were significantly correlated to BMI, age and fludrocortisone dose. Of note, the prevalence of hypertension in CAH patients decreases with age, becoming comparable to the general population (below 5%) at the age of 18 years. In keeping with this, BP values were significantly lower in a French National survey on men with classic 21-OH deficiency compared to healthy population (10), even though other studies reported elevated BP in men (8) or women (4, 9) with CAH, possibly due to differences in treatment regimens among different studies. Finally, significant alterations in BP in CAH patients have been confirmed in the recent meta-analysis by Tamhane et al. (6).

Few studies investigated gender differences, most reporting a similar prevalence of hypertension between males and females with CAH (8, 21, 25, 46, 47). Only in two studies, evaluating morning blood pressure (22) or 24-h BP monitoring (27), CAH females seemed more affected then males, likely due to excess androgen exposure.

Taken together, current findings suggest a higher prevalence of hypertension in CAH children and adolescents, with a selective increase in systolic rather than diastolic BP values. In addition to obesity, the excess of glucocorticoid and mineralocorticoid doses also play a key pathogenic role, especially in the first years of life, when higher doses and salt supplementation are often needed, in order to counteract the physiologic state of relatively higher resistance to mineralocorticoids and the higher renal salt wasting.

NON-TRADITIONAL CV RISK FACTORS

Circulating Cytokines

Adipose tissue represents an important source of inflammatory cytokines and thus, a few studies have evaluated inflammatory markers in CAH patients. A study by Charmandari et al. (36) including 18 classic CAH patients (age range 2–12 years) showed significantly higher leptin concentrations than healthy controls, regardless of BMI and sex. Leptin concentrations were negatively correlated with epinephrine and free metanephrine concentrations, and are likely related to reduced inhibitory effect of catecholamines on leptin secretion. Loss of gender dimorphism in leptin concentrations was also observed in the

TABLE 3 | Summary of main studies investigating hypertension in CAH patients.

Study	Patients and CAH form	Age range (years)	Mean HC dose (mg/m^2/day)	Mean FC dose (μg/m^2/day)	Methods	Outcomes
Roche et al. (21)	-38 Classic SW	6.1–18.2	17.5 (prepubertal) 17.3 (pubertal) 17.8 (postpubertal)	120 (prepubertal) 113 (pubertal) 103 (postpubertal)	24-h BP	↑ BMI vs. normal population ↑ SPB and DBP vs. normal population ↓ Nocturnal dip in BP No difference between males and females
Bonfig et al. (22)	-716 Classic (571 SW, 145 SV)	3–18	14.4	72.7	Morning BP	↑ BMI in SW vs. SV ↑ prevalence of hypertension in 12.5% of children ↑ BP in SW vs. SV More elevated SBP than DBP At the pubertal age↑prevalence of hypertension in females than in males (12 vs. 5.3%)
Subbarayan et al. (25)	-107 Classic (79 SW, 28 SV)	0.4–20.5	13.3	102	Mean of four measurements (every 6 h)	↑ prevalence of obesity (23.6%) vs. normal population (14.8–17.1%) ↑ SBP vs. normal population 9.5% high plasma triglycerides No difference between males and females ↓ HOMA-IR vs. controls
Mooij et al. (23)	-26 Classic (24 SW, 2 SV)	8–16	11.2	98.5	HOMA	↑ BMI vs. reference population (25.9% obese and 14.8% overweight) 44.4% of patients HOMA-IR > 75th and 29.6% HOMA-IR >90th percentile ↑ SBP in 18.5%, lower than reference population 48.1% ↓ nocturnal dip
Akyürek et al. (43)	-25 Classic SW -25 Controls	5–15	17.03	120	Morning BP 24-h BP	↑ BMI vs. control Comparable HOMA-IR between CAH and controls ↑ DBP vs. controls ↓ Nocturnal dip of SBP and DBP vs. controls Comparable lipids between CAH and controls ↑ IMT vs. controls
Nebesio and Eugster (45)	-91 Classic	n.a.	16.4	90 μg/day	n.a.	↑ prevalence of hypertension vs. normal population
Völkl et al. (46)	55 Classic (45 SW, 10 SV)	5.3–19	14.6	47	24-h BP	↑ BMI ↑ daytime and nighttime SBP ↓ nocturnal dip of DBP No difference between males and females in ambulatory 24-h BP
Hoepffner et al. (47)	-23 Classic	6–17	18.7	70.8	Morning BP 24-h BP	↑ SPB and DBP in hospitalized vs. outpatients No difference between males and females
Janus et al. (27)	-61 Classic (51 SW, 10SV) 9 Non-classic	3–17.9	17.2 (SW) 19.5 (SV) 11.9 (NC)	66.5 (SW) 28.6 (SV)	24-h BP	Normal BMI, no differences in body composition between classic and NC forms No overt hypertension but ambulatory 24-h SBP and Night SBP were higher in females than males
Finkielstain et al. (8)	-139 Classic (97 SW, 42 SV) -31 Non-classic	0.6–17	15	n.a.	n.a.	↑ Prevalence of obesity (35%) vs. normal pediatric population (17%), with no difference between Classic and NC CAH and between males and females ↑ HOMA-IR ↑ BP (about 40% Classic and 20% NC CAH)

CAH, Congenital Adrenal Hyperplasia; HC, hydrocortisone; FC, fludrocortisones; SW, salt wasting; BP, blood pressure; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; SV, simple virilizing; HOMA, homeostatic model assessment; HOMA-IR, HOMA insulin resistance; IMT, intima media thickness; NC, non-classic; HOMA-IS, HOMA insulin sensitivity; n.a., not available/non-applicable. ↑ increased; ↓ decreased.

CAH group, possibly due to exposure to androgens excess in females.

Increased leptin concentrations and a strong correlation between leptin, obesity (8, 23, 33) and HOMA-IR (23) have also been documented in studies, enrolling both classic and NC CAH patients.

Conversely, other studies (19, 26, 31) found leptin concentrations in CAH children and adolescents comparable to controls, even in the face of higher BMI and body fat ratio (26). However, significantly lower concentrations of soluble leptin receptor (sOB-R) (which binds circulating leptin, regulating its half-life) were found in CAH patients compared to matched controls, predicting a higher amount of free (unbound) serum leptin (19).

The role of adiponectin on CV health of CAH children is still unclear. Higher adiponectin concentrations than

matched controls, with no alteration in serum leptin and adiponectin/leptin ratio, were found in a study on 51 CAH patients, aged 5.6 to 19.6 years, regardless of sex (48). Adiponectin was negatively correlated with BMI, serum DHEA-S and testosterone, but no clear relationship with hydrocortisone and fludrocortisone dosage (48).

A few studies have also investigated the role of other inflammatory markers in CAH patients. In particular, high sensitivity C reactive protein (hs-CRP), interleukin-6 (IL-6), and plasminogen activator inhibitor-1 (PAI-1) have been found to be normal in two studies (31, 33), while tissue plasminogen activator (tPA) concentrations were higher than controls (23). In addition, a study by Metwalley et al. (49) demonstrated that, compared to controls, classic CAH patients ($n = 32$, mean age 13.6 ± 2.5 years) have higher concentrations of hs-CRP as well as of circulating endothelial cells (CECs), which represent an indicator of vascular endothelial injury. Both hs-CRP and CECs were correlated to poor disease control, IMT and indexes of diastolic dysfunction.

Finally, homocysteine concentrations were also higher than controls (despite comparable folate and vitamin B12 levels), especially in poorly controlled CAH patients aged 5–12 years (50).

In conclusion, a few data suggest that CAH may be associated with an unbalanced profile of adipokines, inflammatory markers and homocysteine, which are all considered early markers of CV morbidity. Further research is needed to unravel whether these alterations represent the consequence of poor disease control, unfavorable metabolic profile or are intrinsic to CAH.

Vascular Function and Intima-Media Thickness

Intima-media thickness (IMT), a surrogate marker of atherosclerosis, has been evaluated in several studies (23, 29, 33, 42, 51), yielding contrasting results. A few studies found normal values of carotid-IMT (C-IMT), comparable to controls (23, 29, 33). However, a loss of sexual dimorphism in females exposed to excess androgens and a correlation between C-IMT, 17-hydroxyprogesterone (17-OHP), and androstenedione concentration were observed (29).

In contrast, significant differences in IMT values between CAH patients and controls have been reported in other studies (6, 24, 42, 43, 49, 51, 52) with no differences between SV and SW forms (24), or normal weight and overweight subjects (52). In the study by Akyürek et al. (43), C-IMT values were greater in hypertensive compared to normotensive CAH patients, and were negatively correlated to nocturnal dipping of DBP. A significant correlation was also found between C-IMT and indexes of disease control, such as duration of treatment, and concentrations of 17-OHP and testosterone (49), suggesting that increased androgen levels may contribute to an increased risk of vascular dysfunction. Janus and coworkers (27) evaluated arterial ambulatory stiffness index (AASI), derived by 24-h blood pressure measurement, reporting higher values in SW forms with genotypes Del/Del and Del/I2G compared to other forms, especially in females. This further suggests a detrimental role of excess androgens or treatment on arterial wall function. Indeed, AASI was related to urinary cortisol, free androgen index and bone age advancement. Positive correlations between carotid and femoral IMT with BMI (51), aortic and carotid IMT with serum triglycerides, and aortic IMT with cumulative glucocorticoid doses (42), suggest how subclinical atherogenesis in CAH children may arise from a complex interplay between overweight, dyslipidemia and hypercortisolism.

Furthermore, Özdemir et al. (53) has recently reported increased stiffness index and elastic modulus of the aorta and the carotid artery, with lower aortic and carotid distensibility in CAH children and adolescents. Multivariate regression analysis identified BMI as the only independent variable for C-IMT and aortic stiffness index.

Finally, classic CAH patients may also exhibit impaired endothelial function, evaluated by flow-mediated dilatation (FMD) and glyceryl trinitrate-mediated dilatation (GTN), in comparison to healthy controls (37). In this study, BMI was comparable between CAH children and controls, thus suggesting that other factors, beyond increased adiposity, are likely to contribute to the development of endothelial dysfunction.

Although current studies are often limited by small sample size, heterogeneous population and study designs, preliminary evidences suggest that CAH may be associated with vascular dysfunction and increased IMT already in childhood. Obesity, hypertension, glucocorticoid overtreatment, as well as prolonged exposure to androgen excess, may all contribute to an increased risk of early atherosclerosis, even though the role of each contributing factors is far from being clear.

CARDIAC MORPHOLOGY AND FUNCTION

Adults affected by CAH may have increased CV morbidity (4) even though the pathogenic mechanism is still unclear. Recent studies showed that alterations in cardiac morphology and function in CAH may be detected already in childhood (30, 49, 53, 54).

We recently demonstrated that classic CAH adolescent males may have signs of mild subclinical diastolic dysfunction, consisting in a significant prolongation of isovolumetric relaxation time (IVRT) and mitral deceleration time (MDT), compared to matched controls (30). These indexes of diastolic dysfunction correlated negatively with testosterone concentrations, which were, in turn, negatively related to cumulative hydrocortisone dose in the 3 years before the study. This led to the hypothesis that excess of glucocorticoid treatment during adolescence may affect cardiac function through either direct detrimental effect on muscle performance (55) or through induction of mild hypogonadism.

Our results have been confirmed by other studies (49, 53, 54). Left ventricular diastolic dysfunction, characterized by higher late diastolic myocardial velocity (Am) and significantly lower early diastolic myocardial velocity (Em)/Am ratio in comparison to controls, was documented by Özdemir et al. (53). In addition to mild diastolic dysfunction, Metwalley et al. (49) also reported higher LV mass (LVM) in classic CAH adolescents

compared to matched controls, which was more pronounced in patients with clinical signs of androgen excess and high serum 17-OHP.

Conversely, normal LV mass was reported in a cohort of 20 classic CAH patients; however, no data on LV cavity size or functional data were provided and no comparison with a control group was made (56). Mooij et al. (57) recently documented, in a cohort of 27 CAH patients (mean age 11.7 years), lower IVR and LV posterior wall thickness, and higher prevalence of incomplete right bundle branch block, compared to matched controls. Shorter exposure to excess androgens (due to neonatal screening allowing early diagnosis) and/or better control of the disease in this cohort have been proposed to explain differences from previous studies (57).

Exercise capacity in CAH adolescents has been investigated in very small studies performing both short-term high-intensity (58) and long-term moderate-intensity exercise (59). In these two studies, exercise capacity and systolic BP at peak exercise were similar to controls; however, epinephrine and metanephrine concentrations at baseline and peak exercise, as well as glucose values throughout exercise and recovery, were lower in CAH patients than in healthy controls (58, 59). Although lower concentrations of epinephrine and metanephrine have been detected in CAH patients compared with healthy controls (60), quality of current evidences does not allow for identification of a clear role of adrenomedullary dysfunction in exercise performance.

Recently, we reported that adolescents affected with classic CAH (age range 13.6 ± 2.5 years) have impaired exercise capacity, with reduced peak workload and exaggerated systolic BP at peak exertion during an incremental exercise test on a bicycle ergometer (30). Unfortunately, we did not measure epinephrine and metanephrine levels, and thus, we could not evaluate the role of adrenomedullary dysfunction in impaired exercise performance in our patients. However, we documented cardiac alterations and impaired exercise capacity, similar to CAH, in a group of adolescents with juvenile idiopathic arthritis who were treated with comparable doses of glucocorticoids, thus suggesting a pathophysiological link between glucocorticoid replacement and CV abnormalities.

In summary, current evidences suggest that CAH adolescents display subclinical diastolic dysfunction, LV hypertrophy and impaired exercise performance, with possible negative consequences on their quality of life. Further studies on larger cohorts are necessary to define the mechanisms underlying these abnormalities and the role of over- and under-treatment.

CONCLUSIONS

Increasing evidence indicates that CAH individuals are prone to develop a number of early CV risk factors, such as obesity, hypertension, insulin resistance, low-grade inflammation, increased IMT and subclinical cardiac dysfunction, already in childhood.

The therapeutic management of patients with CAH remains a challenge and current treatment regimens do not always allow optimal biochemical control. Overexposure to glucocorticoids and mineralocorticoids as well as to androgens may contribute to the development of unfavorable metabolic and CV changes, even though metabolic derangement in CAH patients may also result from other still unraveled risk factors.

At present, there is insufficient evidence to recommend regular monitoring of early metabolic markers of CV disease in all CAH children. However, lifestyle counseling to avoid overweight and other related metabolic consequences, and regular assessment of blood pressure at all ages should be recommended in the management of CAH children. Monitoring for other metabolic and CV abnormalities should be tailored to individual patient's needs.

Long-term prospective studies on large cohorts of patients are necessary to better clarify the pathophysiology of metabolic alterations associated with CAH. In the meantime, further efforts should be made in order to optimize treatment, and identify new therapeutic approaches to prevent metabolic derangement and improve long-term health outcomes of CAH patients.

AUTHOR CONTRIBUTIONS

NI, MS, and DC ideated the manuscript. NI, FB, GC, and DC drafted the manuscript. DC and MS revised the manuscript for intellectual contents. All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Management of the Female With Non-classical Congenital Adrenal Hyperplasia (NCCAH): A Patient-Oriented Approach

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Non-classical congenital adrenal hyperplasia (NCCAH) is considered to be a common monogenic inherited disease, with an incidence range from 1:500 to 1:100 births worldwide. However, despite the high incidence, there is a low genotype-phenotype correlation, which explains why NCCAH diagnosis is usually delayed or even never carried out, since many patients remain asymptomatic or are misdiagnosed as suffering from other hyperandrogenic disorders. For affected adolescent and adult women, it is crucial to investigate any suspicion of NCCAH and determine a firm and accurate diagnosis. The Synacthen test is a prerequisite in the event of clinical suspicion, and molecular testing will establish the diagnosis. In most cases occurring under 8 years of age, the first symptom is premature pubarche. In some cases, due to advanced bone age and/or severe signs of hyperandrogenism, initiation of hydrocortisone treatment prepubertally may be considered. Our unifying theory of the hyperandrogenic signs system and its regulation by internal (hormones, enzymes, tissue sensitivity) and external (stress, insulin resistance, epigenetic, endocrine disruptors) factors is presented in an attempt to elucidate both the prominent genotype-phenotype heterogeneity of this disease and the resultant wide variation of clinical findings. Treatment should be initiated not only to address the main cause of the patient's visit but additionally to decrease abnormally elevated hormone concentrations. Goals of treatment include restoration of regular menstrual cyclicity, slowing the progression of hirsutism and acne, and improvement of fertility. Hydrocortisone supplementation, though not dexamethasone administration, could, as a general rule, be helpful, however, at minimum doses, and also for a short period of time and, most likely, not lifelong. On the other hand, in cases where severe hirsutism and/or acne are present, prescription of oral contraceptives and/or antiandrogens may be advisable. Furthermore, women with NCCAH commonly experience subfertility, therefore, there will be analysis of the appropriate approach for these patients, including during pregnancy, based mainly on genotype. Besides, we should keep in mind that since the same patient will have changing requirements through the years, the attending physician should undertake a tailor-made approach in order to cover her specific needs at different stages of life.

Keywords: premature pubarche, PCOS, androstenedione, 17OH progesterone, CYP21A2

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DEFINITIONS AND PREVALENCE

Congenital adrenal hyperplasia (CAH) encompasses a family of autosomal recessive disorders characterized by mild to acutely impaired cortisol synthesis due to a deficiency in one of the five adrenal steroidogenic enzymes required for cortisol production (1, 2). Conventionally, CAH is divided into (a) classical (CCAH), presenting with salt-wasting or the simple virilizing form that is manifest at birth and/or in the neonatal period, and (b) non-classical (NCCAH), representing a less severe form of the disorder which lacks genital ambiguity, is not immediately life-threatening, and presents later in life, or remains asymptomatic, or is misdiagnosed as a different disease (3). However, the boundaries across the different forms of CAH are not strictly defined, which tends to increase the challenges associated with this disorder. It is therefore advisable to consider CAH as a continuum of phenotypes, from severe to mild or else asymptomatic (3).

The most common form of CAH is caused by 21-hydroxylase deficiency (21OHD), resulting in impaired or no conversion of 17 hydroxyprogesterone (17 OHP) to 11 deoxycortisol and of progesterone to deoxycorticosterone. The blockade of steroid conversion results in increased production of androgen precursors, under CRH-ACTH stimulation, leading to biochemical hyperandrogenism, marked by elevated 17-OHP levels. The classical form of the disease, occurs in 1 out of 16,000 live births worldwide. However, it should be mentioned that the perceived incidence of the disease is related to the screening method used. For example, it has been reported that the incidence of the classical form of CAH in Sweden is 1:11,500 when a case survey approach is used, which figure however drops to 1:9,800 when hormonal screening is applied. Likewise, the incidence in France and Switzerland ranges between 1:15,472 and 1:23,000 and 1:10,749 and 1:11,661 when the different methods of screening are used (4).

NCCAH is much more frequent, occurring in approximately 1 out of 1,000 Caucasians and more commonly in certain ethnic groups, such as Ashkenazi Jews (1:27), Hispanics (1:53), Yugoslavs (1:62), and Italians (1:300) (5, 6). Nevertheless, data on the prevalence of NCCAH based on different estimation methods (case-survey, hormonal screening, and molecular testing) are lacking. NCCAH is considered the most common autosomal recessive endocrine disorder with a carrier frequency of 1:25 to 1:10. In NCCAH due to 21-OHD, the residual enzymatic activity is estimated to be about 10–70% (7–9).

DIAGNOSIS

In comparison to the diagnosis of the classical form of the disease, which is made at birth or during the neonatal period because of genital ambiguity and/or salt-wasting symptoms or through screening programs employed in some countries most cases of NCCAH are not easily detectable (4, 10). Additionally, many individuals remain asymptomatic during childhood and adolescence, have normal reproductive function, and only become aware of NCCAH due to the diagnosis of another family member and consequent testing (11). However, most women with NCCAH seek medical assistance when they experience symptoms of androgen excess and, when clinical suspicion prompts testing, elevated basal 17 OHP levels will more likely than not point to a diagnosis of NCCAH.

Indeed, the clinical guidelines proposed by the Endocrine Society recommend a baseline non-stimulated value of 17 OHP as the screening tool for NCCAH. Morning 17 OHP levels >6 nmol/L in the follicular phase in menstruating females should prompt further evaluation, since it has been shown that levels above 6 nmol/L capture 90% of NCCAH individuals (12, 13). Random measurements of 17 OHP have not been shown to be helpful, since these often yield normal levels in patients with NCCAH, and they are, moreover, extremely high in the luteal phase in half of healthy females (13). However, in our data derived from 280 subjects with the disease, six patients (2.1%) had a baseline 17 OHP value < 6 nmol/L (14). Additionally, Bidet et al., in a large cohort of women with NCCAH verified by molecular techniques also found basal 17 OHP values lower than 6 nmol/L in 8% of the subjects studied (15). Finally, based on data collected by Speiser et al., 9% of individuals with NCCAH displayed 17 OHP values lower than 2 ng/ml (that corresponds to 6nmol/L). According to other studies, a baseline value of 17 OHP between 5.1 and 9 nmol/L is sufficient for the diagnosis of NCCAH (13, 16, 17). Recently, a level of basal 17 OHP of 4.6 nmol/L was suggested as a threshold for ACTH testing to predict NCCAH in subjects with premature adrenarche during childhood (18).

The sum total of these findings and suggestions indicates that the selection of patients who will undergo a Synacthen stimulation test should be evaluated case by case. A 17 OHP post-stimulation level over 3 nmol/L is required for the diagnosis (19). Heterozygotes carrying one CYP21A2 mutation exhibit slightly elevated 17 OHP levels post ACTH stimulation, though there is overlap in unaffected subjects (9). However, as Dacou-Voutetakis et al. have pointed out, if the sum of basal and post-stimulation 17 OHP values exceeds 1.5 nmol/L, then the possibility of heterozygosity is exceptionally high (20).

Another important consideration regards the techniques used for 17 OHP evaluation. 17 OHP levels are measured by a variety of immunoassay methods, but as has recently been shown, the most accurate and reliable results were achieved by the implementation of the combination of liquid chromatography with mass spectrometry (LC-MS/MS). Indeed, many false positive 17 OHP measurements were found when LC-MS/Ms measurements were compared with standard methods (21) Nevertheless, the latter procedures are not universally used, in the event of an uncertain diagnosis, they will provide more precise results. Of note, the screening and diagnostic thresholds for LC-MS/MS quantified 17 OHP are yet to be defined. These thresholds are likely to be lower than those established with immunoassays due to the enhanced specificity of LC-MS/MS, a method less prone to cross-reactivity and interferences (22, 23). Whether a urinary steroid profile is required for the definitive diagnosis remains to be elucidated (22). In borderline cases, it is advisable to obtain a complete adrenocortical profile after the ACTH stimulation test to differentiate 21-hydroxylase deficiency from other enzyme defects and establish a firm diagnosis.

Specifically, a complete steroid profile should be performed in equivocal cases to confirm 21-hydroxylase deficiency and exclude other enzymatic detects. Inclusion of 17 OHP, cortisol, 11-deoxycorticosterone,11-deoxycortisol,17-OH-pregnenolone, Dehydroepiandrosterone, and androstenedione in a serum steroid profile would be useful to exclude other causes of CAH such as 11β-hydroxylase deficiency and, more rarely, 3β-hydroxysteroid dehydrogenase deficiency or P450 oxidoreductase deficiency (24). While genetic testing is not considered to be a primary diagnostic tool for NCCAH at this time, it is mandatory for diagnosis confirmation and for genetic counseling (3).

As far as the cortisol levels are concerned, generally a post-stimulation value of atleast 496 nmol/L is expected. Of note, according to Stoupa et al., 60% of 47 children with NCCAH as a result of 21 OHD had low cortisol values after the stimulation, a finding pointing to the need for increased surveillance for the development of adrenal insufficiency during major stressor events (25).

NCCAH MANIFESTATIONS IN WOMEN

The clinical expression of NCCAH is characterized by a high level of polymorphism as concerns not only age of onset but also the different signs and symptoms. It is reported that the first clinical presentation of NCCAH is in 11% of cases before the age of 10 years and in 80% between the ages of 10 and 40 years (12). The genotype-phenotype correlation in CAH and NCCAH has not as yet been elucidated. Speiser et al. suggest that most but not all of the phenotypic variability in 21-hydroxylase deficiency results from allelic variation in CYP21A2 (26).

Manifestations in Childhood

In the newborn period, typically NCCAH females remain asymptomatic and have normal external genitalia. The earliest case of NCCAH reported is a 6 month old girl who developed pubic hair (11). Usually, clinical findings and symptoms in NCCAH cases start from the age of 5 years or even later and are related to increased androgen levels, though mild cortisol deficiency can also occur in some cases (8). In 92% of NCCAH cases, the first symptom that manifests is premature pubarche (PP), which occurs under 8 years of age in girls and under 9 years in boys (12) with an estimated prevalence of between 10 and 11.3% (15). Of note, in children referred with PP, according to different studies, the incidence of NCCAH ranges from 5 to 30% (20, 27). However, based on our analysis of 280 patients with molecular confirmation of NCCAH, we found that the incidence of PP in 94 females younger than 8 years was as much as 88%. On the other hand, an analysis of 45 males with NCCAH identified PP in only 29% of subjects (14). Furthermore, we should keep in mind that about half of subjects with PP may be heterozygote carriers of a CYP21A2 mutation.

Another aspect that needs clarification is the relationship between PP and precocious puberty. It is hypothesized that in some cases, the peripheral aromatization of adrenal androgens to estrogens may activate the hypothalamic-pituitary-gonadal axis, leading to secondary precocious puberty (28). In such an instance regular follow-up is needed. Some children develop hirsutism, acne, and/or rapid growth and manifest tall structure and bone age advancement (29). The latter can result in truncated final height as a consequence of rapid epiphyseal fusion. Other rare clinical findings during childhood include labial adhesion, perianal hair, clitoromegaly, and hoarseness of voice (30–32).

Manifestations in Adolescence and Adult Life

During adolescence and adulthood, NCCAH in women presents with hyperandrogenic symptoms resembling polycystic ovary syndrome (PCOS) (33). Symptoms include hirsutism, acne, androgenic alopecia, anovulation, menstrual dysfunction, and infertility. In a multicenter study, the most common symptoms among adolescent and adult women were hirsutism (59%), oligomenorrhea (54%), and acne (33%). Among 161 women with NCCAH, presenting symptoms were hirsutism (78%), menstrual dysfunction (54.7%), and decreased fertility (12%) (34). In our group of 161 women with the disease 63% of the patient presented with a polycystic ovary-like phenotype (14). This finding has a *vice versa* implication, since the incidence of NCCAH is high in women with hyperandrogenaemia. Certainly, the study by Witcel et al. reporting an incidence of NCCAH ranging from 1 to 33% in hyperandrogenic women is of great interest. In another study of 950 women who were referred for evaluation of androgen excess, NCCAH was detected in 4.2% of them. However, the NCCAH patients were younger and more hirsute compared with the other groups of hyperandrogenic patients. They were also characterized by significantly higher levels of testosterone, free testosterone, and 17 OHP than patients with other hyperandrogenic syndromes. In ovarian ultrasound, 77% of them displayed polycystic ovaries and 41% increased ovarian size. In sum, they fulfilled the PCOS criteria according to NIH and Rotterdam at a percentage of 56 and 72.8%, respectively, (35).

The progressive nature of the disease is highlighted by the fact that the prevalence of hirsutism has been shown to increase with age and has been observed to be rare before puberty. Hirsutism is the most common clinical manifestation reported in patients with NCCAH, ranging from 71 to 96% (28, 36). Pubertal girls with NCCAH typically present with hirsutism (37, 38). At the other end of the spectrum is the issue of alopecia. Male pattern balding is reported in NCCAH patients. The prevalence of alopecia also appeared to increase with age, from 6% in patients during the second decade of their life to 19% in the fifth, indicating again the progressive nature of the disease (39). Acne is reported in almost 33% of NCCAH subjects (36). Remarkably, mutations of the CYP21A2 gene causing NCCAH were detected in 4.9% of 123 adult females presenting with severe acne (40).

Menstrual irregularities including oligomenorrhea or secondary amenorrhea can often be the presenting sign of NCCAH in post menarche individuals. Additionally, 8–9% of cases also experience primary amenorrhea and, for this reason, seek medical advice for the first time. Moreover, among NCCAH individuals, oligomenorrhea is more common than primary amenorrhea. For example, in a series from Moran et al.,

among NCCAH women aged 10 to 19 years, oligomenorrhea was present in 56% of the cases in comparison to only 9% of adolescence with primary amenorrhea (12). To conclude, so many NCCAH symptoms resemble those of PCOS that it is not surprising that NCCAH has been dubbed the big mimicker of PCOS. In any case, a diagnosis of NCCAH should be considered during the evaluation of any young woman who is referred for hyperandrogenic symptoms.

In a study by Arlt et al., patients with NCCAH were characterized by significantly higher BMI and lower final height in comparison to matched controls (41). These data, in conjunction with a few studies that indicate the presence of insulin insensitivity, suggest an unfavorable metabolic profile that could be pathophysiologically explained by the effect of elevated androgen concentrations and/or as a result of glucocorticoid therapy (42, 43). Osteoporosis can also be detected in these subjects, probably as a consequence of the corticosteroid therapy (34).

NCCAH MANAGEMENT FROM THE FIRST MANIFESTATION TO THE ADULT LIFE

Once the diagnosis of NCCAH has been established, several issues related to subsequent treatment recommendations warrant consideration. The first question to be addressed is whether glucocorticoid (GC) therapy is indicated. The general reasoning behind this approach is that by providing sufficient cortisol concentrations to the patient, her daily needs are covered and, consequently, CRH-ACTH axis stimulation will be tapered, leading to decreased adrenal androgen production. The general rule concerning this approach is that GCs are given at replacement and not pharmacological doses, while the influence of age, gender, laboratory data, patient-specific recommendations, and goals of treatment on glucocorticoid replacement therapy are seriously taken into consideration. However, we should also be aware that prolonged GC substitution therapy may lead to hypercortisolemia, with the resultant well-documented adverse effects on every aspect of metabolism, especially growth, fat distribution, and insulin resistance as well as on psychological profile. Another major disadvantage of this approach is the lack of an adequate clinical index or biochemical marker of adequate replacement dosage, such as exist regarding TSH values in hypothyroidism.

Finally, the fact that there is no consensus as to which GC should be used and the absence of long-term data regarding different modalities of GC administration further complicate attending physicians' decisions on and selection of the optimal choice for each patient. Hydrocortisone is typically used in children, as it most closely resembles the natural hormone (cortisol), but it is not considered a suitable approach in adolescents and young females due to the need for multiple daily dosing. Hence, most adult endocrinologists prefer either dexamethasone or prednisolone, at appropriate doses. On the other hand, it must be pointed out that the equivalence of different GCs is based on their anti-inflammatory action and not on different aspects of human metabolism. Since cortisol affects almost 20% of the human genome, diverse responses of different GCs are expected in various tissues. Corroborating this perception is the fact that the administration of dexamethasone to patients with CAH has shown deterioration of indices of insulin resistance in comparison to other GCs (44). Additionally, the administration of 2.5–7.5 mg of prednisolone, a dose considered as normal, exerts a longstanding negative impact on bone metabolism (45). Therefore, hydrocortisone should be considered the best form of treatment in cases of GC supplementation therapy. The advent of the newly synthesized hydrocortisone formula with one pill per day and its initial positive results in patients with CAH shows much promise for the future (46).

Management During Childhood

NCCAH patients who are diagnosed during childhood with signs of PP may be treated with hydrocortisone with the aim of suppressing the adrenal hormones and preventing rapid advancement of bone age that could affect final height. In those patients in whom hydrocortisone treatment was initiated 1 year before the onset of puberty and who had a bone age below 9 years, final height remained within the genetic potential (47). Available studies indicate that adult height approached the expected target height in patients that were closely monitored and who strictly complied with medication plans (48–50). A recent small study of five cases (patients 6.1–9.2 years of age) demonstrated that an ultralow dose of dexamethasone is a promising option to reduce endogenous stress and its effects. Whether this constitutes a realistic approach and how much the administered dose should be remain to be elucidated (51). A study by Nebesio et al. compared the hormonal effects and pharmacokinetics of hydrocortisone, prednisolone, and dexamethasone in 9 prepubertal patients with CCAH. They showed that dexamethasone was more potent that the other forms in achieving significantly lower adrenal hormone levels, hence suggesting that dexamethasone is more efficacious for the suppression of adrenal androgen production (46). Of course, further studies are needed to verify or reject this finding. Furthermore, in some cases, elevated androgen concentrations may lead to secondary stimulation of the GnRH axis, leading to premature puberty. In such a case, a parallel course with GnRH analog may be prescribed if bone age is significantly higher than chronological age and/or projected final height is disproportionate to target height.

Another important consideration regards cases of concomitant GH deficiency in the event that treatment with GnRH analogs and GH prescription resulted in attainment of target height (52). According to the current guidelines, the above-described therapy is cautiously recommended only for those cases in which the predicted height SD is $-2.25 <$ the target height or even lower (53). An alternative indication for starting hydrocortisone treatment is an inadequate cortisol response post ACTH stimulation (36).

The preferred GC treatment in children is usually hydrocortisone 10–15 mg/m^2, divided into three doses. Often, lower doses have also proved effective, starting from 6 mg/m^2/day (34). Overdosing should be avoided, considering

it that can result in poor growth as well as Cushingoid features. Regular growth pattern, a bone age compatible to chronological age, and absence of central obesity may also serve as clinical indices for appropriate management. Regarding biochemical/hormonal profile, androstenedione and testosterone levels in the mid to upper ranges for bone age are considered better markers of adequate GC replacement therapy in children with NCCAH. However, suppressed testosterone levels were found in 10% of NCCAH patients, whereas another 28% of patients had increased testosterone concentrations, this phenomenon possibly being attributable to hydrocortisone variability (54, 55). DHEAS values are decreased with very small doses, whereas the suppression of 17 OHP and progesterone levels requires very high GC doses. It should be noted that 17 OHP values are crucial for the diagnosis, but not helpful during follow–up. Of note, blood sampling for hormonal evaluation must be carried out without cessation of therapy. However, due to the perplexity of the disease and its multifaceted nature, there are no specific guidelines for the timing of regimen changes or cessation of glucocorticoid therapy in children.

Management During Adolescence

Patients Treated Since Childhood

Until the establishment of the normal menstrual pattern in NCCAH girls, the continuation of GCs that started during childhood is highly recommended (56). However, adolescent patients frequently do not show sufficient compliance with chronic administration of drugs and often omit doses. Additionally, during puberty, the half-life of hydrocortisone falls by 50% as a result of increased IGF-1 levels, which diminishes 11βOHSD activity, as well as due to increased cortisol clearance stemming from amplification of glomerular filtration rate (57). In the young female, 2 years post menarche and if normal ovulatory cycles have been recorded, a patient-centered approach toward the hyperandrogenic symptoms that may appear is highly recommended.

If there are severe hyperandrogenic findings, such as hirsutism, acne, and/or oligomenorrhea, continuation of treatment will be considered. It is important to remember the fragile sensibilities of a young adolescent, which will be seriously damaged by the "repellent" signs of hyperandrogenism. The combination of hyperandrogenic signs, menstrual disorders, and poor quality of life is well documented and it is particularly high in younger women. Another point to be considered is the response of adrenal reserve post ACTH stimulation. Given that peak cortisol of pubertal and adult females after stimulation is below 496 nmol/L, in cases of stress, steroid treatment should be administered (58). On the other hand, if none of the above symptoms are encountered in the young patient, GC treatment may be discontinued, after which regular follow-up is advised.

Females With First Manifestation During Adolescence or Hyperandrogenic Symptoms After Treatment Discontinuation

In the presence of hyperandrogenic symptoms, a patient-oriented approach is highly recommended, focusing on the main complaints of the patient. This is far better than a general universal *modus operandi*, since, with the latter approach, the young patient will be disappointed, this leading to treatment discontinuation and, as a result, to adverse health outcomes in adulthood. In cases with severe hirsutism, a course of 6 to 12 months with oral contraceptive pills (OCPs) constitutes a reasonable procedure given the beneficial effects of OCPs on SHBG liver production and the decrease of androgen release from the ovary. Clinical improvement will be expected following at least 2–3 months of OCPs initiation, while the concurrent use of a progestagen with minimal androgenic properties is highly recommended. If OCPs fail as the first line approach, antiandrogens (spironolactone, flutamide, bicalutamide, cyproterone acetate, and finasteride) may be added to the treatment. In our experience, the administration of bicalutamide has achieved significant improvement in cases of severe acne, but similar results were not obtained in cases with severe hirsutism. Cosmetic approaches such as laser application and depilatories can also be suggested for women complaining of excessive or unwanted hair growth or scalp hair loss (androgenic alopecia) (32, 34, 57).

In patients with inadequate cortisol secretion after stimulation, or if OCPs and antiandrogens cannot be tolerated, a course with GCs is highly recommended (57). Data from New et al. indicate that irregular menses and acne are reversed within 3 months after the initiation of the glucocorticoid therapy, whereas hirsutism requires nearly 30 months (59). By contrast to childhood, in adolescence, longer-acting steroids are often used and regimens of 5 mg of prednisolone or 0.25 mg of dexamethasone are recommended (53). However, in the real world clinical data have shown a variety of different regimens applied in NCCAH management (41).

The primary goal of treatment should be the patient's relief from the hyperandrogenic symptoms. Thus, among individuals with NCCAH diagnosed based solely on laboratory results, clinical features should guide management recommendations, since hormonal and molecular findings do not necessarily predict clinical severity. According to the Endocrine Society guidelines, NCCAH patients should be given the option to discontinue GC therapy when symptoms resolve (60). Of course, these patients should not be lost to follow-up, while treatment should be reinitiated in the event of recurrence of the symptoms. Further, in the case of discontinuation, patients should be informed about the possibility of infertility and should be encouraged to seek medical advice if they wish to conceive (19). Of note, the appropriate transfer of the patient from the pediatric to the adult endocrinologist should be carried out, optimally after 1 year of synchronized monitoring (53, 61).

NCCAH AND REPRODUCTION

Subfertility

Subfertility is commonly reported in classical forms of CAH, which is mainly due to menstrual disorders, chronic anovulation, and anatomical deformities. The birth rate has been estimated at 17% in comparison to 65% of the control population (62). Meanwhile, data regarding fertility in women with NCCAH have recently been assessed in detail and the estimated infertility

incidence is 11% among NCCAH women, that is, relatively milder than in CAH, and in many cases is easily resolved. Bidet et al. evaluated fertility in 190 women suffering from NCCAH, 95 of whom wanted to become pregnant. In this population, 187 pregnancies in 85 women were reported, which resulted in 141 births in 82 individuals. It must be highlighted that 99 of the pregnancies (52.9%) occurred before the diagnosis of NCCAH, three of them with the application of ovulation induction protocols, the rest being spontaneous, while 88 pregnancies took place post NCCAH diagnosis. In the vast majority of them the pregnancy developed after the institution of therapy with hydrocortisone, whereas in 11 women it happened spontaneously (63, 64). In another report of 22 observed NCCAH women desiring pregnancy, 12 pregnancies ensued with prednisone (65).

Miscarriages

In the cohort of Bidet et al., the miscarriage rate was 6.5 and 26.3% in patients treated with GCs and untreated patients, respectively (64). The outcome of the pregnancy may even be successful without any glucocorticoid treatment in cases where NCCAH was not yet diagnosed, as also reported in a case report by Cuhaci et al. Nevertheless, the same couple experienced two miscarriages and reported subfertility after this first pregnancy (66). As far as the possibility of preterm pregnancies is concerned, as assessed by Bidet et al., it does not differ significantly from that of the rest of the population (15).

Fertility Planning

For those women with symptomatic hyperandrogenism or with reported infertility but who wish to conceive, GC therapy is highly recommended. During this period, the glucocorticoids used are prednisone or hydrocortisone. The most important action, because it will guide the subsequent steps, is genetic testing of the prospective parents. Females with NCCAH who desire to conceive should be aware of the risk of giving birth to an infant with the classical form of the disease. The outcome of pregnancies among women with NCCAH, and more specifically the incidence of infants born with CCAH, is estimated in recent studies to be between 1.5 and 2.5%, with NCCAH at about 15% (36, 64). Of course, this frequency depends on the reference population, a higher incidence occurring in populations with high intermarriage rates (36). The predicted chances of parents giving birth to a child with CAH is 1 in 120 for unknown paternal genotype, zero if the father has no relevant mutation, and 1 in 4 if he has a heterozygous mutation (19). As a result, genetic testing of the partners of these women is essential to assess the risk of giving birth to a child with the classical form of CAH (64, 67).

In the case that the prospective mother carries two NC mutations (**Table 1**), there is no absolute need for genetic testing in the future father, since, if he is heterozygous for the CYP12A2 mutations, the offspring is at risk of developing the non-classical form of the disease in the future. However, it should be noted that carrier status is 2–15% in different populations and half of these individuals carry a severe mutation. Furthermore, definitive recommendations regarding situations when genetic testing is not required are difficult given the imperfect genotype-phenotype correlation, particularly for milder mutations [81].

TABLE 1 | Mutations of CYP21A2 with minimal (C) or moderate (NC) residual enzyme activity (14, 68, 69).

Classic (C) mutations estimated residual activity 2–10%	Non-classic (NC) mutations estimated residual activity 10–78%
E380D	G424S
I235N	G375S
I172N	H62L
IVS2-13A/C>G	K121Q
G292S	P453S
Q318X	P30L
R354C	P482S
R356W	P105L
R426C	R339H
R483Q	V281L
V236E	V304M
W22X	
W406X	
Microconversions	
Deletions (30-KB DEL, 8-BP DEL)	
Large gene conversions	

For example, the P30L mutation, most frequently associated with NCCAH, does also occur in patients with classical CAH [82]. A large multicentre European study recently showed that this was the case in 78% of patients [81]. In this study, the mild V218L mutation was associated with Classical rather than NCCAH in 30% of cases. Hence all patients with NCCAH should be offered genetic counseling and molecular assessment of reproductive partners.

By contrast, in the event that the prospective mother is a compound heterozygote with one severe (C) and one NC mutation, then genetic testing of the future father is mandatory. We should keep in mind that data originating from studies using immunoassays or the more accurate LC-MS/MS procedures, consistently highlight the inability to reliably exclude heterozygosity using basal or ACTH stimulated 17 OHP values, due to the significant overlap with the normal range. One may suggest the use of a Synacthen test, and if it is not compatible with heterozygosity (sum of basal and peak stimulated 17 OHP values < 1.5 nmol/L), then DNA testing could be avoided. However, we should be very cautious in the interpretation of this test, since it has not been validated in other studies. Hence, the use of ACTH stimulated 21-deoxycortisol either singularly or as part of a steroid ratio or steroid profile, may facilitate the biochemical identification of heterozygotes in the future, particularly as LC-MS/MS becomes more widely available [83–85]. If the prospective father carry a NC mutation, then nothing else is needed. Conversely, if he is a carrier of a Classical mutation, the question concerning prenatal treatment of the fetus will arise. All possible genotype combinations and the suggested procedures are presented in **Table 2**.

Prenatal Treatment of CAH

The aim of prenatal treatment of CAH is the prevention of genital virilization of the fetus, but also alleviation of the stress felt

TABLE 2 | Planning of suggested procedures during pregnancy based on prospective parents genotype.

Genotypes		Procedures
Mother	**Father**	
NC/NC	?	None
NC/NC	Normal	None
NC/C	?	DNA testing of future father Fetal sampling? Prenatal treatment??
NC/C	Normal	None
NC/C	NC/Normal	None
NC/C	C/Normal	Fetal sampling Prenatal treatment?

? indicate the unknown values.

by the parents who are likely to have a child with ambiguous genitalia (70). Dexamethasone is used because of its ability to cross the placenta and because it is not deactivated from the placental 11β OH steroid dehydrogenase and binds only minimally to the mother's blood cortisol-binding globulin (CBG). Dexamethasone, by suppressing fetal ACTH secretion, decreases elevated fetal androgen production. The treatment must be discontinued in the event that karyotype or DNA analysis reveals a male or an unaffected female, respectively (71). Nevertheless, although fetal genital virilization has already started at 6–7 weeks post conception, chorionic villous biopsies for genetic diagnosis cannot be obtained before the 10th−12th week. This time interval suggests that all pregnancies at risk for virilizing CAH should be treated, even though only 1 out of 8 fetuses is affected and female (19, 34, 70).

Whether this exposure of the fetus to dexamethasone for preventive measures is medically and ethically acceptable remains controversial (70). Animal studies have demonstrated that first-trimester dexamethasone decreases birthweight, affects renal pancreatic beta cell function and brain development, and predisposes to anxiety, hypertension, and hyperglycemia in adulthood (70). Furthermore, 10–20% of pregnant women using dexamethasone treatment during pregnancy complain of weight gain, increased appetite, mood swings, insomnia, edemas, hypertension, hyperglycemia, and striae. However, New et al. found a lower than average Prader score in fetuses treated with dexamethasone prenatally, but no difference in long-term outcomes (72). Additionally, in a review by Merce Fernandez-Balsells et al., dexamethasone was shown to be associated with reduction in fetus virilization without significant maternal or fetal adverse effects (63). However, the authors of the abovementioned review as well as the consecutive guidelines of the Endocrine Society point out that because of the small sample sizes in the whole body of the literature, the subject remains uncertain and further investigation is clearly needed.

The decision about initiating treatment should be undertaken only in large centers with an experienced team and protocols approved by Institutional Review Boards and based on the family's values and preferences and with their written informed consent as a prerequisite (19, 63). Several specialists in the field indicate that a higher value should be placed on preventing unnecessary prenatal exposure of mother and fetus to dexamethasone rather than imposing the emotional toll of ambiguous genitalia on parents and patients. Most importantly, it should be clarified to parents that dexamethasone administration does not modify patient status but is directed toward reducing the need for surgery rather than preserving life or intellectual capacity. They must also know that the probability that their child will suffer from the classical form of the disease is high, despite treatment. Most crucially, meanwhile, the exposure of a young organism to a very potent GC during a particularly sensitive period of fetal programming and growth, which might well prove useless in the case of a fetus with the XY karyotype, is not at present supported by robust and unquestionable data.

Ideally, there should be an early diagnosis, this performed via a non-invasive technique and before the beginning of the genital virilization of the fetus. Working in this direction, novel studies point to the use of cell-free fetal DNA obtained from maternal plasma as a promising method that will allow the determination of fetal gender and the diagnosis of CAH at an early gestational age (<9 weeks). If this procedure is widely implemented in clinical practice, unnecessary prenatal dexamethasone treatment will be avoided (73).

Treatment During Pregnancy

During pregnancy, women with NCCAH should preferably be treated with hydrocortisone, not only because pregnancy is typically accompanied by elevated stress levels, but also as a preventive measure against the abovementioned high incidence of miscarriages. This form of glucocorticoid is favored due to its metabolization by placental enzyme 11β OH steroid dehydrogenase II, which prevents glucocorticoid from having any effect on the fetus (71). At the time of conception, the hydrocortisone dose should be increased to 20–25 mg/day and dose modifications are carried out every 6–8 weeks with the aim of keeping testosterone levels at the upper normal levels of the pregnancy trimester (74). As an example, the testosterone and D4 values of a patient with NCCAH from conception up to 3 years post-delivery of two healthy twins are shown (**Figure 1**).

OTHER SPECIAL ISSUES

Stress Management in NCCAH

During major life-threatening stress, surgery, or serious illness, patients with NCCAH who are glucocorticoid-treated may require larger or more frequent doses of glucocorticoids given that their adrenal function is iatrogenically suppressed. It is therefore crucial to educate the parents of young children, as well as to re-educate patients on their transition to adult care, about stress dosing. For NCCAH patients who are not treated with GCs or in the event of discontinuation, cortisol response to ACTH should be assessed. Almost one third of NCCAH patients respond inadequately to the stimulation (15, 75). For those who respond normally to ACTH stimulation no treatment with stress doses is recommended (19). Mineralocorticoid therapy is not required in any of the cases, as these patients have

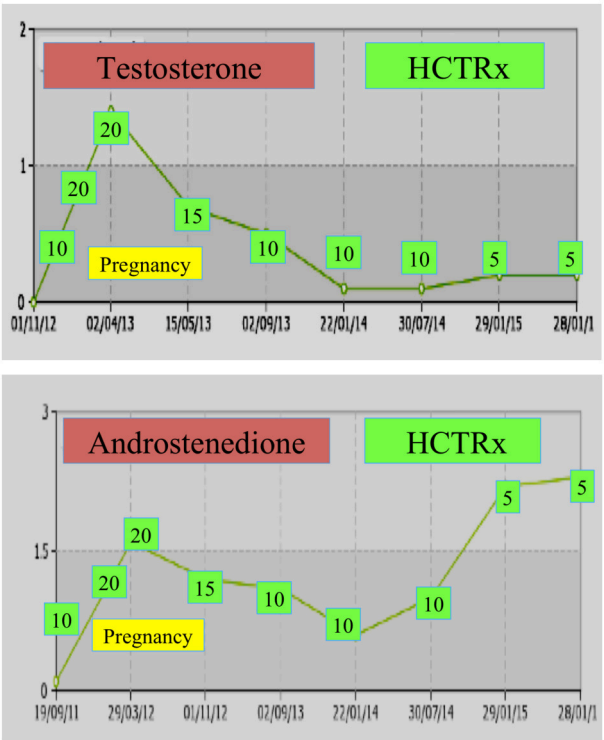

FIGURE 1 | Testosterone and andostenedione levels and subsequent hydrocortisone dosing (HCTRx), from conception until 3 years post-delivery administered to a patient with NCCAH.

normal aldosterone secretion and do not develop salt-wasting (58). Of note, in the case of hyperthyroidism or an increase in levothyroxine treatment, cortisol clearance is increased and an adrenal crisis may occur (58, 76).

Psychobiological Aspects in NCCAH

Meyer-Bahlburg et al. have in several studies reported impaired psychological profile among patients with NCCAH due to 21OH deficiency. More specifically, the affected women had a higher masculinization/defeminization score on several measures of gender-related behavior when compared with normal control women, although markedly less so than in women with classical CAH. They also had significantly increased bisexual or homosexual orientation. Retrospective clinical-qualitative interviews with these women revealed a history of discomfort and social stress related to their pre-treatment experiences with androgen-dependent signs, such as acne, hirsutism, and conception difficulties (59, 77, 78). Similarly, Arlt et al. showed that subjective health status was significantly impaired across all eight domains of a short-term health survey, with the most prominent differences, as compared with age- and sex-matched controls, relating to the domains general health, vitality, and role limitations due to emotional problems. The Hospital Anxiety and Depression Scale (HADS) questionnaire also revealed increased anxiety scores (41). Bearing in mind these findings, psychological parameters to guide therapy should be considered in women with NCCAH and, in the context of the patient-oriented approach, a psychological diagnosis and support need to be offered.

CONCLUSIONS

NCCAH is considered as the more frequent and milder form of CAH because of retention of 20–50% enzyme activity. Most patients may seek medical advice at any stage of their life due to symptoms related to androgen excess. These include premature pubarche, rapid growth, hirsutism, acne, menstrual irregularities, or infertility, as well as many other less prominent manifestations. Early morning baseline values of 17 OHP as a good initial screening test and further evaluation with ACTH stimulation and, in the case of borderline results, genetic testing, is recommended. As a general rule, androstenedione and not 17 OHP levels should be used during follow-up.

The treatment decision should be based on assessment of the facts and should follow an individualized approach. Treatment is not always indicated unless the patient is symptomatic, for example, children with early onset and rapid progression of pubic and body hair, rapid growth, and/or skeletal advancement, or women with oligomenorrhea, acne, hirsutism, infertility, or a combination of these and others of the abovementioned symptoms. Genetic counseling is strongly advised in NCCAH women who wish to conceive, as well as genotyping of the father. The overall management of the patient additionally includes management of the probable complications of glucocorticoid therapy or metabolism-related manifestations of the disease. Lastly, given that many therapeutic issues related to the appropriate management of these patients have not as yet been elucidated, it is very important for the attending physician to keep up to date with all developments in this field and to integrate the new data into his clinical practice. Certainly, further clinical studies in this area are essential.

To sum up, for the NCCAH woman, the ideal approach is a tailor-made one, incorporating a smooth transition of her management once she is referred from the pediatric to the adult endocrinologist, along with symptom-oriented treatment that will accompany her throughout her life. Given the multiple factors affecting the hyperandrogenic system, it is advisable to encourage patients to adopt a healthier lifestyle by improving their dietary habits, increasing exercise, and aiming at weight reduction. Furthermore, in certain cases, psychological support is often beneficial. Moreover, specialists in fields involved in the treatment of this disorder, such as dermatologists, gynecologists, and psychologists, need in-depth understanding about the management of suspicious or already diagnosed cases of NCCAH. To conclude, let us keep in mind the insightful quote of American astronomer Vera Cooper Rubin: "Science progresses best when observations force us to alter our preconceptions."

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Polycystic Ovary Syndrome and NC-CAH: Distinct Characteristics and Common Findings. A Systematic Review

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Background: Twenty-one-hydroxylase–deficient non-classic adrenal hyperplasia (NC-CAH) is a very common autosomal recessive syndrome with prevalence between 1:1,000 and 1:2,000 individuals and the frequency varies according to ethnicity. On the other hand, polycystic ovary syndrome has a familial basis and it is inherited under a complex hereditary trait. This syndrome affects 6 to 10% of women in reproductive age and it is the most common endocrine disorder in young women. Our aim was to investigate, through a systematic review, the distinct characteristics and common findings of these syndromes.

Methods: The search period covered January 1970 to November 2018, using the scientific databases PubMed. Inclusion criteria were adult women patients with PCOS or NC-CAH. Search terms were "polycystic ovary syndrome," "PCOS," "non-classical adrenal hyperplasia," "NC-CAH," "21-hydroxylase deficiency." From an initial 16,255 titles, the evaluations led to the final inclusion of 97 papers.

Results: The clinical features of NC-CAH are hirsutism and ovulatory and menstrual dysfunction therefore; differentiation between these two syndromes is difficult based on clinical grounds only. Additionally, NC-CAH and PCOS are both associated with obesity, insulin resistance, and dyslipidaemia. Reproductive abnormalities are also common between these hyperandrogenemic disorders since in patients with NC-CAH polycystic ovarian morphology and subfertility are present as they are in women with PCOS. The diagnosis of PCOS, is confirmed once other disorders that mimic PCOS have been excluded e.g., conditions that are related to oligoovulation or anovulation and/or hyperandrogenism, such as hyperprolactinaemia, thyroid disorders, non-classic congenital adrenal hyperplasia, and androgen-producing neoplasms.

Conclusions: The screening tool to distinguish non-classic adrenal hyperplasia from PCOS is the measurement of 17-hydroxyprogesterone levels. The basal levels of 17-hydroxyprogesterone may overlap, but ACTH stimulation testing can distinguish the two entities. In this review these two common endocrine disorders are discussed in an effort to unveil their commonalities and to illuminate their shadowed distinctive characteristics.

Keywords: non-classic adrenal hyperplasia, NC-CAH polycystic ovary syndrome, PCOS, 17-hydroxyprogesterone, 17-OHP, 21- hydroxylase deficiency, 21-OHD

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INTRODUCTION

Rationale

Twenty-one-hydroxylase–deficient non-classic adrenal hyperplasia (NC-CAH) is a relatively common autosomal recessive disorder with prevalence between 1: 1000 and 1: 2000 individuals and the frequency varies by ethnicity (1). On the other hand, polycystic ovary syndrome has a familial predisposition and is inherited under a complex genetic mechanism. Polycystic ovary syndrome affects 6 to 10% of reproductive-aged women and is one of the most common endocrine disorders (2). The clinical features of NC-CAH are hirsutism and ovulatory and menstrual dysfunction as well as insulin resistance and polycystic ovarian morphology (3).

Objectives

Women with NC-CAH present with similar symptoms as with PCOS women and therefore, it is difficult to differentiate the two disorders based on clinical grounds solidly (4). This difficulty becomes apparent on several studies from different parts of the world, as in Turkey (5, 6), and in Greece (7), where women with NC-CAH were diagnosed as PCOS women at the beginning. About 1–4% of women in USA with clinical signs of androgen excess have NC-CAH (8–10). The main objective of this review is to find the common characteristics of the two syndromes.

Research Question

The main search question of this review is to underline the main differences of the two syndromes and to discuss the methods to differentiate them.

METHODS

This review was performed following the preferred reporting items for systematic reviews and meta-analyses (PRISMA) guidelines (11).

Criteria for Selection

Articles describing the main characteristics of PCOS and NC-CAH were considered, including case-control, cross-sectional, and cohort studies.

Search Strategy

Studies in English that met the above criteria were collected by searching the Pubmed database: The MeSH terms ("polycystic ovary syndrome" OR "PCOS") AND ("non-classical adrenal hyperplasia" OR "NC-CAH" OR "21- hydroxylase deficiency" OR "21-OHD"), complemented with manual searching, for publications listed up until November 2018. Two independent investigators conducted the searches. The list of recognized articles was scanned, and the reference lists of all related reviews and main articles were searched manually for more references. To decrease bias, two authors conducted the searches independently, and any disagreement between them was debated in a group discussion until a consensus was achieved.

Study Selection

The authors independently assessed the titles and abstracts of all identified studies. Full reports were obtained for those studies that appeared to meet the inclusion criteria.

Data Extraction and Management

The following data were recorded from each study: authors, country of origin, study type, the main outcome measures, and the outcomes.

Inclusion and Exclusion Criteria

For further review, the authors screened abstracts and titles. Since every screened study was included in this systematic review, the researchers attempted to evaluate the relationship between PCOS and NC-CAH and underline their common findings and their differences. Studies on non-human creatures (i.e., animal studies), those published in languages other than English, those that were meta-analyses or systematic considerations, and those that presented insufficient data or were duplicate publications were also excluded. The research was conducted in conformity with the ethical standards of the field.

RESULTS

A total of 16,255 papers were initially identified. Of these, 801 were excluded because they were duplicates or had irrelevant contents. A total 9,642 more articles were excluded after titer and abstract screening and 5,812 articles were retrieved. A total of 5,715 articles were also excluded after full text screen and during data extraction. Finally, only 97 articles were included in this systematic review, as shown in Prisma Flow Diagram (**Figure 1**).

DISCUSSION

The main findings of our search were categorized according to epidemiology, genetics, pathophysiology, and clinical parameters. The main different characteristics and differentiated criteria were also described as well the different treatment options.

EPIDEMIOLOGY OF NC-CAH AND PCOS

The NC-CAH is a common autosomal recessive disease, and the frequency changes in different ethnicities. The prevalence is as high as 1:1000 to 1:100 in white population (12–14), and even higher in women with Mediterranean, Hispanic or Eastern European Jew origin (8). Non-classical 21-hydroxylase deficiency (21-OHD) can be diagnosed by the elevation of 17-OHP that plot a nomogram between the range of unaffected persons and the levels of patients with the classical form of CAH. Similar to CAH, non-classical 21-OHD can cause premature growth of pubertal hair, acne, advanced bone age, accelerated linear growth velocity and reduced final height in both sexes (15). The classic form can be diagnosed in the neonatal screening test with a very high 17-hydroxyprogesterone (17-OHP) (16). It appears that the false negative rate is at least one-third in children with the moderate form of CAH. On the other hand, polycystic ovary syndrome

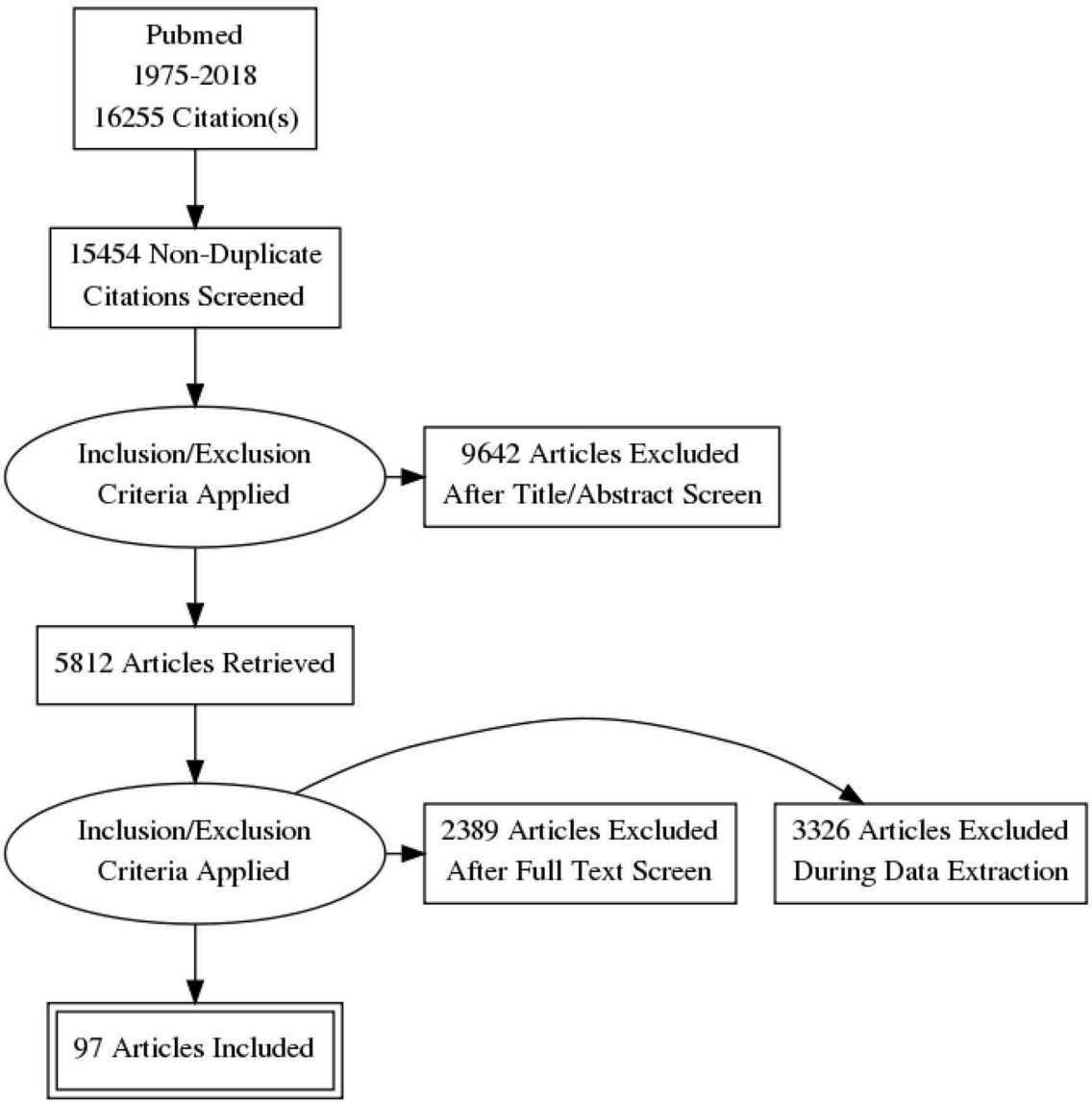

FIGURE 1 | Flow diagram showing an overview of the articles selection process.

(PCOS) appears in 6–10% of the women in reproductive years and it is the most common endocrine disorder in these women (2), but according to Rotterdam criteria, the prevalence can be as high as 10% (17).

DISTINCT CHARACTERISTICS

Genetics

The etiology of PCOS remains equivocal; however, genetic, environmental and lifestyle factors interact each other and predispose to the syndrome. PCOS is considered to have a complex genetic background (2, 18, 19). The pathogenetic mechanisms of PCOS are connected to genetic and environmental parameters. An increased familial incidence implies that a complex genetic trait possible plays a role (20–23). Different genes are involved that are related to different hormonal and biochemical paths such as steroid and androgen synthesis, insulin production, folliculogenesis, gonadotropins, and weight control (24). Chromosomes such as chr 8 p 23.1, chr 11 p 14.1, and chr 9 q 22.32 have been associated with polycystic ovary syndrom, as well as chr 11p14.1 SNP, rs11031006 of the FSHB gene, have been directly associated with polycystic ovary syndrome and the synthesis of LH (19).

Nevertheless, NC-CAH is characterized by CYP21A2 deficiency, which is transmitted as an autosomal recessive disorder. The two CYP21A genes are both located in a 35-kilobase region of chromosome 6p21.3 within the major histocompatibility locus, and one gene is a non-functional

pseudo-gene and the other an active gene. These two genes have a high degree of homology (up to 90%) and exchanges of DNA parts are often during meiosis. The exchange of small amounts of material can result in hybrid CYP21A1/CYP21A2 gene products. The enzyme activity is reduced at about 20–60%. A patient who is heterozygous for such mutations may have non-classic CYP21A2 deficiency.

Genetic testing is an alternative diagnostic tool for NC-CAH and it can be used when biochemical results are uncertain or when genetic counseling is necessary prior to conception. CYP21A2 genotyping should be performed to identify heterozygote carriers. On the other hand, there is no specific genetic test to determine the risk of developing PCOS in the female offspring or to diagnose the PCOS syndrome.

Pathophysiology

In patients with CYP21A2 deficiency the cortisol synthesis is decreased because of the defective conversion of 17-OHP to 11-deoxycortisol. This biochemical path is determined by 21-hydroxylase and is defective because of mutation in the gene of CYP21A2. Only 20–60% of 21-hydroxylase enzyme activity is preserved. As a result, the corticotropin (ACTH) secretion is increased and that leads to increased adrenal stimulation and increased production of androgens. The enzyme activity in patients with non-classic form is reduced but remains sufficient to maintain the balance of glucocorticoid and mineralocorticoid production, at the expense of excessive androgen production.

On the other hand, PCOS has a puzzling pathophysiologic and biochemical basis (25), and genetic and environmental parameters interact with genetic factors generating aberrations related to metabolism and reproduction (26–28). The ovarian dysfunction is involved in the pathophysiology of polycystic ovary syndrome combined with hyperinsulinaemia and malfunction of the hypothalamic-pituitary-ovarian axis. The hypersecretion of luteinizing hormone (LH), which affects ovarian androgen production and oocyte development, as well as the insulin resistance at the peripheral tissues and the pancreatic β-cell dysfunction are also common features in PCOS. Moreover, the advanced glycation end- products (AGES) are involved in the pathogenic mechanisms of polycystic ovary syndrome. The chronic inflammation and the increased oxidative stress are related to AGES interaction and the reproductive and metabolic derangements of polycystic ovary syndrome (29, 30). Moreover, environmental toxins and endocrine disruptors, and in particular bispenol A, may predispose additional impacts on the syndrome as it has been shown, that they are related to metabolic and reproductive aberrations in PCOS (31, 32).

COMMON FINDINGS

Hyperandrogenism

Hirsutism affects between 5 and 10% of women of reproductive age (33). Other common findings of hyperandrogonaemia are acne, alopecia, ovulatory dysfunction, and virilization and masculinization in most severe androgen excess. The virilization, the deepening of the voice and the clitoromegaly, are relatively rare findings and can be related to others sources of androgens hyperproduction, such as ovarian hyperthecosis or androgen-secreting neoplasms, although women with NC-CAH can more often present with minimal clitoromegaly (4).

PCOS is one of the most frequent causes of hyperandrogenism, with a prevalence of 50 to 80% of women with this sign (34, 35), whereas in NC-CAH it affects between 1 and 10% of these patients (34–37). Nevertheless, the frequency of PCOS is about 40–50 times higher than the frequency of NC-CAH in women in reproductive age or among women with hyperandrogonaemia (4).

Hirsutism constitutes the most common physical manifestation of hyperandrogenism (60–70%) in women with PCOS (38) and hyperandrogenaemia is the most characteristic hormonal alteration of PCOS. It has a multifactorial cause attributed mostly to the ovaries with a concomitant substantial contribution from the adrenals and a minor contribution from fatty tissue. Hyperandrogenism may be involved in deteriorating insulin resistance and in the concurrent obesity in women with polycystic ovary syndrome. In fact, androgen excess appears to participate as an additional factor, which deteriorates the cardio-metabolic profile of women with PCOS (39, 40).

Hirsutism can be milder in PCOS as they grow older, whereas, the prevalence of hirsutism increased in older women with NC-CAH and reaches about 90% in women over 40 years old. The degree of hirsutism does not differentiate between the two disorders neither in young nor in older ages.

Polycystic Morphology

The PCOS morphology has been characterized by the presence of twelve or more follicles with a diameter of 2–9 mm, and/or an enlarged ovarian total volume of more than ten mL in one ovary, excluding a dominant follicle or a cyst (41). In PCOS polycystic ovaries on ultrasound are more common than in NC-CAH (70 vs. 40%) (3, 4). Polycystic ovaries are observed in about 75% in PCOS women (42). Similarly, 30–40% of patients with NC-CAH demonstrate polycystic ovarian morphology (PCO) (9, 43) with one other study suggesting higher percentage up to 82% (44).

Nevertheless, the ultrasound appearance of PCO is not a specific feature in PCOS syndrome. In the NIH 1990 diagnostic criteria, the PCOS was not considered a pathognomic criterion (45). Polycystic morphology was evident in 92% of female patients with hirsutism (46), in 87% of women presenting with symptoms of oligomenorrhea (46), in 26% of women presenting with symptoms of amenorrhea (46) and in 67% of women presenting with androgenic alopecia (47). Moreover, 82% of diabetic premenopausal female patients (48) and 40% of women with a history of gestational diabetes mellitus (49) have polycystic ovaries. Polycystic ovarian morphology up to 23% was also common in healthy women with regular menstrual cycles (50). Therefore, it is clear that PCO morphology by ultrasound is not a distinct characteristic between PCOS and NC-CAH its presence or absence does not serve any diagnostic outcome or further treatment choice.

Ovulatory Disorders and Fertility

PCOS accounts for 70–90% of ovulatory disorders (51). Anovulation can present as frequent bleeding at intervals sooner

than 21 days or non-frequent bleeding at intervals that arrive later than 35 days. Additionally, PCOS accounts for 70–90% of ovulatory disorders and consists the most frequent cause of ovulatory disorders (51). Moreover, about 50% of PCOS women present with primary infertility, and 25% with secondary infertility (52).

NC-CAH patients were less possible to present with anovulation, and more than 70% of women had a normal menstrual cycle and ovulation (24) and only 17% of NC-CAH women have menstrual irregularities (53). The subfertility in women with NC-CAH is milder and many women conceive spontaneously, the infertility may be up to 13%. Combined treatment for ovulation induction is also possible in these women. However, the different hormonal contribution of two conditions on ovulatory dysfunction may prove to be of major clinical significance on therapeutic decision, due to the specific role of glucocorticoids on NC-CAH (54).

Pregnancy Complications

Women with polycystic ovary syndrome are at high risk of preterm delivery, pre-eclampsia and gestational diabetes during pregnancy (55). The spontaneous abortion rate in women with PCOS is 20–40% higher than healthy women (56). The connection between PCOS and gestational diabetes mellitus was initially supported by retrospective data (57). There is also a significant risk of spontaneous abortion in NC-CAH patients (25%) (58–60). Treatment with glucocorticoids seems to contribute to a decrease this risk (60). Most women with NC-CAH conceive with ovulation induction, with the treatment of glucocorticoids alone or combined with clomiphene citrate, and the rate of early pregnancy loss is equal to healthy women.

Genetic counseling in women with NC-CAH is also useful. Genotyping should be suggested in patients who seek fertility because approximately two-thirds of patients with non-classic 21-OHD carry a severe mutation. The total risk of severe (classic) 21-OHD in an offspring of a patient with the non-classic type is about 2.5%, whereas the risk of non-classic deficiency is ∼15%. Knowledge of the father's genotype can help assess these risks more precisely (61, 62). Therefore, the distinction between PCOS and NC-CAH is mandatory for genetic counseling on several aspects including future generations.

Metabolic Parameters

Metabolic syndrome encompasses a mixture of: increased insulin resistance, high lipids level, increased cardiovascular risk, and increased central obesity. Women with polycystic ovary syndrome are also at high risk of having metabolic syndrome (63).

Obesity is more common in women with polycystic ovary disease than other hyperandrogenic syndromes (3, 10) and the percentage of central obesity among women with PCOS ranges between 20 and 85.5% (64). Nevertheless, NC-CAH is also related to increased obesity, up to 41% (65).

Patients with polycystic ovary syndrome have also a higher risk for diabetes mellitus type 2. The prevalence of insulin resistance has been noted in 60–80% of obese women with PCOS (66, 67). Earlier studies reported a prevalence of impaired glucose tolerance of 35% and diabetes mellitus type 2 of 10% in female patients presenting with PCOS (68, 69). Moreover, affected women have noticeable insulin resistance, which is irrelevant of obesity (70).

Insulin resistance is very common characteristic in PCOS women, found up 60–80% in lean and 95% in obese women with the syndrome (66). Insulin resistance is described as the condition in which a cell, tissue or organism needs above-normal amounts of insulin to respond normally to a certain glucose load. It is related with increased insulin secretion by pancreatic β-cells and compensatory hyperinsulinemia, whereas the blood glucose remains normal. Insulin resistance can be calculated by HOMA (Homeostasis Model Assessment) INDEX (product of fasting plasma insulin [mU/L] and glucose [mmol/L] concentrations divided by 22.5). Insulin resistance itself is connected to altered large artery compliance and endothelial function. Insulin resistance may be more severe, but probably not more common in polycystic ovarian syndrome than in NC-CAH (3, 4, 65, 71). The degree of hyperinsulinemia was higher in female patients with PCOS and central obesity, whereas in lean women with PCOS the metabolic abnormalities equal often and severe as in female patients with non-classic adrenal hyperplasia (53).

The hyperinsulinemia contributes to the augmented production of androgens by the adrenal glands (72) and the ovaries. This is achieved with the activation of P450c17a (CYP17 mRNA and protein expression) which increases the effect of the CYP21A deficiency and so he steroidogenic precursors are diverted to the path of androgen production (73). Moreover, hyperinsulinaemia obstructs liver production of SHGB, and so the testosterone availability increases.

Women with PCOS are at higher risk for impaired glucose tolerance and diabetes mellitus type 2 compared to healthy women (69, 74, 75). A diagnosis of PCOS increases the risk of developing type 2 diabetes mellitus up to 5- to 10-fold (69, 74, 75). The overall prevalence of glucose intolerance in PCOS was 30–35, and 3–10% for type two diabetes mellitus, depending on the degree of obesity as well as ethnicity. Non-obese female patients with PCOS had a 10–15% prevalence of impaired glucose tolerance and a 1–2% prevalence of type two diabetes mellitus (69, 74, 75).

About 70% of PCOS patients present abnormal serum lipid levels such high low-density lipoprotein (LDL) and triglyceride levels and low high-density lipoprotein (HDL) levels (28). The lipids elevation is regardless of body mass index (BMI) (18, 76). Moreover, hypercholesterolemia is also common in women with NC-CAH (up to 46%) (65).

Cardiovascular Risk

PCOS incorporates many metabolic abnormalities that result in high risk for cardiovascular diseases. The metabolic aberrations include the impaired glucose tolerance, the dyslipidaemia, the low-grade inflammation and the increased coagulation factors (10, 63). Moreover, the active AGE-RAGE axis contributes to the atherosclerosis as well as the endothelial dysfunction (77). Although all these predisposing factor to cardiovascular disease

TABLE 1 | Common and different characteristics of the two syndromes.

	NC-CAH	PCOS
Prevalence	**Rare syndrome**	**Common syndrome**
Prevalence in reproductive age women (4)	0.1–0.05%	4–6%
Prevalence in hyperandrogenic patients (4)	1–10%	50–80%
Difference in prevalence according to ethnicity	Major differences High-risk group: women with Ashkenazi Jewish, Hispanic, and Mediterranean origin	Only minor differences
Pathophysiology	**Defective enzymatic activity**	**Genetic and environmental factors**
Hyperandrogenaemia manifestations	**Common**	**Common**
Hirsutism	Common (59%)	Common (60–70%)
Acne (71, 88, 89)	Common (33%)	Common (14–25%)
Clinical presentation of hirsutism as woman gets older	Similar or increase	Milder
Gynecological problems	**Common**	**More common**
Menstrual irregularities (53)	Common (17%)	Very common (90%)
Polycystic ovaries (3, 71)	Common (40%)	Very common (70%)
Infertility (52)	Yes, milder (13%)	Yes (25–50%)
Pregnancy complications (56, 58–60)	Yes, spontaneous abortions: common (25%)	Yes, spontaneous abortions: common (20–40%)
Metabolic aberrations	**Common**	**More common, more severe**
Type 2 diabetes mellitus (35, 69, 74, 75)	<4%	3–10%
Obesity (10, 65, 67)	Common (12.2–41%)	Very common (28.4–85%)
Insulin resistance (65, 66)	Common (29%)	Very common, more severe (60–80%)
Dyslipidaemia (28, 65)	Common (46%)	Very common (70%)
Mood disorders/depression (81–84)	**Common (50%)**	**Common (21–64%)**
Inheritance mechanism (4)	**Autosomal recessive**	**Unclear**
Special test for differential diagnosis	**Yes**	**Exclusion of other conditions**
Basal 17-OHP >2 ng/mL (53)	87%	25%
Specific Hormonal diagnosis (4)	ACTH-stimulated 17-OHP	None
Other test		
LH/FSH >2 (53)	Not very common (9%)	Common (22–29%)
DHEAS (53)	Elevated or very elevated	Elevated
Testosterone (53)	Elevated	Equally elevated
Therapy options	**OCS, glucocorticoids, antiandrogens, clomiphene citrate**	**OCS, weight loss, antiandrogens, metformin, clomiphene citrate**

it remains uncertain whether they result in a higher mortality rate (78, 79).

On the other hand, the same cardiovascular factors are met also in NC-CAH women. Patients with NC-CAH present also very often with obesity, insulin resistance and higher lipids level. However, it remains uncertain whether women with NC-CAH have actually a higher cardiovascular risk and higher mortality when compared with female patients without hyperandrogonaemia (24).

Mood Disturbances

Women with PCOS present increased risk for depression and this is independent of androgen levels, hirsutism, acne, obesity, and infertility (80). The prevalence of depression in PCOS women in different studies is high reaching up to 64% (81–83). Likewise, depression and anxiety symptoms are frequent in female patients with NC-CAH, up to 50% (84). Therefore, it is important to monitor the patient's mood symptoms during treatment. Some studies suggest that treatment of the hirsutism can improve quality of life and reduce depression and anxiety symptoms (85, 86).

Hormonal and Biochemical Parameters

A high ratio of luteinizing hormone (LH) to follicle-stimulating hormone (FSH) can be found in PCOS women, as well in women with NC-CAH but to a lesser extend (4, 43).

Patients with PCOS and NC-CAH do not differ in their hormonal parameters. The testosterone levels are elevated in both syndromes often to a similar degree (4, 43) or can be higher in NC-CAH women (3, 10). The DHEAS levels can be equally elevated in both syndromes (43, 87), or can be higher in NC-CAH (3, 4, 10).

The **Table 1** summarizes the main differences and common characteristics of the two syndromes.

In conclusion and clearly PCOS is a more common syndrome. The pathophysiology of PCOS is multifactorial, whereas in NC-CAH there is a clear mechanism of a defective enzymatic activity duo to specific genes. Moreover, PCOS present more often with the metabolic and gynecological aberrations, although these are also present with different degree of frequency in NC-CAH women.

DIFFERENTIAL DIAGNOSIS

According to the 1990 National Institute of Health (NIH) criteria, the existence of oligoovulation and/or anovulation and clinical and/or biochemical indication of hyperandrogenaemia are necessary, irrespective of the existence of polycystic ovaries on U/S examination (43). The 2004 Rotterdam criteria proposed that PCOS should be defined when at least two of the three aforementioned criteria exist, and other diseases or disorders that can resemble the polycystic ovary syndrome can be excluded (90). Among these are the thyroid disease, hyperprolactinemia, and non-classic congenital adrenal hyperplasia. In women with more severe phenotypes, a further evaluation is necessary in order to exclude other causes, such as Cushing's syndrome and acromegaly. Based on the Androgen Excess PCOS Society Criteria (AE-PCOS Society Task Force) PCOS should be defined when the following are present: hyperandrogenaemia (clinical and/or biochemical), ovarian dysfunction (oligo-anovulation and/or polycystic ovarian morphology), while related disorders are excluded (91). **Table 2** summarizes the different diagnostic criteria for PCOS.

TABLE 2 | Summarizes the different diagnostic criteria for PCOS.

A comparison of diagnostic criteria for polycystic ovary syndrome
1990 National Institute of Child Health and Human Development (NICHD) diagnostic criteria: 1. Clinical and/or biochemical signs of hyperandrogenism 2. Oligo- or chronic anovulation Other reasons for androgen excess and annovulatory infertility are excluded
2003 European Society for Human Reproduction and Embryology and American Society for Reproductive Medicine (ESHRE/ASRM or Rotterdam) Criteria: 1. Oligo- or chronic anovulation 2. Clinical and/or biochemical signs of hyperandrogenism 3. Polycystic ovaries Other reasons for androgen excess and annovulatory infertility are excluded
2006 Androgen Excess Society (AES) crieteria: 1. Hirsutism and/or hyperandrogenemia 2. Oligo-anovulation and/or polycystic ovaries Other reasons for androgen excess and annovulatory infertility are excluded

PCOS is 40 to 50 times more frequent than that of NC-CAH in reproductive aged women or among hyperandrogenic women (4). NC-CAH is uncommon in women of African-American and Hispanic-Puerto Rican origin (1, 71). Nevertheless, distinguishing NC-CAH from PCOS is recommended in all female patients of Eastern European Jewish origin (prevalence 1:27), and women of Hispanic (prevalence 1:40), Slavic (prevalence 1:50) or Italian origin (prevalence 1:300) (92). Nevertheless, it is suggested to distinguish NC-CAH from PCOS in all female patients with apparent PCOS. Instead the prevalence of PCOS according to ethnicity has a minor variation (93).

In women with hyperandrogonenism (hirsutism and/or acne) and oligomenorrhea the non-classic type of NC-CAH should be distinguished from polycystic ovary syndrome. PCOS is much more common than NC-CAH. The basal levels of 17-OHP may overlap, but ACTH stimulation testing can distinguish the two entities (53).

When the hormonal and biochemical results are borderline a genetic test for NC-CAH can be done. Genetic counseling is also needed prior to conception. When a parent has NC-CAH the risk for the child to develop (classic) 21-OHD is ∼2.5%, while the risk of non-classic deficiency is ∼15% (59). The risk can be more precisely assessed when testing the partner's genotype.

The prevalence of NC-CAH in women who present with PCOS-type picture depends on the population. High-risk groups include women with Mediterranean, Hispanic, and Ashkenazi Jewish origin. Testing for NC-CAH with measurement of a basal 17-OHP is recommended in populations of high risk, as well in all women who present with clinical picture compatible with PCOS (94).

A basal 17-OHP should be measured at around 8 am during the follicular phase of the cycle. For a woman with irregular or no menses a random blood sample can be drawn. A basal 17-OHP more than 200 ng/dL (6 nmol/L) is diagnostic for NC-CAH is strongly suggested and further evaluation is required, whereas a value <200 ng/dL (6 nmol/L) suggest that the diagnosis is unlikely. The ACTH stimulation test confirms the diagnosis.

When the basal 17-OHP is more than 6 nmol/L, a Synachten test should follow. A high dose of ACTH is used (250 mcg). The diagnosis of NC-CAH is confirmed when the 17-OHP reached or exceeds 1,500 ng/dL (43 nmol/L) after Synachten test (8, 9). When the stimulated values in 1 h are between 1,000 and 1,500 ng/dL (30 to 43 nmol/L) a genotyping is recommended to confirm the diagnosis.

The basal 17-OHP increases during the preovulatory or luteal phase of menstrual cycle. Therefore, the sample should be obtain within the 10 first days after the beginning of the menstruation or any time when the patient in amenorrheic. A serum progesterone can be also measured to exclude that the blood was not drawn during the luteal phase of the menstrual cycle. A serum progesteron >400 ng/dL (12.7 nmol/L) indicates a luteal phase.

About 20% of patients with PCOS have also elevated 17-OHP values. A value of 200 ng/ml might be suggestive of NC-CAH but in order to distinguish the two syndromes, it is suggested to perform an ACTH stimulation test for values <1,000 ng/ml in order to confirm the diagnosis of NC-CAH.

Almost two-thirds of patients with non-classic 21-OHD are compound heterozygotes, characterized by a severe or mild mutation on their alleles. Genotyping is therefore useful for patients who seek fertility (61, 62). Men's genotyping helps to assess the risk. Men with NC-CAH are asymptomatic. The criteria for diagnosis are the same as in women and the

diagnosis is usually made for a family evaluation (95). When one patient has the non-classical form the risk for the child for classic 21-hydroxylase form is 2.5% and for the non-classic 15% (59).

APPROACH TO TREATMENT

The oral contraceptives and the anti-androgen therapy is the primary therapy treatment for the symptoms of hyperandrogonaemia in adult women with either PCOS or non-classic 21-OHD who are not pursuing fertility. The role of glucocorticoid therapy is more documented for NC-CAH but it may be used for hyperandrogenic symptoms and menstrual cycle management in women who do not take or tolerate oral contraceptives or antiandrogens, such as spironolactone, therapy.

The glucocorticoids reduce the androgen production from the adrenal glands by suppressing the corticotropin-releasing hormone (CRH) and the corticotropin (ACTH). Nevertheless, hydrocortisone and dexamethasone seem to be more effective than oral contraceptives for suppressing serum adrenal androgen concentrations but less effective for decreasing clinical apparent hirsutism. Moreover, the glucocorticoids even in mild excess have many potential risks and side effects (96).

Considering that oral contraceptives suppress ovarian and adrenal androgens and ACTH. They have been accepted as the first-line therapy in adults because they seem to be more effective for hirsutism than glucocorticoids. Glucocorticoids can be prescribed to women with NC-CAH who cannot tolerate or don't respond to or oral contraceptives and antiandrogen therapies.

Antiandrogens, such as spironolactone, are also effective, though antiandrogen monotherapy is not recommended because of possible teratogenicity. Oral contraceptives can be started and spironolactone can be added after 6 months if the cosmetic response with an oral contraceptive alone has not been adequate. Dexamethasone crosses the placenta and therefore is not suggested in sexually active women, and instead, hydrocortisone, prednisone, or prednisolone are preferred. Oral contraceptives are suggested instead of glucocorticoids for menstrual cycle management. Glucocorticoids can be used for ovulation induction in anovulary women who seek fertility, and clomiphene citrate can be also added.

The treatment of PCOS is based also in oral contraceptives as basic treatment for the disorders of menstruation and the clinical hirsutism and acne. The contraindications of oral contraceptives should be also taken into account. Oral contraceptives protect also the endometrium and offer contraception. When oral contraceptives are contraindicated a progestin pills or cyclical progestins can be provided. Exercise and diet can improve the metabolic parameters as well the reproductive dysfunction. Weight reduction is probable useful for the reproductive and metabolic disorders of women with PCOS, whereas weight reduction is probable unsatisfactory as a treatment for patients with normal body weight. Metformin is a second-line treatment for the regulation of metabolic parameters and menstrual irregularity. Metformin is recommended for women with PCOS who have type 2 diabetes mellitus or impaired glucose tolerance (IGT) who fail lifestyle modification. Pioglitazone has also been used in women with PCOS, providing more metabolic and reproductive benefits and possibly protection from developing diabetes and cardiovascular problem. Inositols are second messengers for insulin, and their deficiency contributes to the various features of PCOS and when given to PCOS women they can alleviate the metabolic, menstrual/ovulatory, and cutaneous hyperandrogenic features of the syndrome. Clomiphene citrate (or the estrogen modulator letrozole) can be used as the primary therapy for the infertility (94). Alternative options for infertility treatment in anovulatory women are the gonadotropins and the *in vitro* fertilization. Metformin is not suggested for ovulation induction, whereas the laparoscopic diathermy of the ovaries may be used under specific circumstances. The management of PCOS encompass a tailored approach to individual needs of each patient (97).

In both syndromes, the cardiometabolic alterations require regular screening and therefore statins and anti-obesity drugs can be helpful for the metabolic parameters. Bariatric surgery is recommended only in severe obese female patients with PCOS or NC-CAH. Hirsutism can be approached with cosmetic procedures in both PCOS and NC-CAH patients. For women with patient-important hirsutism possible solutions are direct hair removal and chemical depilatory agents that dissolve the hair. The plucking and waxing, are quite cheap and safe methods, although they can be unpleasant.

Limitations

The strength of this systematic review is that we compared two syndromes an effort was made to illuminate their hidden characteristics. Clinicians should recognize their main differences and proceed to the necessary tests in order to differentiate them. This knowledge can be implemented in the clinical practice. Limitation of this study is that we used only one database, Pubmed, and only the English written articles.

CONCLUSIONS

Women with NC-CAH due to 21-OHD and women with PCOS have similar clinical presentation, with hyperandrogenism, oligomenorrhea, and polycystic ovaries. Insulin resistance, hyperinsulinism, and polycystic ovarian morphology were all detected in a great number of NC-CAH women. PCOS is more common, but NC-CAH should be also excluded by measuring the serum 17-OHP during the first days of follicular phase (94).

NC-CAH and PCOS present with analogous clinical characteristics and augmented androgen levels. In NC-CAH the androgens are as high as in obese PCOS women, but the metabolic profile is similar to lean PCOS women. Women with PCOS present more often with oligomenorrhea or

amenorrhea and polycystic ovarian morphology. Moreover, they present with a LH/FSH ratio more than 2:1 (53). The screening tool to distinguish non-classic adrenal hyperplasia from PCOS is the basal 17-OHP levels and the acute ACTH stimulation test. Genetic screening may also be necessary in difficult cases of PCOS and NC-CAH, when their commonalities on clinical and hormonal grounds, even unveiled cannot illuminate their shadowed distinctive characteristics.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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The Complexities in Genotyping of Congenital Adrenal Hyperplasia: 21-Hydroxylase Deficiency

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The deficiency of 21-hydroxylase due to *CYP21A2* pathogenic variants is a rather frequent disease with serious consequences, going from a real mortality risk to infertility and to milder symptoms, nevertheless important for affecting the patients' self-esteem. In the most severe cases life-threatening adrenal salt wasting crises may occur. Significant morbidity including the possibility of mistaken gender determination, precocious puberty, infertility and growth arrest with consequent short stature may also affect these patients. In the less severe cases milder symptoms like hirsutism will likely affect the image of the self with strong psychological consequences. Its diagnosis is confirmed by 17OH-progesterone dosages exceeding the cut-off value of 10/15 ng/ml but genotyping is progressively assuming an essential role in the study of these patients particularly in confirming difficult cases, determining some aspects of the prognosis and allowing a correct genetic counseling. Genotyping is a difficult process due to the occurrence of both a gene and a highly homologous pseudo gene. However, new tools are opening new possibilities to this analysis and improving the chances of a correct diagnosis and better understanding of the underlying mechanisms of the disease. Beyond the 10 classic pathogenic variants usually searched for in most laboratories, a correct analysis of 21OH-deficiency cases implies completely sequencing of the entire gene and the determination of gene duplications. These are now recognized to occur frequently and can be responsible for some false positive cases. And finally, because gene conversions can include several pathogenic variants one cannot be certain of identifying that both alleles are affected without studying parental DNA samples. A complete genotype characterization should be considered essential in the preparation for pregnancy, even in the case of parents with milder forms of the disease, or even just carriers, since it has been reported that giving birth to progeny with the severe classic forms occurs with a much higher frequency than expected.

Keywords: 21OH deficiency, CAH—congenital adrenal hyperplasia, adrenal cortex, androgen excess syndromes, genotyping, endocrine genetics, rare diseases, disorders of sex development

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INTRODUCTION

The congenital adrenal hyperplasias (CAH) are a group of autosomal recessive disorders that are caused by decreased activity of one of the enzymes involved in the steroidogenic pathway of the adrenal cortex, leading to impaired synthesis of cortisol by the adrenal gland. The vast majority of the cases of CAH (95%) are due to 21-hydroxylase deficiency and associated with pathogenic variants in the 21-hydroxylase (*CYP21A2*) gene. This form of CAH will be the major focus of this article. Most affected individuals are compound heterozygotes, presenting different pathogenic variants on each allele rather than being homozygous for the same pathogenic variant. Although there seem to be some exceptions, most heterozygotes/carriers are asymptomatic.

Complete loss of function pathogenic variants of the CYP21A2 gene are associated with impaired cortisol and aldosterone synthesis. The accumulation of steroid precursor molecules leads to increased adrenal androgen production utilizing the delta-5 pathway and CYP17A1. Decreased cortisol concentrations result in loss of the negative feedback inhibition leading to a compensatory increase of adrenocorticotropic secretion (ACTH) and hypertrophy of the adrenal cortex.

The clinical importance of CAH results from the possible occurrence of adrenal insufficiency, genital ambiguity, short stature, androgen excess syndromes and infertility.

With increased awareness of the signs and symptoms of CAH, morbidity and mortality has decreased. Hormone replacement therapy is beneficial, but affected individuals require very precise and personalized treatment regimens for optimal outcomes.

Thus, clinicians need to be aware of the potential consequences and complications of CAH. Specific issues include concerns regarding genital ambiguity in affected females, premature pubarche, accelerated skeletal maturation with reduced final height, bone health, adrenal tumors, and testicular adrenal rests tumors (TARTs). Although more common among affected females, infertility can affect both genders. Genetic counseling is essential, especially since this disease affects many individuals at reproductive age (1–3).

In spite of the fact that this disorder results from a continuum of enzymatic deficiencies, congenital adrenal hyperplasia has been classified into three main forms (**Table 1**).

1 The salt-wasting or salt-losing form is associated with complete loss of 21-hydroxylase activity leading to deficient cortisol and aldosterone biosynthesis.

Prior to newborn screening programs, affected females were more rapidly identified due to the simultaneous presence of genital ambiguity. The genital ambiguity involves enlargement of the phallus, varying degrees of fusion of the labioscrotal folds, and non-palpable gonads. Affected males typically presented within the first 2 weeks of life with failure to thrive, vomiting, hypotension, hyponatremia, and hyperkalemia.

2 The simple virilizing form often presents with genital ambiguity without overt salt loss in affected females. This simple virilizing form may present with phallic enlargement, premature development of sexual hair, and initially tall stature, accompanied by advanced skeletal maturation resulting in final short stature. Children with simple virilizing CAH generally synthesize sufficient aldosterone and so they are not overt salt-losers.

Salt-losing and simple virilizing CAH are often grouped together as the classic forms. The incidence of classic CAH is reported as being of 1:15,000 live births. Consequently, the carrier frequency is ∼1:60 (4–9). In black Americans the incidence is much lower, going from 1:25,000 to 1:42,000 in different studies (10, 11).

3 The most common form of CAH is the non-classic or late onset form (NCAH). The characteristic features of NCAH, hirsutism, irregular menses, and infertility, lead to an ascertainment bias favoring diagnosis of affected females. Affected males are usually only identified through family studies. Overt glucocorticoid and mineralocorticoid deficiency are unusual. Although patients with NCAH usually have no evidence of ACTH or CRH excess, some may demonstrate an increased glucocorticoid response to ACTH stimulation, possibly reflective of subtle adrenal hyperplasia (12–14). The reason for the existence of increased androgen production by the adrenals without an increase in ACTH has been attributed to an altered enzymatic kinetic of *CYP21A2* (15). The elevated androgen levels in NCAH may also result from ovarian hypersecretion since the ovaries in NCAH women are frequently polycystic (15, 16), and from peripheral conversion of precursors.

NCAH affects between 0.1 and 1% of the general population. Among hirsute women its prevalence reaches between 1 and 10%

TABLE 1 | Phenotypes of 21-hydroxylase deficiency.

Classic Salt-Wasting—very severe (0% enzymatic activity)
Failure to thrive
Cortisol deficiency
Mineralocorticoid deficiency
Hyponatremia
Hyperkalemia
High PRA
Hypovolemic shock
Excess androgen production, early in life
Virilization of external genitalia in females
Classic Simple Virilizing—intermediate severity (1–2% enzymatic activity)
Virilization of external genitalia in females
Progressive Premature Pubarche
Progressive virilization with clitoromegaly (female) or increased penile size (male)
Elevated androgen levels cause accelerated growth velocity and advanced bone age but premature fusion of the epiphyses is also observed causing final short stature.
Non-Classic Adrenal Hyperplasia—milder form (20–50% enzymatic activity)
Between asymptomatic or with signs of androgen excess appearing later in life (acne; hirsutism; menstrual irregularities; anovulation; infertility).

(16–20). The most recent meta-analysis indicated the prevalence of 4.2% among women with androgen excess worldwide (21). One clinical study based on ACTH-stimulated 17-OHP concentrations reported the incidence to be highest among Ashkenazi Jewish populations (22).

The signs and symptoms of NCAH are similar to those of Polycystic Ovary Syndrome (PCOS) (16). Since the treatment, potential complications, and genetic implications differ between these 2 syndromes, accurate diagnosis is important and that may impose a complete differential diagnosis in every case of hirsutism and a surveillance of metabolic dysfunction (e.g., insulin resistance) and subsequent prevention of the increased cardiovascular risk not only in PCOS but also in NCAH cases (16).

MOLECULAR GENETIC TESTING

CYP21 Gene Structure

The *CYP21A2* gene is located in the long arm of chromosome 6, within the major human histocompatibility complex (HLA), a region that displays a complex organization of genes with a great variability in gene size and copy numbers (2, 3, 23, 24). Approximately 30 kb from the *CYP21A2* gene there is a non-functional pseudogene—*CYP21A1P*. Both, the functional gene and the pseudogene comprise ten exons and share a high level of nucleotide sequence identity of 98% in their exons and 96% in their introns (25, 26). The pseudogene *CYP21A1P* is inactive because of the presence of multiple pathogenic variants, small insertions or deletions and point pathogenic variants that prevent the synthesis of a functional protein. The location and high rate of homology between the two genes facilitates misalignment that results in recombination events between the gene and the pseudogene (**Figure 1**). These events that are called gene conversions constitute a mutagenesis mechanism that contributes to the majority of the point pathogenic variants in the *CYP21A2* gene.

Neighboring the *CYP21A2* and the *CYP21A1P* genes there are three other genes, *RP1*, *C4*, *TNXB* and two truncated pseudogenes, *RP2* and *TNXA*, that together, constitute a genetic unit designated RCCX module (RP-C4-CYP21-TNX) (**Figure 2**) and correspond to a highly variable stretch of DNA of ∼30 Kb (28). The genes *C4B* and *C4A* encode for the fourth component of serum complement (29, 30) the gene *TNXB* for an extracellular matrix protein termed tenascin-X^{23} and the *RP1* gene for a serine/threonine nuclear protein kinase (28). The usual organization is bimodular, and consists of two RCCX modules, one with the *CYP21A1P* pseudogene and the other with the *CYP21A2* gene, where the orientation of the genes from telomere to centromere is: *RP1-C4A-CYP21A1P-TNXA-RP2-C4B-CYP21A2-TNXB*. This bimodular haplotype is present in about 69% of the Caucasian population, while a monomodular haplotype occurs with a frequency of 17% and a "three modular" haplotype in about 14% of the cases (**Figure 2**) (28, 31). The majority of the trimodular haplotypes carry two copies of the *CYP21A1P* pseudogene and one copy of the *CYP21A2* gene, but the haplotype with two copies of the *CYP21A2* gene and one copy of the *CYP21A1P* pseudogene is also possible and has been described in carriers of the p.(Gln319*) pathogenic variant and of chimeric *CYP21A1P*/*CYP21A2* genes (31–34).

The frequent existence of copy number variations together with the large number of possible genetic variants makes the characterization of *CYP21A2* alleles rather difficult. Pathogenic variants have been identified both in the coding and non-coding regions of the gene inclusively in the 5′UTR and the 3′UTR regions. Consequently, it is important to screen all coding exons, as well as intron-exon boundaries of the gene.

CYP21A2 Pathogenic Alterations

Due to gene and pseudogene location and the highly polymorphic complexity of the region, recombination events are the major cause of *CYP21A2* pathogenic variants.

Intergenic recombinations are responsible for more than 95% of the pathogenic variants causing 21OHD. Approximately 75% of the deleterious variants are transferred by small conversions from the pseudogene during meiosis. These conversions can involve one or more pseudogene variants. They are called "microconversions," when they are limited to a single point variant.

In the remaining 20–25% of the cases, CAH is due to gross misalignment owing to unequal crossing over during meiosis that can lead to gene deletions, gene duplications and deletions involving *CYP21A2* and other contiguous genes (35, 36). CAH can also be caused by uniparental isodisomy but this is rare (37).

To date more than 1,300 genetic variants have been reported but only 230 affecting human health (38). The majority of these pathogenic variants result in classic form cases (156 in the total 230) (38). Nineteen genetic variants have been described in the non-translated regions of the gene. Of these, 4 affect the promoter, resulting in promoter conversion: c.(-126C>T; −113G>A; −110T>C; and−103A>G). c-126C>T was reported to cause NCAH (39).

One hundred and fifty three of the 230 genetic variants were demonstrated to be missense mutations (38). These can result in all forms of the disease while nonsense and frameshift mutations always result in the classic forms.

The real life situation, however, can be much simplified as there is a small group of pathogenic variants that account for the great majority of affected alleles ($n = 10$) (**Figure 3**). The screening strategy to search for those most common pathogenic variants is an usual practice among molecular geneticists as the process is less expensive and less time consuming.

Whenever possible familial segregation studies should be done, in which both parents are studied together with the proband, so that one may know if two detected pathogenic variants affect the 2 alleles (*trans* configuration) or are located in the same allele (*cis* configuration). In this last situation there is only one allele with mutations and the other allele is normal. That person will not be clinically affected in spite of having 2 pathogenic variants on the CYP21A2 gene.

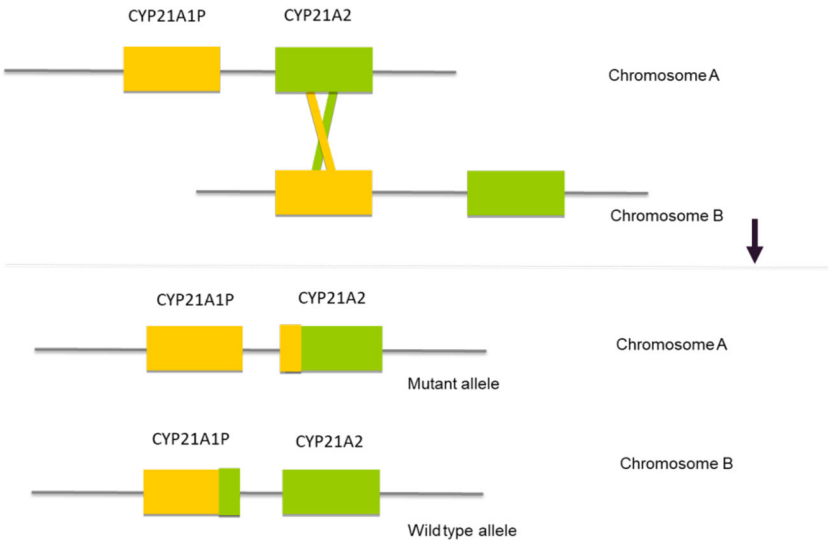

FIGURE 1 | Schematic representation of the mechanism of gene conversion, where a misalignment between the two DNA sequences results in a recombination between the *CYP21A2* gene and the *CYP21A1P* pseudogene.

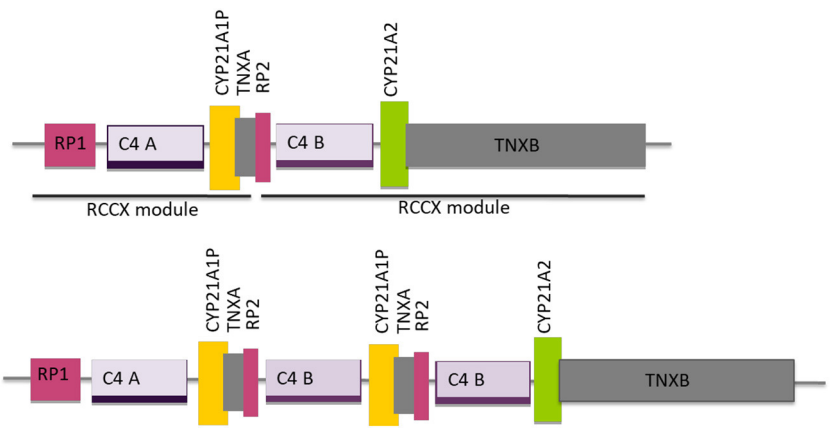

FIGURE 2 | Schematic diagram of the organization of the *RCCX* modules, one with the *CYP21A1P* pseudogene and the other with the *CYP21A2* gene for the most common bimodular haplotype and for the three modular haplotype with two modules harboring the *CYP21A1P* pseudogene and one the *CYP21A2* gene. Adapted from Sweeten et al. (27).

Molecular genetic testing of the CYP21A2 gene should be considered essential since it allows the establishment of correlations between genotype and phenotype, confirming the clinical and biochemical diagnosis, inferring about the severity status of the patients, distinguishing between severe and milder cases and, very importantly, allowing a correct genetic counseling for any couple at risk and their relatives.

CYP21A2 Genetic Variants

Two types of recombination can be considered: one is the result of an unequal crossing over during meiosis, with the production of large rearrangements and the other consists of smaller gene conversions where a segment of the functional *CYP21A2* gene is replaced by a segment copied from the *CYP21A1P* pseudogene (**Figure 1**). The segment of the converted *CYP21A2* gene will carry either a few nucleotides from CYP21A1P (microconversions) or a short sequence affecting one or more exons (25, 40–42). The converted sequences harbor pathologic variants so that they will inactivate or at least significantly modify the normal *CYP21A2* gene translation of the protein.

Large Deletions and Gene Conversions

Large gene conversions and large deletions, sometimes involving *C4B* and *CYP21A2* with the formation of *CYP21A1P*/*CYP21A2* chimeric genes comprise ∼20% of the pathogenic variants. In the last situation a 26 or 32 Kb deletion (depending on whether *C4B* is the long or short gene), involving the

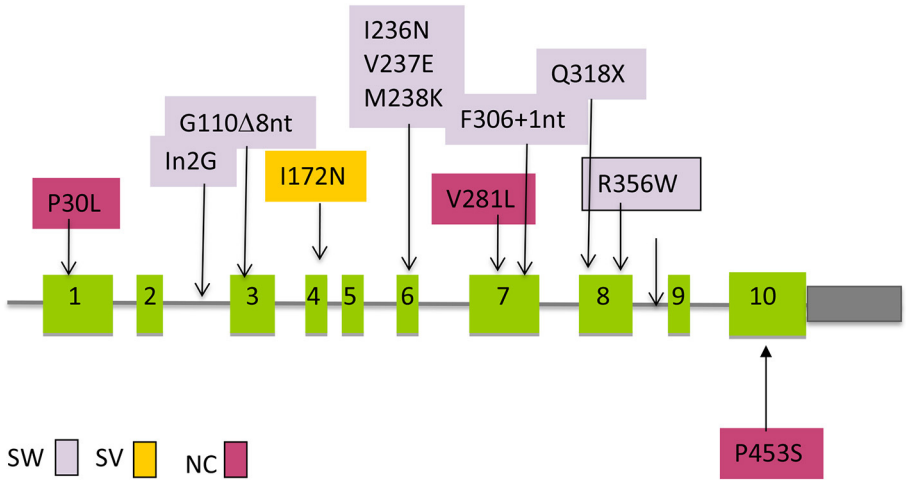

FIGURE 3 | Distribution of the most common mutations along the *CYP21A2* gene that are transferred by gene conversion and the association with clinical severity. SW, salt wasting; SV, simple virilizing; NC, Non-classic.

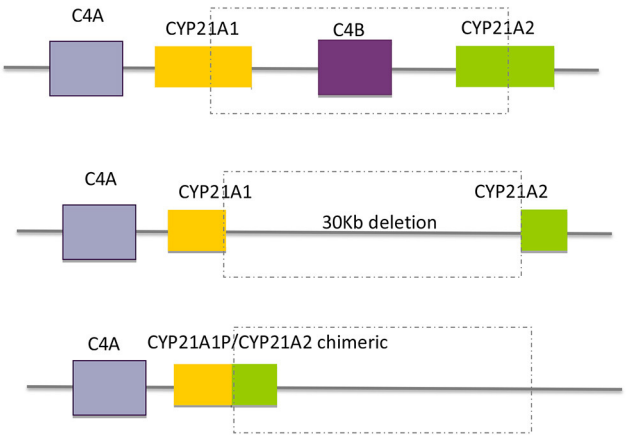

FIGURE 4 | Schematic diagram of the formation of chimeric genes by large gene deletions.

3′ end of *CYP21A1P*, all of the *C4B* gene, and the 5′ end of the *CYP21A2* gene, produces a single non-functional chimeric gene with its 5′ and 3′ ends corresponding to *CYP21A1P* and *CYP21A2*, respectively (**Figure 4**). Several different chimeric *CYP21A1P*/*CYP21A2* genes have been found and characterized (43–47).

Point Pathogenic Variants and Small Deletions/Insertions

Approximately 75% of the intergenic recombinations correspond to pathogenic variants normally present in the pseudogene that are transferred to the functional gene by microconversion events (**Figure 3**) (41). Other rearrangements, such as a deletion of 10 nucleotides in exon 8 and a duplication of 16 nucleotides in exon 9 have also been reported.

P30L: Pro-30Leu (p.(Pro31Leu))

This pathogenic variant yields an enzyme with 20–60% of normal activity when expressed in cultured cells (48). However, enzymatic activity is rapidly lost when the cells are lysed, suggesting that the enzyme is relatively unstable. Patients carrying this pathogenic variant tend to have more severe signs of androgen excess than patients carrying the more common non-classic pathogenic variant V281L (p.(Val282Leu) (48, 49). This pathogenic variant is found in approximately one-sixth of alleles in patients with non-classic disease, but it may comprise a higher percentage in Japan (50).

IVS2-13A/C>G: A or C-G Pathogenic Variant in Intron 2 (c.293-13A/C>G)

This pathogenic variant is characterized by the substitution of A or C nucleotide at 13 bp before the end of intron 2 (nt 656) to G. This pathogenic variant causes aberrant splicing of intron 2 with retention of 19 nucleotides normally spliced out of mRNA, resulting in a shift in the translational reading frame (51, 52).

G110Δ8nt (p.(Gly111Valfs*21))

This deletion of eight nucleotides (8-nt) in exon 3 prevents the synthesis of the protein by a frameshift and causes a salt-wasting type of CAH (40). It is present in about 8% of the salt-wasting cases.

I172N: Ile-172Asn (p.(Ile173Asn))

This pathogenic variant results in an enzyme with ∼1% of normal activity (53, 54) and has been specifically associated with the simple virilizing form of the disease; however it has also been described in the salt wasting form (55).

Cluster in Exon 6: I236N/V237E/M238K: Ile-Val-Met-236–237-238-Asn-Glu-Lys

(p.(Ile237Asn), p.(Val238Glu), p.(Met239Lys))

This cluster of three missense pathogenic variants in the G helix also abolishes enzymatic activity possibly by interference with substrate binding (52, 54).

V281L: Val-281Leu (p.(Val282Leu))

This pathogenic variant results in an enzyme with 50% of normal activity when 17-OHP is the substrate but only 20% of normal activity for progesterone (54, 56). V281L occurs in the majority of patients with non-classic 21-hydroxylase deficiency who carry the HLA haplotype B14; DR1, an association that is consistent to a founder effect (57). Overall, ∼70% of all non-classic alleles carry the V281L pathogenic variant (58, 59).

F306+T: L306insT (p.(Leu307Phefs*5))

This 1-nt insertion in exon 7 of *CYP21A1P* has generally been described not as an independent pathogenic variant but in a cluster of pseudogene derived pathogenic variants in exons 7 and 8 particularly in Dutch patients (60).

Q318X: Gln-318-Term (p.(Gln319*))

A nonsense pathogenic variant in codon 318 (Q318X) where the CAG, encoding glutamine changes to TAG, a nonsense codon that is predicted to result in a completely non-functional enzyme due to premature termination of translation (61).

R356W: Arg-356Trp (p.(Arg357Trp))

This pathogenic variant abolishes enzymatic activity when expressed in mammalian cells (52, 53). It is located in a region of the gene encoding the K helix of the enzyme, which suggests that the pathogenic variant affects interactions with the cytochrome P450 reductase (POR), but this has not been demonstrated experimentally (62).

P453S: Pro-453Ser (p.(Pro454Ser))

This missense pathogenic variant results from a transition of a CCC to a TCC and was initially described as not present in the pseudogene (63, 64). Although the functional mechanism is not clearly explained it corresponds to a decrease of 50–68% of 17-OHP and 20–46% of progesterone utilization (65). It occurs in a number of different populations and suggests that *CYP21A1P* may carry P453S as an occasional polymorphism and that this pathogenic variant is transferred to *CYP21*A2 in the same way as the other pathogenic variants frequently causing 21-hydroxylase deficiency (2).

Other Pathogenic Variants

More than 200 different pathogenic variants have been described and this number is increasing with the improvements of molecular diagnosis (see http://www.cypalleles.ki.se/cyp21.htm and http://www.hgmd.cf.ac.uk). Some of these pathogenic variants have been reported in several cases, but most of them are private family pathogenic variants, which means that they were described only in one family. Except for the nonsense, frameshift, and rearrangement alterations that are deduced as severe, most of these pathogenic variants are missense, and require functional studies to be classified.

Less than 5% of the pathogenic variants in the *CYP21A2* gene are not caused by gene conversions and possibly are not present in the pseudogene (66, 67).

In a study trying to establish a phenotype-genotype correlation of 13 rare *CYP21A2* pathogenic variants (68) it was demonstrated that some were associated with the severe SW form (L167P, G291S, G292D, and R354H), some with the SV form (I77T, E320K, R341P, and G424S) and with the NC form (I230T and R233K) but at the same time it was observed that some of these pathogenic variants conferred different phenotypes depending on if they were isolated or associated with another pathogenic variant. This was the case of the pathogenic variant I230T responsible for a NC form that if associated with the V281L pathogenic variant corresponded to a more severe phenotype. This synergistic effect that results in a different phenotype has also been described for other pathogenic variants, such as H62L (35), R339H (69), or P105L (65) with P453S.

Polymorphisms

Some variants do not affect the protein production and are considered normal polymorphisms (2). One of these variants, D183E is also present in the *CYP21A1P* gene and represents a gene conversion that does not affect the enzyme activity while others, like K102R, S268T, and N493S have been described only in the *CYP21A2* gene.

GENOTYPING AND PREGNANCY

Genotyping of *CYP21A2* gene is strongly recommended particularly in couples that have the intention to conceive, both to confirm the diagnosis in difficult cases but mostly to be able to do a correct genetic counseling. Although the correlation between genotype and phenotype is high, sometimes the interpretation of the genotypes is rather difficult as we will demonstrate.

The risk for a woman with CAH to have an infant with CAH depends on her partner's genotype. If her partner does not carry a mutant *CYP21A2* allele, all of her children will be carriers but will not have the disease. If the woman is homozygous for a mild pathogenic variant, such as V281L (p.(Val282Leu)) and her partner carries a *CYP21A2* pathogenic variant, the probability is that 50% of her children will have NCAH. Since the probability of a person in the general population being a carrier for a severe pathogenic variant is 1.7% (1 in 60) (22) and the probability of a patient with NCAH having a severe pathogenic variant together with a mild one is ∼60% (as this occurs in 2/3 of the cases) (70), the risk for having a child with classic CAH would be expected to be 1:600. However, it was demonstrated that the real frequency is closer to 2.5% (71) and this increased risk was attributed to presumably higher carrier frequencies in certain ethnic groups. Thus, the genotyping of both parents should be a component of the pre-natal study protocol for families in which one parent has CAH (71).

GENOTYPE-PHENOTYPE CORRELATIONS

In general terms there is a good correlation between genotype and phenotype (90–95%).

Some pathogenic variants translate into the most severe forms of the disease (enzymatic deficiency of almost 100% or, in other terms no 21-hydroxylase activity) resulting in the salt-wasting forms of CAH. These pathogenic variants are called Severe pathogenic variants (**Table 2**).

Missense pathogenic variant I172N (p.(Ile173Asn)) confers around 1–2% 21-hydroxylase activity. This results in a near normal aldosterone synthesis and so it is associated with the simple virilizing form of CAH. These are called the Intermediate pathogenic variants (**Table 2**).

A third group of pathogenic variants including P30L (p.(Pro31Leu)), P453S (p.(Pro454Ser), R339H (p.(Arg340His)), R369W (p.(Arg370Trp)), I230T (p.(Ile231Thr)) (68), and V281L (p.(Val282Leu)) (clearly the most frequent pathogenic variant in NCAH cases in every series) result in a more substantial preservation of enzymatic activity (20–60%) and are associated with the NCAH forms (Mild pathogenic variants) (**Table 2**).

Since most of 21-hydroxylase deficient CAH patients are compound heterozygotes:

(1) The most severe phenotypes (the classic forms) must have two severe pathogenic variants and no mild pathogenic variants

(2) NCAH patients may have two mild pathogenic variants (25–50%) or one mild and one more severe pathogenic variant (50–75%). The mild pathogenic variant allows the synthesis of 21-hydroxylase up to 50% of the normal activity in spite of the fact that the severe pathogenic variant would not contribute to any synthesis.

These pathogenic variants are substantially correlated with the clinical severity and with the different clinical forms of disease—salt-wasting, simple virilising, and non-classical. This is particularly true in patients with severe pathogenic variants. A greater diversity of clinical phenotypes can be observed in patients with less severe pathogenic variants where although the phenotype can be predicted to correspond to the less severely affected allele, the presence of a second allele with a severe or an intermediate pathogenic variant, can result in a more severe clinical phenotype (65, 68, 69, 72, 73).

It was also reported that NCAH patients with a mild plus a severe pathogenic variant had more intense degrees of hirsutism and higher 17OHP levels both basal and after ACTH stimulation than those with two mild pathogenic variants (70, 74).

Heterozygotes, also, have 17OH-progesterone responses to ACTH stimulation that are clearly above normal even though not attaining the levels of patients bearing the disease. These results await further developments.

There are several examples that, in spite of the general assumption that there is a relatively high concordance between genotype and phenotype there is some variability, particularly in the moderately affected patients (75–77). The pathogenic variants designated as *IVS2-13* (c.293-13A/C>G) and *I172N* (p.(Ile173Asn)) for instance result in variable degrees of 21-hydroxylase activity (possibly through alternative splicing) hence explaining that patients that would generally be expected to be Simple Virilizing cases may sometimes be Salt-Wasting and others also be closer to NCAH (51, 60). Another example is the P30L (p.(Pro31Leu)) pathogenic variant which can be associated with cases of NCAH as well as cases of SV-CAH (64).

TABLE 2 | Genotype-phenotype correlation for the most common pathogenic variants, according to the percentage of enzyme activity.

	Variant	% Enzyme active	Phenotype
Severe	Gene deletions and Large gene conversions	0%	Classic—Salt wasting CAH
	8bp del		
	E6 cluster		
	Q318X (p.(Gln319*))		
	R356W (p.Arg357Trp)		
Intermediate	I172N (p.(Ile 173 Asn))	1–2%	Classic—Simple virilizing CAH
Mild	P30L(p.(Pro31Leu))	20–60%	Non-classic CAH
	P453S (p.(Pro454Ser)		
	R339H (p.(Arg340His))		
	R369W (p.(Arg370Trp)		
	I230T (p.(Ile231Thr))		
	V281L (p.(Val282Leu))		

An explanation for some lack of correlation between genotype and phenotype may result from not sequencing the whole gene in most studies hence not having a full picture of the total number of pathogenic variants.

In conclusion, the clinical condition related to 21-hydroxylase deficiency represents a continuum of reductions in enzyme activity of which the 3 levels of severity generally considered, represent merely a systematization to guide and facilitate the clinical practice (15). Finally, it was also recognized that the phenotype can change with time which implies the impossibility of a perfect correlation between genotype and phenotype.

PREVALENCE IN DIFFERENT ETHNIC POPULATIONS

Reports regarding the incidence and percentage of specific pathogenic variants among different ethnic groups have been published (78). The V281L (p.(Pro282Leu)) pathogenic variant is the most common in Ashkenazi Jews (allelic frequency of 63%). Large deletions are frequent in Anglo-Saxons (28%). The Q318X (p.(Gln319*)) was found in 16% of the East Indians. In Croatians, the R356W (p.Arg356Trp)) pathogenic variant was the most frequent (14%).

V281L (p.(Val282Leu), the most common pathogenic variant in most of the European populations, was not detected in Yupik-speaking, Eskimos of Western Alaska, Native Americans, East Indians and Asians. The Yupik Eskimos, representing an isolated geographic population with founder effect, carry the IVS2-13A/C>G: A or C-G pathogenic variant in intron 2 (c.293-13A/C>G).

In a study of a large French population (68) the frequency of the most common pathogenic variants was, for the classical form: int2 (c.293-13A/C>G) (30%), large rearrangements (25%), I172N (p.(Ile173Asn)) (17%), and Q318X (p.(Gln319*)) (7%) and for the NC form V281L (p.(Val282Leu) (55%), int2 (c.293-13A/C>G) (9%) large rearrangements (8%), I172N (p.(Ile173Asn)) (4%), and Q318X (p.(Gln319*)) (3%).

Three novel pathogenic variants, an insertion 1,003^1,004 insA in exon 4, a C>T transition in codon 408 (p.(Arg408Cys)) and a A>G transition in the intron IVS2-2A>G were described in Brazil and suggested to be due to a founder effect, as was previously found for another pathogenic variant (G424S) in the same population (79, 80).

In Finland, there seem to be multiple independent founder *CYP21A2* gene pathogenic variants, each one associated with a different haplotype, where some are identical to those observed in other Europe populations, probably introduced by immigrants from Scandinavian or Baltic origin during the first centuries AD and others found only locally and with a more recent origin. The study of this diversity provided important informations about migrations between and within populations (81, 82).

In Tunisia, there is a high prevalence of the pathogenic variant Q318X (p.(Gln319*)) (35.8%) (83).

A study in Iran, on the contrary, demonstrated that the most frequent pathogenic variants in the CYP21 gene were in2G, del-CYP21A2, and I172N (p.(Ile 173 Asn)). Unlike in other ethnic groups, there was no R356W (p.(Arg357Trp)) pathogenic variant, and a higher rate of del-8bp (10%) was found (84).

In Lebanese, for the classical forms, the most frequent pathogenic variant was the splice site pathogenic variant in intron 2 accounting for 39% of the disease alleles, gene conversions accounted for 14% of the alleles, but no large deletions were found. In non-classical forms, the V281L (p.(Val282Leu)) pathogenic variant in exon 7 represented 86% of the tested alleles (85).

De novo deletions and *de novo* apparent conversions have been reported, comprising about 1% of 21-hydroxylase-deficient alleles. The allele frequency of de novo gene conversion in intron 2 in the general population is estimated 1 in 2×10^4 (2).

Different chimeric *CYP21A1P*/*CYP21A2* genes have been described in different populations, some of them in Taiwanese (45, 46, 86), and the others in patients of Caucasian origin (43, 44, 47).

GENETIC TESTING

PCR-based mutation-detection methods with sequencing of the entire gene and multiplex ligation-dependent probe amplification are nowadays the golden standard for genotyping the *CYP 21A2* gene.

General Considerations

The specific gene amplification by PCR has dramatically improved the sensitivity of the different techniques to detect *CYP21A2* gene pathogenic variants. However, it was initially difficult to use PCR because of the paucity of primers that would amplify *CYP21A2* without amplifying the highly homologous *CYP21A1P* pseudogene, which carries most of the pathogenic variants of interest.

With time, however, PCR conditions were identified that permitted gene-specific amplification of *CYP21A2* in two segments.

PCR-based diagnosis may be complicated by failure to amplify one haplotype and result in misdiagnosis. Examination of flanking microsatellite markers in all family members can minimize this problem.

If only a DNA sample from the patient is analyzed, it is impossible to distinguish between compound heterozygosity for different pathogenic variants in *trans* and the presence of 2 pathogenic variants in the same allele allele (*cis*). Therefore, ideally both parents should also be analyzed so as to most reliably determine the phase of different pathogenic variants (i.e., whether they lie on the same or opposite alleles). Analysis of parental alleles also permits homozygotes and hemizygotes (i.e., individuals who have a pathogenic variant on one chromosome and a deletion on the other) to be distinguished.

In the approach of genetic testing for CAH caused by *CYP21A2* pathogenic variants we can consider three groups of studies:

1- Targeted analysis by screening of the most common *CYP21A2* pathogenic variants
2- Duplications and deletions
3- Whole gene sequencing.

TARGETED PATHOGENIC VARIANT ANALYSIS

This approach is designed to detect the most common pathogenic variants described above. A number of different methods and strategies have been described that cover a variable range of pathogenic variants (87, 88).

Allele-Specific Oligonucleotide Hybridization

This method is based on the hybridization with allele-specific oligonucleotide (ASO) probes, which are short (typically 19–21 nucleotides) single-stranded DNA segments with the specific sequence of each polymorphic or mutant nucleotide in the gene. These probes are usually radioactively labeled. DNA amplified by PCR is dotted on filters and hybridized with the probes corresponding to the normal and mutant sequences for each of the frequently occurring gene conversions (**Figure 5**) (40, 63, 89).

Amplification-Created Restriction Sites

Several pathogenic variants causing 21-hydroxylase deficiency (e.g., V281L and Q318X) create or destroy restriction sites and can thus be detected after digestion of a PCR-amplified DNA fragment with a restriction enzyme and subsequent analysis in agarose gels stained with ethidium bromide. If a restriction site does not exist it can be created by changing the sequence

during the PCR with a modified primer and introduce a polymorphic restriction site into the amplified segment. This method thus involves a series of second round PCRs and several different restriction digests but does not require radioactivity or specialized equipment (**Figure 6**) (89, 90).

Single-Stranded Conformation Polymorphisms (SSCP)

If double stranded DNA is denatured and then quickly returned to native conditions, it will remain in a single-stranded state with a characteristic conformation that can be detected by a change in the mobility of the segment during polyacrylamide gel electrophoresis under non-denaturing conditions. This method can detect novel pathogenic variant that would be missed by allele-specific approaches, but has some complexity in execution and interpretation (**Figure 7**) (74, 92).

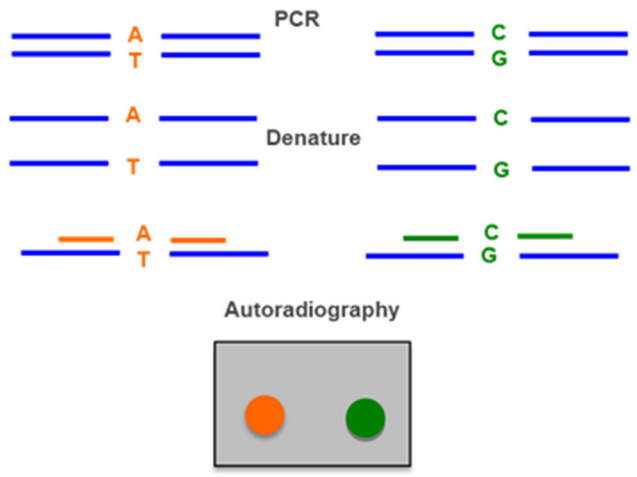

FIGURE 5 | Allele-specific oligonucleotide hybridization (ASO) uses two radiolabeled probes, one for the wild type allele (A–T) and one for the allele with the mutation (C–G). DNA amplified by PCR is denatured, applied in a membrane and hybridized with the two probes. The result is detected by autoraradiography.

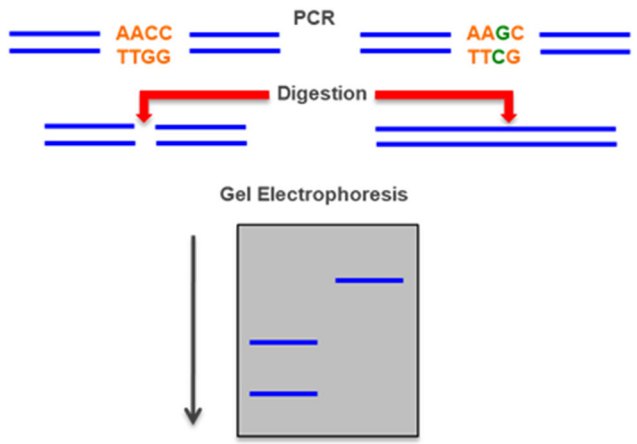

FIGURE 6 | Restriction Fragment Length Polymorphism (RFLP)—a mutation can create or destroy a site that is digested by a specific a restriction enzyme. The mutation can be detected by the different length of a PCR-amplified DNA fragment between the normal and the mutant allele after separation in an electrophoresis gel.

Allele-Specific PCR (ARMS)

In this method two alternative reactions are done for each pathogenic variant. Both PCR reactions use the same primer on one end, but at the other end each reaction uses a primer that corresponds to either the normal or mutant sequence. This technique has similar advantages to the amplification-created restriction site approach. The main differences are that it requires more PCR reactions but does not involve restriction digests (**Figure 8**) (93).

Ligation Detection Reaction (LDR)

DNA ligase can discriminate point pathogenic variant by sequential rounds of linear template dependent ligation and preferentially seal adjacent oligonucleotides hybridized to target DNA in which there is perfect complementarity at the nick junction. A single base mismatch at the nick junction inhibits ligation and permits sequence discrimination at the single nucleotide level by the mobility on a sequencing gel. If the oligonucleotides are fluorescently labeled, the entire genotyping can be performed on an automated DNA sequencer (**Figure 9**) (94).

D-HPLC

Denaturing high pressure liquid chromatography (DHPLC) is a relatively new technique, which uses heteroduplex formation between wild-type and mutated DNA strands to identify

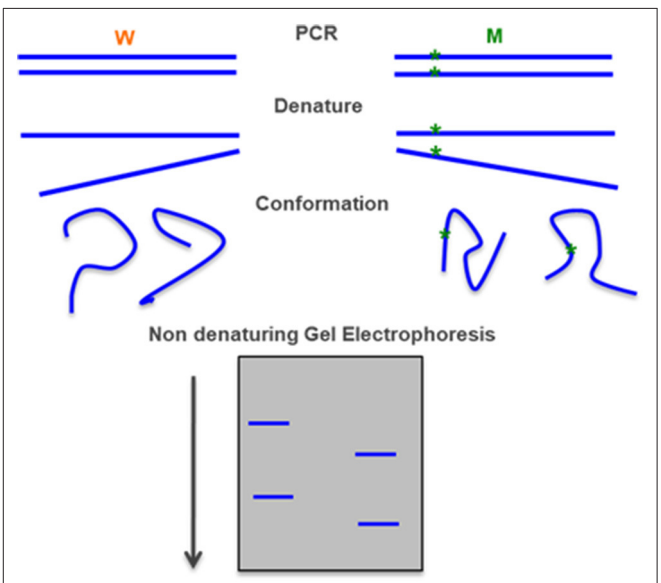

FIGURE 7 | Single-stranded conformation polymorphism—a PCR-amplified DNA fragment is denatured in particular conditions and each the single-stranded fragment assumes a characteristic conformation that can be detected in a polyacrylamide gel electrophoresis. Adapted form Gasser et al. (91).

pathogenic variant. Heteroduplex molecules are separated from homoduplex molecules by ion-pair, reverse-phase liquid chromatography on a special column matrix with partial heat denaturation of the DNA strands (**Figure 10**) (95, 96).

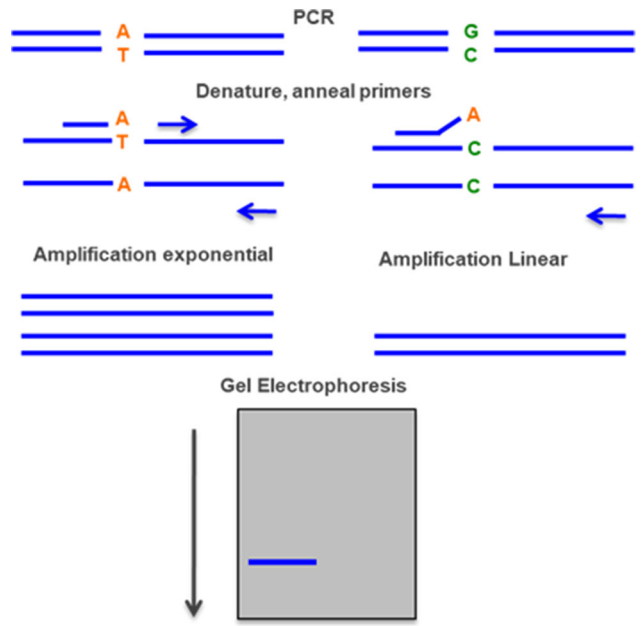

FIGURE 8 | Allele-specific PCR—two PCR reactions are done simultaneously, with the same primer in one end and two different primers on the other end, one with the normal sequence and the other with the mutant. The rate of amplification is much higher with the specific primer and can be detected by gel electrophoresis.

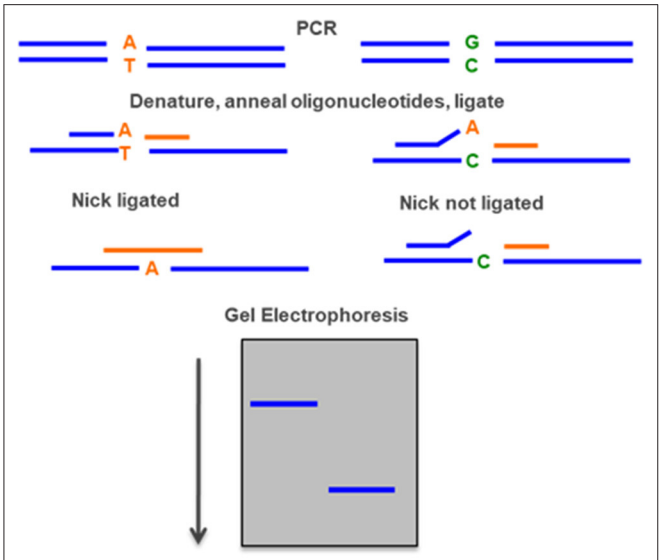

FIGURE 9 | Ligase detection reaction—DNA ligase preferentially seal adjacent oligonucleotides hybridized to a DNA sequence when there is a perfect complementarity at the nick junction. A single base mismatch generates a different fragment detected by gel electrophoresis.

Minisequencing and Multiplex Minisequencing

In minisequencing, a primer is hybridized to DNA next to a variant nucleotide site and extended with DNA polymerase by a single appropriate dideoxyribonucleotide triphosphate (ddNTP) that matches the nucleotide at the target site. This method can be used in a multiplex reaction with primers elongated at the 5′ end with a poly(T) track of different sizes to facilitate electrophoretic separation of the diagnostic products (**Figure 11**) (97).

DUPLICATIONS AND DELETIONS

A variety of methods are also available that can detect large deletions or duplications not only in the exonic or intronic regions of the CYP21A2 gene but also in the promoter and in contiguous regions as for the C4B gene.

Southern-Blot

This is a method that combines the transfer of electrophoresis and separation of DNA fragments to a filter membrane and subsequent fragment detection by probe hybridization. It usually uses genomic DNA, previously digested with restriction enzymes, to determine the number of sequences (e.g., gene copies) in a genome. Because it is a time-consuming and laborious method that uses radioactively labeled probes and requires a large amount of DNA it has been replaced by other techniques (**Figure 12**) (44, 98).

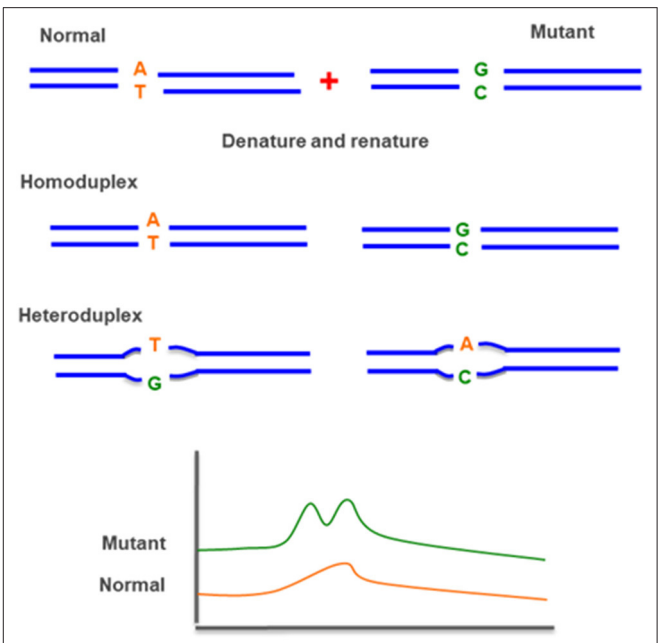

FIGURE 10 | Denaturing high pressure liquid chromatography (D-HPLC)—uses heteroduplex formation between normal and mutated DNA strands. The different conformations are detected by ion-pair reverse-phase liquid chromatography.

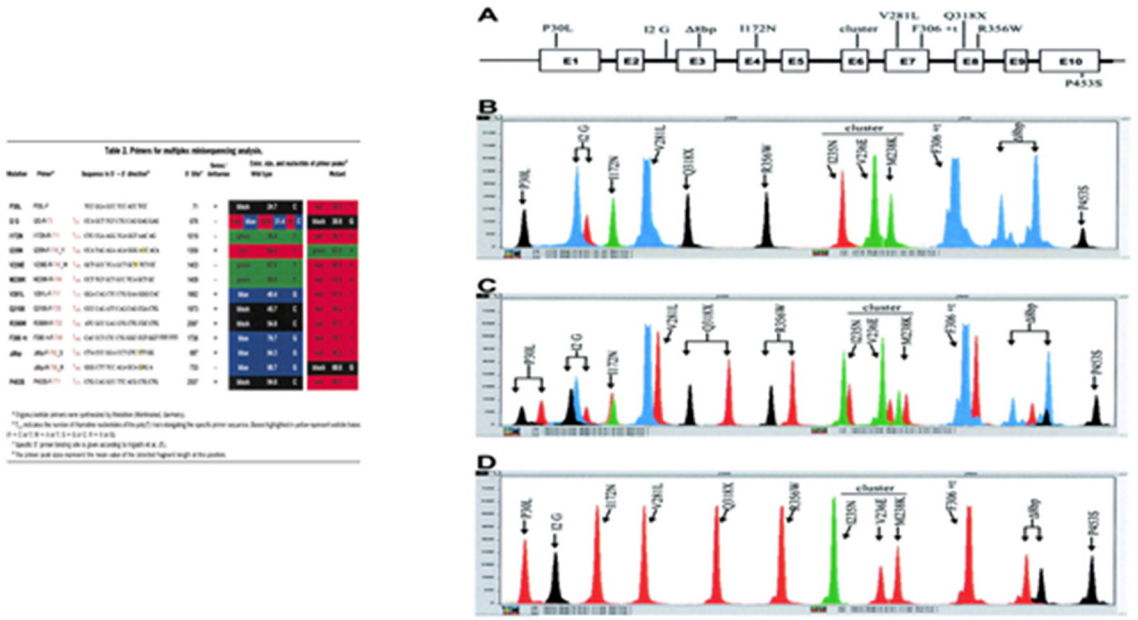

FIGURE 11 | Minisequencing—a primer is hybridized to DNA next to a variant nucleotide site and extended with DNA polymerase by a single appropriate dideoxyribonucleotide triphosphate (ddNTP) that matches the nucleotide at the target site. A poly(T) sequence with different sizes is included in each primer at the 5′ end to facilitate the electrophoretic identification. Adapted from Krone et al. (97). **(A)** The CYP21 gene shown schematically with the nine most common mutations, transferred by apparent gene conversions from the CYP21P pseudogene. The P453S mutation is not present in the pseudogene, but occurs in 1–2% of mutant alleles. **(B)** CYP21 wild-type gene with heterozygosity for the A/C polymorphism at the intron 2 splice site (I2 G) position. **(C)** Mixture of CYP21 and CYP21P gene fragments demonstrating the detection of heterozygous mutations at every peak position. **(D)** CYP21P pseudogene amplicon with all common CYP21-inactivating mutations, demonstrating the detection of all mutations in a homozygous state.

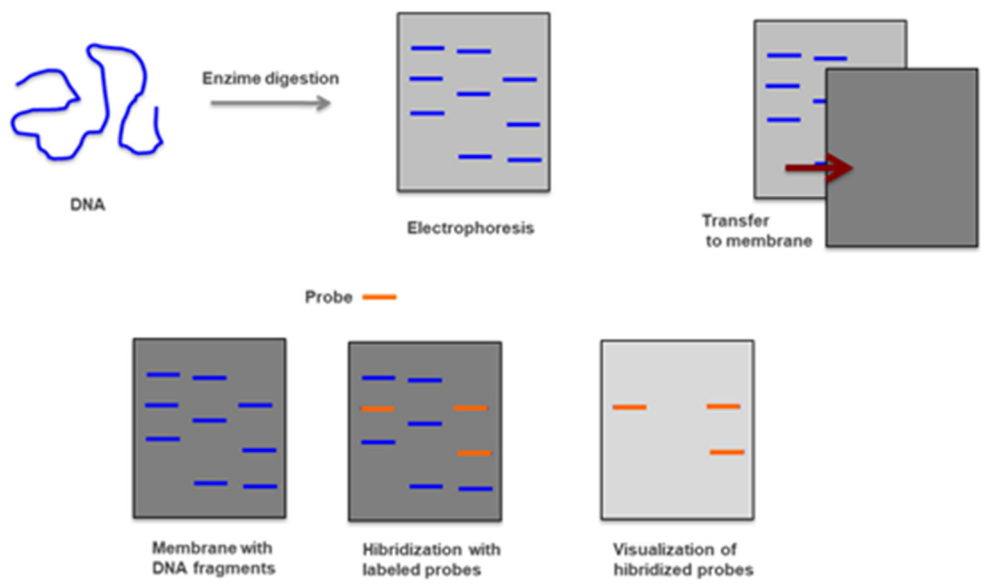

FIGURE 12 | Southern-blot—Genomic DNA is digested with restriction enzymes and smaller fragments are obtained and separated by electrophoresis. After being transferred to a membrane and hybridized with radioactively labeled probes are detected by autoradiography.

Real Time PCR

Real time PCR is a technique where the progressions of a PCR reaction can be monitored in real time and simultaneously quantify the amount of product amplified. The method is based on the detection of the fluorescence produced by a reporter molecule which increases, as the reaction proceeds.

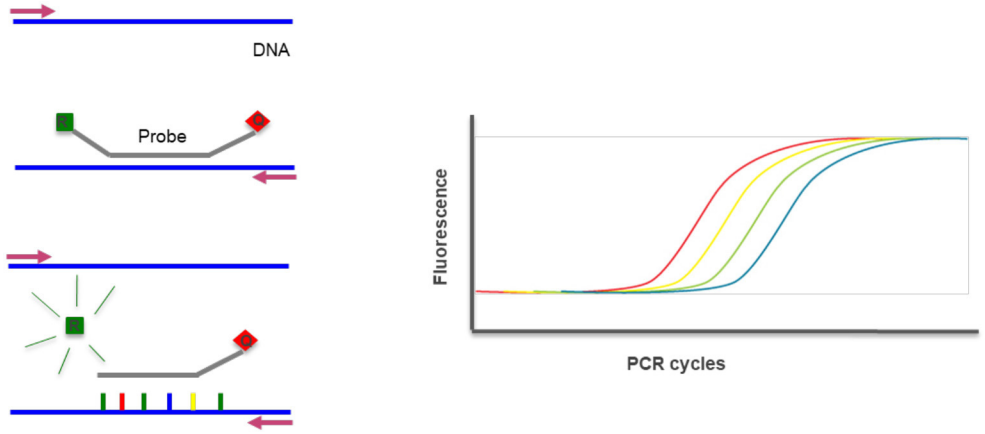

FIGURE 13 | Real-time PCR—the method uses DNA probes with a fluorescent reporter at one end and quencher of fluorescence at the opposite end. The close proximity of the reporter to the quencher prevents detection of its fluorescence. During a PCR reaction, in each cycle the probe hibridyzes and elongates releasing the reporter that produces fluorescence detected and measured in real-time. The increase of fluorescence reflects the increase of product and can be quantified. R, Reporter; Q, Quencher.

This quantification can be used to assess gene copy number variations through a co-amplification of a control gene (**Figure 13**) (23).

MLPA

Multiplex Ligation-dependent Probe Amplification (MLPA) assay is a technique that enables the detection of variations in the copy number of several human genes. Due to this ability, MLPA can be used for several molecular diagnosis of several different genetic diseases whose pathogenesis is related to the presence of deletions or duplications of specific genes. Moreover, MLPA assay can also be used in the molecular diagnosis of genetic diseases characterized by the presence of abnormal DNA methylation. Due to the large number of genes or genetic sequences that can be simultaneously analyzed by a single technique, MLPA assay represents the gold standard for molecular analysis of all pathologies derived from the presence of gene copy number variation. Detection of deletions and duplications of the *CYP21A2* gene and *CYP21A1P* pseudogene is currently performed by Multiplex Ligation—dependent Probe Amplification (MLPA), using the P050- CAH Kit (MRC-Holland). This high resolution method to detect copy number variation in genomic sequences uses only a single pair of PCR primers and the specificity relies on the use of progressively longer oligonucleotide probes in order to generate locus-specific amplicons of increasing size that can be resolved electrophoretically. Comparing the peak pattern obtained to that of the reference samples it is possible to determine which probes/locus show aberrant copy numbers (**Figure 14**) (99, 100).

DNA SANGER SEQUENCING

Nowadays, in many hospitals, a whole gene sequencing together with MLPA has become the standard procedures to genotype the CYP21A2 gene in cases of 21OH Deficiency. This is the method elected to detect pathogenic variants not screened by the targeted analysis and is also able to detect novel sequence variants. It usually covers the coding regions and the flanking intron-exon regions of the gene. *CYP21A2* whole genomic sequence may be performed selecting the functional *CYP21A2* gene and amplifying by PCR into 2 partially overlapping fragments, P1 and P2 (**Figure 16**), respectively with 1 517 and 2 214 base-pairs (bp), avoiding the co-amplification of the pseudogene *CYP21A1P* (101). After selective amplification of the targeted genes and subsequent purification, the PCR product is sequenced with internal primers that cover the entire *CYP21A2* gene (**Figures 15**, **16**) (102).

NEW ASPECTS IN GENOTYPING

A final and promising aspect that can result from Genotyping is prevention. Preimplantation Genetic Diagnosis (PGD) can already be performed and be used to limit the transmission of the disease when used in conjunction with *in vitro* Fertilization (IVF).

For prenatal diagnosis the use of maternal circulating fetal DNA (Cff-DNA) allows early gender determination (*SRY*) in a precocious phase of pregnancy. This allows a timely identification of male fetuses that do not need to be treated prenatally contrarily to female fetuses in which doctors may want to prevent the occurrence of genital ambiguity. It is also possible to do sequencing of the *CYP21A2* gene in the fetal DNA circulating in maternal blood, but the technique is complex and still carries a significant possibility of false positive or false negative diagnoses. Chorionic villus sampling and amniocentesis still gives better outcomes but can only be performed rather late in view of the timing where genetic identification of Classic phenotypes of the disease would mostly benefit the decision process concerning the institution of dexamethasone suppressive treatment during the pregnancy (103).

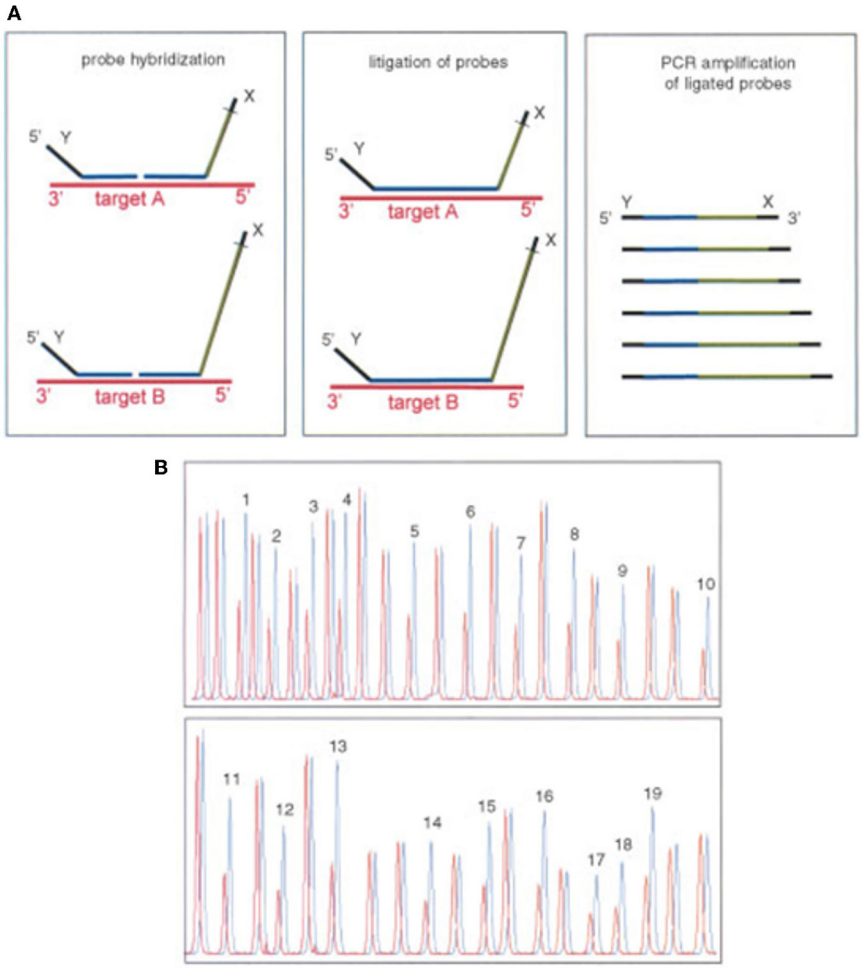

FIGURE 14 | Multiplex ligation-dependent probe amplification (MLPA). Adapted from Schouten et al. (99). **(A)** Multiplex ligation-dependent probe amplification (MLPA) uses a single pair of primers and specific probes with progressive increasing lengths to be identified by electrophoresis. **(B)** Comparing the height of each peak of the sample with a control detects the number of copies.

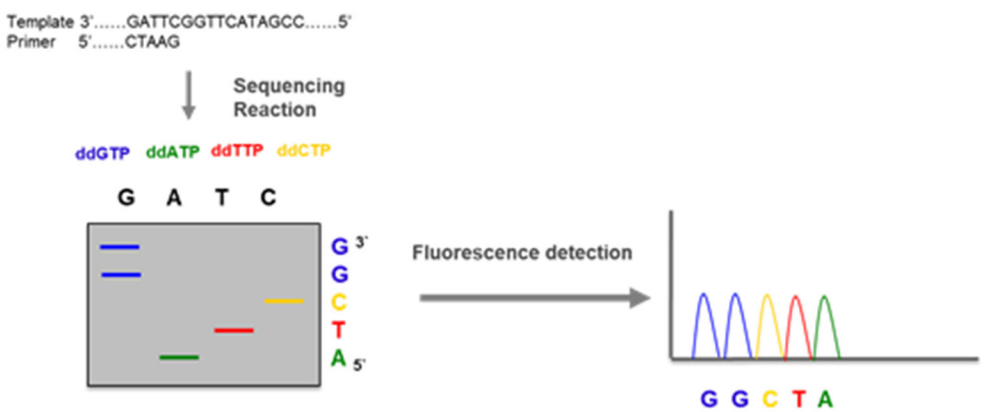

FIGURE 15 | DNA sequencing—a DNA fragment amplified by PCR is used in an amplification reaction that, besides the normal nucleotides dATP, dGTP, dCTP, and dTTP, contains a mix of dye labeled terminator nucleotides (ddATP, ddGTP, ddCTP, and ddTTP). These modified nucleotides do not have the capacity of elongate the DNA chain and terminate the reaction when incorporated. The DNA sequence is obtained by electrophoresis that separates the fragments and fluorescence detection that identifies each of the nucleotides.

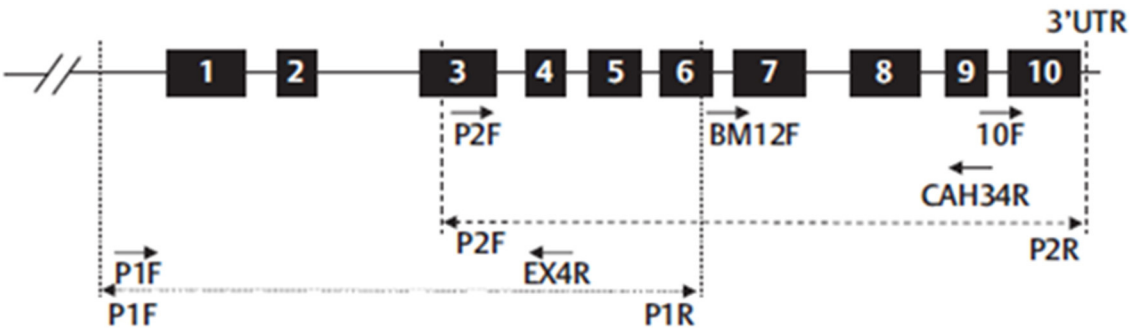

FIGURE 16 | Schematic representation of *CYP21A2* gene structure and the strategy proposed to sequence whole gene. Numbered black boxes represent *CYP21A2* exons. Dot line (...) represent the first PCR-P1-amplification product (1,517 bp) and dash line (- - -) represent the second PCR-P2-amplification product (2,214 bp). Arrows represent the primers used in six different sequencing reactions (P1F and Exon4R for P1 fragment and P2F, BM12F, CAH34R, and 10 F for P2 fragment). Adapted from Carvalho et al. (102).

CONCLUSION

Congenital adrenal hyperplasia due to 21-hydroxylase deficiency are a group of very important diseases due to its high morbidity (the Classic forms) and its high prevalence (the Non-classic forms). They affect patient's life in many ways, going from salt wasting life-threatening crises to genital ambiguity with all its consequences of gender determination and reconstructive surgeries ultimately affecting normal sexuality and reproduction. This already highlights the importance of having a correct diagnosis to the level of a complete genetic characterization.

Perhaps more importantly than being infertile many of these patients often do not even attempt to conceive, but in those who wish to do it, genetic counseling is of particular importance.

The consequences of these diseases go still beyond, affecting growth and final height, body image, impacting on self-esteem and other psychological consequences including depression and anxiety. Attention to the consequences of overtreatment as well as under-treatment should always be present. In adults transitioning from pediatric care, adrenal crises and cardiovascular consequences together with the psychological well-being become the principal focus.

The diagnosis is first confirmed through 17OH-progesterone determinations which can be very high, moderately high or even normal at basal conditions needing confirmation through an ACTH stimulation test. The defining cut-off is generally considered to be between 10 and 15 ng/ml, either basally or post-ACTH. In the case of women these dosages should be done in the follicular phase of their menstrual cycles and in every patient the blood samples should be collected early in the morning. Newborn screening programs are very important as they permit the identification of severe cases at the ideal time for treatment thus being life-saving in many situations. In consequence of this screening programs, survival is no longer the major issue and has been replaced by the need to improve the patients' quality of life.

Suspected cases and even confirmed ones should be genotyped to completely characterize the pathogenic genetic variants. Both parents should also be analyzed to confirm that the pathogenic genetic variants affect both alleles. The actual recommendation involves the entire gene sequencing whenever that is possible. The main objectives are to confirm the diagnosis, delineate personalized therapeutic strategies and allow a correct genetic counseling.

AUTHOR CONTRIBUTIONS

DP and AP designed the paper. DP, BC, AP, AB, SG, and DM wrote and reviewed the article.

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CORRECTION
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Corrigendum: The Complexities in Genotyping of Congenital Adrenal Hyperplasia: 21-Hydroxylase Deficiency

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In the article, an author's name was incorrectly spelled as **Djuro Maçut**. The correct spelling is **Djuro Macut**. In addition there was an error in affiliation **7**. Instead of "**Department of Endocrinology, Diabetes and Metabolic Diseases, Faculty of Medicine, University of Belgrade, Belgrade, Serbia,**" it should be "**Clinic of Endocrinology, Diabetes and Metabolic Diseases, Faculty of Medicine, University of Belgrade, Belgrade, Serbia**."

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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REVIEW
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Metabolic Perspectives for Non-classical Congenital Adrenal Hyperplasia With Relation to the Classical Form of the Disease

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Non-classical congenital adrenal hyperplasia (NC-CAH) represents mild form of CAH with the prevalence of 0. 6 to 9% in women with androgen excess. Clinical and hormonal findings in females with NC-CAH are overlapping with other hyperandrogenic entities such as polycystic ovary syndrome hence causing difficulties in diagnostic approach. Metabolic consequences in subjects with NC-CAH are relatively unknown. We are lacking longitudinal follow of these patients regarding natural course of the disease or the therapeutic effects of the different drug regiments. Patients with NC-CAH similarly to those with classical form are characterized with deteriorated cardiovascular risk factors that are probably translated into cardiometabolic diseases and events. An increased preponderance of obesity and insulin resistance in patients with NC-CAH begin at young age could result in increased rates of metabolic sequelae and cardiovascular disease later during adulthood in both sexes. On the other hand, growth disorder was not proven in patients with NC-CAH in comparison to CAH patients of both gender characterized with reduced final adult height. Similarly, decreased bone mineral density and osteoporosis are not constant findings in patients with NC-CAH and could depend on the sex, and type or dose of corticosteroids applied. It could be concluded that NC-CAH represent a particular form of CAH that is characterized with specificities in clinical presentation, diagnosis, therapeutic approach and metabolic outcomes.

Keywords: non-classical congenital adrenal hyperplasia, metabolism, obesity, cardiovascular risk, stature, osteoporosis, glucocorticoids, antiandrogens

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INTRODUCTION

The non-classical form of congenital adrenal hyperplasia (NC-CAH) was initially called late-onset as clinical presentation was observed in adolescents and adults. Presentation of NC-CAH is subtle and diagnosis requires implementation of different tests to exclude other problems. Moreover, clinical expression of NC-CAH is variable in patients carrying the same mutation. This suggests that additional factors may modify the clinical expression of the disease including age, steroid metabolic

pathways, variation in androgen production, individual sensitivity to androgens, differences in skin sensitivity to androgens and possibly the existence of other genes modifying 21-hydroxylase activity (1, 2). In patients with NC-CAH predominant signs are those of androgen excess including premature pubarche, acne, hirsutism, polycystic ovary syndrome (PCOS) and subfertility (3).

NC-CAH is more frequently observed in females. The preponderance of NC-CAH in women with androgen excess, and according to ethnicity and genotype, is ranging from 0.6 to 9% (2). Males with NC-CAH are diagnosed significantly less often than females due to less frequently presented and recognizedsigns of androgen excess (4). One of the scarce and small studies showed in men with NC-CAH premature pubarche in 29% before 9 years of age (5). Gynecomastia has been reported as the presenting symptom in two male adolescents with non-classical congenital adrenal hyperplasia. Considering diagnosing NC-CAH, basal values of 17-OHP below 6 nmol/L were found in 2.1% of subjects with disease while concentrations of over 30 nmol/L after ACTH test is confirmatory for diagnosis (5).

As CAH is considered as disease spectrum, disease phenotype is ascertained by the less severe mutation with the highest residual enzymatic activity of CYP21A2 (6). The prevalence of classical CAH is from 1:10,000 to 1:20,000 births (7, 8) while in the non-classical CAH it is 1:1,000 births (9) and occurs in about 6% of hirsute women (10) (**Supplementary Table 1**).

Metabolic consequences in subjects with NC-CAH are relatively unknown and we are missing longitudinal follow up data of these patients regarding either the natural course of the disease or the outcomes of therapeutic regiments mainly based on glucocorticoids. Therefore, metabolic consequences in NC-CAH subjects could be analyzed from scarce clinical studies (11) or extrapolated from as well scarce clinical follow-up of patients with classical CAH.

CLINICAL CHARACTERISTICS IN DIFFERENT AGE PERIODS

Growth Differences

Patients With Non-classical CAH

Both boys and girls with NC-CAH could be characterized with fast linear growth, advanced bone age with consequent tall stature (4, 12). On the other hand, short stature was reported in subjects with NC-CAH as well particularly in patients on glucocorticoid therapy that commenced before the start of puberty. However, risk for short stature is relatively small with majority of children having almost normal final height (13).

Patients With Classical CAH

Birth length of salt wasting (SW) boys and girls at neonatal screening is above the average at birth while during infancy their height velocity declines by the age of 1.5 years in boys, and by the age of 3 years in girls (14). Patients with simple virilizing (SV) form of CAH have relative androgen insensitivity during the first year of life with consequent absence of increased height velocity (15). Before puberty, patients with SW are growing close to the reference values. True pubertal growth spurt is not noticeable in both forms and genders (16).

The first meta-analysis on the height outcome in classical CAH showed similar mean final height SD score (SDS) of −1.37 (∼10 cm) in both genders compared to their target height (17). Studies on the outcomes in adults with classical CAH showed those on smaller glucocorticoid doses had slightly better SDS outcome of −1 below the target height. Patients with both CAH forms had reduced final adult height. However, women with simple virilizing form of CAH had shorter stature in comparison to those with salt wasting phenotype (18) (**Supplementary Table 1**).

Sexual Maturation Characteristics

Howig et al. reported that the mean age for the onset of puberty was 9.8 years for girls with simple virilizing form, and 10.3 years for girls with SW CAH, and similar age at menarche of 13.3 and 13.7 years, respectively. In boys with SV form the onset of puberty was at 9.8, and 10.6 years for those with SW (19). Moreover, dissociation of pubarche and adrenarche was shown in classical CAH. Namely, in both boys and girls an earlier pubarche, gonadarche, and thelarche was confirmed with the absence of typical signs of adrenarche. Children having advanced skeletal maturation are at risk for early pubertal development (20). Elevated androgens may induce secondary central precocious puberty. High adrenal androgens resulting from non-suppression of HPA axis cause early puberty in both forms, more prevalent in SV than SW forms (21).

In those with NC-CAH pubertal delay, pubertal development progressed well after the initiation of glucocorticoid therapy with attainment of menarche and subsequently regular menstrual cycles (22) (**Supplementary Table 1**).

IMPORTANCE OF DIFFERENTIAL DIAGNOSIS

NC-CAH and Early Adrenarche/Pubarche

Adrenarche is characterized with elevated secretion of adrenal androgen precursors (AAPs), namely, dehydroepiandrosterone (DHEA) and DHEA-sulfate (DHEAS) that occurs at 5–8 years of age (23). Premature adrenarche (PA) and premature pubarche (PP) are defined as early appearance (before 9 years in boys and 8 years in girls) of axillary and pubic hair, adult type body odor or acne, with the absence of true central puberty. AAPs concentrations are above the prepubertal level with DHEAS $>$ 1 μmol/l (termed as biochemical adrenarche) (24). The mechanisms of PA are not fully elucidated but the obesity and genetic factors seems to contribute in apparently multifactorial etiology (24).

Premature adrenarche is a benign condition but before the diagnosis can be accepted, differential diagnosis should consider defects of cortisol synthesis, androgen-producing tumors originated from adrenals or gonads, central precocious puberty, primary hypothyroidism, Cushing's syndrome, exposure to exogenous androgens, and most importantly NC-CAH (25).

The reported prevalence of CAH in PP patients differs from study populations (0–43% for all types of CAH) (26).

Differential diagnosis between PA and NC-CAH, as well as other genetic reasons for adrenal hyperandrogenism, is not always obvious based on clinical examination. However, rapidly accelerating growth, remarkable androgenic signs and bone age advancement, and a positive family history are clues toward a genetic disorder (27).

NC-CAH and PCOS

Clinical and hormonal findings that are overlapping with other hyperandrogenic entities such as PCOS may cause some difficulties in diagnostic approach in patient with suspected NC-CAH. Therefore, one should question what endocrine tests are appropriate to confirm or exclude NC-CAH, and if genetic analyses are indicated (5, 28, 29).

PCOS is considered frequent endocrine disease of women during reproductive period that is characterized with hyperandrogenism (30). As PCOS is a disease of exclusion, clinicians are obliged to perform necessary basal and functional endocrine testing to exclude other causes of androgen excess. Serum total testosterone determination is the main androgen determinant of PCOS (30). Approach to the hormonal analyses depends on the severity of hyperandrogenism. Determination of total testosterone is requested in patients with regular menstrual cycles and mild hirsutism (30). Determination of total testosterone together with FSH and LH is requested in patients with moderate hirsutism. In subjects with sudden development of hirsutism in adolescents and adults, and high concentrations of testosterone and androstenedione, an additional ACTH stimulation test with determination of 17-OHP together with CYP21A2 genotyping is requested (31).

Gonads could contribute to increase in 17-OHP as they also secrete this hormone. A hyperresponsiveness of 17-OHP to gonadotropin-releasing hormone agonist (GnRHa) is confirming ovarian hyperandrogenism. Hence, PCOS is considered as a result of ovarian thecal cell overactivity associated with 17,20-lyase activity down-regulation (32). As a consequence, hyperandrogenemia in PCOS could lead toward estrogen oversecretion. However, LH to estradiol ratio is kept normal in functional ovarian hyperandrogenism in the presence of elevated 17-OHP (33).

THERAPEUTIC APPROACH

Therapy for NC-CAH

Concerning therapy for NC-CAH patients in contrast to classical CAH patients, adrenal replacement is not required. Therefore, pharmacological treatment is focused on the management of the signs of androgen excess. Use of glucocorticosteroid (GCS) therapy should be reserved for special outcomes such as restauration of fertility. When talking on the long-term outcomes of androgen excess including menstrual cycle derangement, first line options are antiandrogens and oral contraceptives (34) (**Supplementary Table 1**).

Therapy for Classical CAH

Glucocorticoids and All Forms of CAH

Longitudinal growth and bone age development are the most important clinical parameters for monitoring adequate glucocorticoid replacement in children with CAH (35). Serum levels of 17-OHP, androstenedione and testosterone need to be monitored frequently and the hydrocortisone dose adjusted to maintain treatment efficacy. The levels of androstenedione and testosterone are more important in monitoring the glucocorticoid dosage than 17-OHP that should be kept a little above the normal range.

Analysis of growth in CAH patients reveal that the two most rapid phases of growth (first year of life and pubertal growth spurt) are vulnerable ones, during which glucocorticoid overtreatment should be avoided (14, 19). During infancy hydrocortisone dose should be decreased in patients with slower growth velocity, but strict hydrocortisone dose adjustments are necessary during childhood to avoid accelerated growth and advanced skeletal maturation (16). Pharmacokinetics of free cortisol showed shorter half-life in pubertal girls than in pubertal boys (36). Consequently, female patients at puberty need more frequent hydrocortisone replacement regimen. Besides decreased compliance of adolescents with CAH, use of oral contraceptive pills, aromatase inhibitors, thyroxine, or ingestion of hydrocortisone with food could influence cortisol dynamics and altering hydrocortisone dosing (37).

Hydrocortisone three times per day is the mostly prescribed regimen by pediatricians (38). Prednisolone or dexamethasone could be used in children with late puberty and in adults, but are avoided in childhood due to the potent effect on growth. It was observed several rises in cortisol concentrations during the day in children on hydrocortisone therapy. These phased are interchanged with periods of hypocortisolemia between the doses, especially during the night. Reduction of glucocorticoid dose during puberty is of clinical importance as pubertal height gain represent a predictor of final height. On the other hand, low glucocorticoid dose with concomitant hyperandrogenism can lead to premature epiphyseal closure and short stature (17). Other treatment options to improve final height and metabolic outcome might include subcutaneous hydrocortisone infusion through a pump, as well as plenadren (modified-release hydrocortisone) and chronocort (modified-release hydrocortisone formula, under development) that have a delayed and sustained absorption profile (38) (**Supplementary Table 1**).

Mineralocorticoids

Endocrine Society (USA) recommended fludrocortisone (0.05–0.2 mg once or twice per day) to be used in all patients with classical CAH (13). Fludrocortisone is used more frequently and in higher doses in patients with more severe genotypes. Overtreatment, defined by renin concentrations either in the lower reference range or suppressed, was only rarely observed throughout the different mutation groups (22) (**Supplementary Table 1**).

METABOLIC OUTCOMES OF NC-CAH

Obesity and Cardiometabolic Risk During Life

Non-classical CAH

One third of untreated NC-CAH women have insulin resistance (11, 39). In children with both classical CAH and NC-CAH

prevalence of obesity is approximately 35% and exceeds obesity rates in children and adolescent in the general population (18). Obese children with either forms of CAH are hyperinsulinemic and hyperleptinemic but predominantly in those with classical form of CAH (18, 40). However, Saygili et al. did not show difference in leptin concentrations in hyperinsulinemic and hyperandrogenemic adult women with NC-CAH in comparison to controls (39). Moreover, an association of leptin levels with hyperinsulinemia and hyperandrogenism in women with NC-CAH was not established with conclusion that potential relation of leptin with hyperinsulinemia and reproduction in NC-CAH patients need further investigation (39).

We are lacking clinical studies in NC-CAH children regarding obesity, metabolic syndrome and their consequences (41). Concerning adult NC-CAH patients, it was recently shown an increased risk of metabolic and cardiovascular morbidities in both males and females (42).

Adrenal androgen secretion is increased in NC-CAH in the presence of normal levels of ACTH in the majority of subjects (43). Moreover, NC-CAH patients have normal levels of DHEAS while androstenedione and testosterone are similarly elevated to the levels in PCOS women (43, 44). As mentioned previously, altered enzyme kinetics due to CYP21A2 missense mutations is associated with adrenal androgen oversecretion in NC-CAH (2). Another mechanisms contributing to hyperandrogenism in NC-CAH includes ovarian dysfunction and peripheral synthesis of androgens from steroid precursors (41). As a consequence, use of glucocorticoids around puberty in hyperandrogenic state could favor abdominal visceral adiposity, insulin resistance with concomitant metabolic derangement and further exacerbation of androgen production (45). It was recently proposed a backdoor pathway for hyperandrogenism of NC-CAH with transformation of 17-OHP and progesterone into more potent androgens such as dihydrotestosterone (46) **(Supplementary Table 1)**.

Classical CAH

It was observed an existence of higher rates of obesity and insulin resistance in adult patients with CAH (22). Hyperandrogenism was suggested as an intrinsic hormonal imbalance in classical CAH. Life-long glucocorticoid treatments are conferring to the increased risk for obesity and cardiovascular disease (CVD). Moreover, higher doses of glucocorticoids were associated with obesity in adults irrespectively on their family predisposition to obesity (18).

Leptin and other adipokines are elevated in almost all ages of patients with classical CAH, and characterized with abdominal obesity, consequent changes in food consumption, insulin sensitivity, and energy homeostasis. Therefore, it seems that adipokines are involved in the pathogenesis of obesity in patients with CAH (47). The increase in amount of fat commenced during childhood, it is existing even in children adequately treated, and was found in young adults with CAH as well (48). However, we are lacking information on the metabolic activity of abdominal adipose tissue in CAH patients. Recently, Kim et al. (47) showed that CAH adolescents and young adults have increased abdominal adiposity, with a higher proportion of pro inflammatory visceral adipose tissue (VAT) than subcutaneous adipose tissue. This places CAH patients at even greater risk for harmful metabolic sequelae from obesity linked to CVD risk. Moreover, strong correlation was obtained between VAT and adipokines or inflammatory markers (i.e., leptin, PAI-1, and hs-CRP). These findings implicated on the association of insulin resistance and metabolic syndrome in young patients with CAH (49). Consequently, adolescents and young adults with CAH express similar low-grade inflammation as obese individuals without CAH (49).

Clinical studies based on dual x-ray absorptiometry (DXA) showed that either males or females with classic CAH exhibit higher total fat mass and reduced lean body mass than controls. In respect to therapy used, a week correlation was found for cumulative glucocorticoid dose and total fat mass in females only (50). However, there was no observed differences between males and females for body composition or obesity in either classical CAH or NC-CAH (18, 40, 47, 51) (**Supplementary Table 1**).

Impact of Therapies on the Disease Prevalence

In respect to the therapy used, different studies analyzing patients with classical CAH showed variation in prevalence of obesity from 16.8 to 35% on hydrocortisone doses ranging from 13.3 to 15 mg/m^2/day while only one study reported prevalence of obesity of 60.7% on the dose of 19.5 mg/m^2/day (50). As meta-analyses was not performed yet, it could not be suggested a linear correlation between the prevalence of obesity and increasing doses of hydrocortisone as a frequently used therapeutic modality.

Many studies analyzing patients with CAH indicate an increased prevalence of insulin resistance but very few indicated an increased prevalence of diabetes including gestational diabetes. This could be partly explained with CAH studies analyzing patients younger than 50 as age group that is not typical for development of diabetes (4). In respect to glucocorticosteroids used, a few reports on classical CAH patients are giving opposite results with elevated insulin resistance index (HOMA-IR) in 18 and 44.4% on hydrocortisone doses of 19.5 and 11.2 mg/m^2/day, respectively (50).

Androgen Excess and Cardiovascular Risk

Classical CAH as hyperandrogenic state is characterized in women with increased cardiovascular risk (52). Either low or high testosterone levels have an increased risk for CVD independently of age, adiposity, ovarian function, and smoking (53). Traditional and non-traditional cardiovascular risk markers as well as their functional and morphological effects recognized throughout the surrogate indices, imply on the existence of increased cardiovascular risk and subclinical CVD in various types of women with androgen excess (52). Study by Falhammar et al. (54) on small number of patients analyzed cardiovascular and metabolic risk profiles in adult CAH males on lifelong glucocorticoid treatment. The authors showed that younger CAH males did not differ from age-matched controls while risk increased in subjects older than 30 years (54). However, we are lacking data on the frequency of established cardiovascular disease and mortality. It was shown that stroke

is common in women with NC-CAH (odds ratio 5.8) while acute coronary syndrome is characteristic for males with classical CAH (odds ratio 9.9) (42). Hazard ratio of death was higher for females, and with 32% caused by cardiovascular diseases (55).

Higher concentrations of testosterone and DHEA in post-menopausal women are associated with the progression of atherosclerosis and development of hypertension (56). It was recently suggested an association of hyperandrogenism with the occurrence of inflammation and oxidative stress at the level of vascular endothelium, and concomitant renal reabsorption of sodium and water (56). Androgens have an adverse effects on insulin sensitivity, visceral adiposity and lipolysis, low-density-lipoprotein cholesterol (LDL-C) clearance, and high-density-lipoprotein cholesterol (HDL-C) concentrations (57). Moreover, androgen excess is deteriorating lipid profile making it more atherogenic through lower HDL-C, increased LDL-C level as a consequence of blunted LDL-receptor activity, or by enhanced lipoprotein lipase activity (57).

CAH is characterized by an existence of cardiovascular risk factors including dyslipidemia, obesity and insulin resistance, as well as cardiovascular outcomes as hypertension (58). There is a similarity in respect to the presence of cardiovascular risk factors in CAH patients and PCOS patients. Glucocorticoid used in high doses for the androgen suppression could induce Cushing syndrome characteristics with further aggravation of existing cardiovascular risk factors (59). Some data in children's populations showed decreased HDL-C in ∼10% of both classic CAH and NC-CAH subjects (18). In another study, an association of androgen concentrations with dyslipidemia, obesity, and IR was shown in CAH patients (58) as well as elevated triglyceride concentrations confirmed in children with classical CAH on prednisone therapy (60). Moreover, prepubertal children with classical CAH could have elevated leptin and insulin levels constituting a group with increased life-long cardiovascular risk (61).

Hypertension is frequent finding in patients with classical CAH. It could be a consequence of the disease or the effect of therapy. Moreover, hypertension is considered the main cause of cardiovascular morbidity in young patients with CAH (62). In respect to the activity of the disease, elevated blood pressure was more likely diagnosed all patients with classical CAH than in NC-CAH ones, and associated with children of younger age and adult male gender, respectively (18). Moreover, deranged systolic blood pressure in children with classical CAH was characterized with higher daytime values and the absence of the nocturnal descent (63). Monitoring of blood pressure in children is of importance. Namely, it was observed an increased hypertension rate in young patients with classical CAH on fludrocortisone therapy (64). Available studies on CAH patients of different age showed similar prevalence of hypertension between males and females with CAH (18, 40, 63) with few exceptions showing more deteriorated BP in younger females likely to be related to androgen excess (50).

Carotid intima-media thickness (CIMT) is used as surrogate marker of arterial damage. It was shown that CIMT was increased in CAH patients including different age groups and independently of androgen levels, insulin levels or glucocorticoid treatment (65). Moreover, when comparing adolescents with classical CAH and NC-CAH, even those with NC-CAH may be at higher risk of having increased CIMT, possibly related to intermittent iatrogenic hypercortisolism and secondary insulin resistance (66). In respect to gender differences, there was no association between CIMT and androstenedione and 17OH progesterone in exposed females with classical CAH (51) (**Supplementary Table 1**).

Bone Mineral Density and Risk for Osteoporosis

Adult patients with classical CAH are shorter than average individuals in the general population by ∼10 cm (17). This is caused by the parallel effects of earlier exposure to androgens causing accelerated growth rate in childhood and premature epiphyseal closure in the long bones, and suppressive effects of excessive GCS doses on the secretion of growth hormone on the other.

The age and duration-related data on the assessment of bone mineral density (BMD) in patients with CAH are varying. While some authors showed lower BMD in adults with classical CAH and NC-CAH (18) others showed differences in BMD in relation only to glucocorticoid use (67). Curiously, while GCS therapy in children did not show a decrease in BMD, significantly lower BMD was demonstrated in adult patients with CAH on GCS treatment, and specially in those having salt-wasting CAH managed with highest doses of GCS (68, 69). In respect to the drug used, lower BMD values are found in patients managed with longer-acting GCS compared to those managed with hydrocortisone (70). An additional factor influencing decrease in BMD could be the reduced levels of DHEA and DHEAS that was shown in post-menopausal women treated with long-acting GCS (68).

Apart from the assessment of height and BMD using dual energy X-ray absorptiometry, there is not an established role for the bone turnover biochemical markers in patients with CAH (69).

Use of glucocorticoids could result in long-term complications including osteoporosis and fractures (4). In respect to the CAH variant, while some authors reported normal or even better BMD in different age-groups of patients with NC-CAH in comparison to classical CAH (71, 72), other authors found similar frequency of osteoporosis in NCAH in comparison to classical CAH (18).

Osteoporosis could be expected in adults with classical CAH on chronic glucocorticoid therapy. GCS therapy is influencing the activity of osteoblasts with consequent decreased in BMD (67). In line with this is low activity of the markers of bone formation such as osteocalcin, in adults with CAH (73). Apart from the more pronounced decrease in BMD after the age of 30, an increased rate of osteoporotic fractures was shown in women with classic CAH treated with GCS (71). Moreover, males older than 30 years had lower BMD in all measured sites similarly to women of the same age (67, 71). However, in spite of lower BMD and osteocalcin concentrations in males on prednisolone therapy in comparison to the ones hydrocortisone treatment, they did not have higher frequency of fractures (67).

Taking into consideration all the aspects of GCS treatment on BMD, and in spite of decreased bone turnover markers, most patients with CAH have normal BMD. Preservation of bone integrity over GCS treatment could be explained with the commonly increased body mass index, which is protective to bones, as well as with the anabolic effects of androgens in both males and females (73). However, the most likely explanation for the preserved BMD could rely in the positive net effect of the GCS type and dose adjustment during patient's follow-up (**Supplementary Table 1**).

CONCLUSION

Patients with NC-CAH are prone to develop metabolic consequences. Those patients are with higher rates of obesity and insulin resistance, as well as with increased rates of metabolic sequelae and cardiovascular disease during an adult period in both males and females. These patients could be at higher risk of having increased arterial intima-media thickness, possibly related to intermittent iatrogenic hypercortisolism and secondary insulin resistance. Decreased BMD and osteoporosis is not a constant finding in these patients and could depend on the sex, and type or dose of glucocorticosteroids applied.

AUTHOR CONTRIBUTIONS

The present work was designed by DM, VZ, JB-M, GM, and DP. The initial manuscript draft was prepared by DM and subsequently revised by VZ, J-BM, GM, and DP. All the authors approved the final submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00681/full#supplementary-material

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Obesity and Cardiometabolic Risk Factors in Children and Young Adults With Non-classical 21-Hydroxylase Deficiency

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Introduction: Classical congenital adrenal hyperplasia is associated with an increased risk of obesity and cardiometabolic disease. The aim of the study was to determine if this is also true for non-classical congenital adrenal hyperplasia (NCCAH).

Methods: A retrospective, cross-sectional, single-center study design was used. Data were collected on 114 patients (92 female) with NCCAH diagnosed during childhood/adolescence at a tertiary medical center. Patients were classified by treatment status at the last clinic visit. Outcome measures were assessed at diagnosis and the last clinic visit: weight status, body composition, blood pressure, lipid profile, and glucose metabolism. The prevalence of overweight/obesity was compared to the parental prevalence, and for patients aged 11–20 years, to the Israeli National Survey.

Results: Mean age was 7.9 ± 4.2 years at diagnosis and 17.1 ± 6.9 years at the last follow-up. At the last clinic visit, 76 patients were under treatment with glucocorticoids, 27 were off-treatment (previously treated), and 11 had never been treated. The rate of obesity (11.4%) was similar to the parental rates, and the rate of overweight was significantly lower. In patients 11–20 years old, rates of obesity or obesity + overweight were similar to the general Israeli population (11.4 vs. 15.1%, $P = 0.24$ and 34.2 vs. 41.6% $P = 0.18$, respectively). No significant difference was found between glucocorticoid-treated and off-treatment patients in any of the metabolic or anthropometric parameters evaluated, except for a lower mean fat mass (% of body weight) in off-treatment patients (23.0 ± 7.7% vs. 27.8 ± 6.8%, $P = 0.06$). Systolic hypertension was found in 12.2% of NCCAH patients either treated or untreated.

Conclusion: NCCAH diagnosed in childhood, whether treated or untreated, does not pose an increased risk of overweight, obesity, or metabolic derangements in adolescence and early adulthood.

Keywords: cardiometabolic syndrome, children and adolescents, non-classical congenital adrenal hyperplasia, overweight, obesity

INTRODUCTION

Non-classical congenital adrenal hyperplasia (NCCAH) due to 21-hydroxylase deficiency is an autosomal recessive disease of the adrenal cortex caused by mutations in the *CYP21A2* gene. The disease frequency is estimated at 0.1% of the general population, but it is significantly higher in some ethnic groups: 1–2% in Hispanics and Yugoslavians and 3–4% in Ashkenazi (Eastern European) Jews (1, 2).

In childhood, NCCAH may manifest with premature pubarche, signs of hyperandrogenism such as acne, hirsutism, and irregular menses (2–5), and advanced bone age which may lead to short adult height (6, 7). Treatment consists of glucocorticoids in doses aimed at suppressing hyperandrogenism, which are usually higher than the physiologic replacement and cannot mimic the physiological circadian rhythm of cortisol. Given the controversial findings in the literature regarding the need for treatment of NCCAH (2, 8), clinicians need to weigh the benefits against the risks of steroid-induced hyperglycemia, weight gain, increased blood pressure, and hyperlipidemia (9). The presence of three or more of these conditions leads to the metabolic syndrome, increasing the risk of cardiovascular morbidity and mortality. At the same time, chronic androgen excess may contribute to the metabolic disorder by its association with increased visceral adiposity and insulin resistance and their metabolic consequences (10, 11). Adolescents and adults with the classical form of congenital adrenal hyperplasia (CAH) appear to have an increased risk of obesity and cardiometabolic risk factors (12–14). Studies on cardiometabolic risk factors in pediatric and young adult patients with the non-classical form are scarce (15, 16).

The aim of the present study was to investigate the prevalence of overweight and obesity among adolescents and young adults with NCCAH and to assess metabolic risk factors in these patients as well as the association of these risk factors with treatment.

PATIENTS AND METHODS

Study Population

A retrospective, cross-sectional study design was used. The cohort consisted of 114 consecutive unselected patients with NCCAH (92 female) attending a tertiary pediatric endocrinology institute from 1986 to 2017. Inclusion criteria were diagnosis before 18 years of age and diagnosis on the basis of an ACTH stimulated 17-hydroxyprogesterone (17-OHP) serum level of $\geq$45 nmol/L ($n = 100$, molecular confirmation in 67) or a level of >30 nmol/l if confirmed by molecular analysis.

Treatment

Therapy with glucocorticoids was initiated in symptomatic patients, namely children with early onset, and rapid progression of pubarche or bone age advancement and adolescent girls with overt virilization. Doses were titrated according to growth and clinical parameters as well as the hormonal profile in order to maintain androstenedione levels at sex- and age-appropriate levels and 17-OHP levels at <30 nmol/l. Glucocorticoid dosage was expressed as hydrocortisone per square meter of body surface. When glucocorticoid treatment was administered in the form of prednisolone rather than hydrocortisone, the total dose of prednisolone was multiplied by 4 to yield an equivalent dose of hydrocortisone.

Clinical Methodology

Clinical data were extracted from the medical files as follows: age at diagnosis, reason for referral, height, weight, secondary sex characteristics, and laboratory results. Outcome measures were assessed at diagnosis and the last clinic visit: weight status, body composition, blood pressure, lipid profile, and glucose metabolism.

Pubertal stage was classified according to Tanner and Marshall (17, 18) at diagnosis, as follows: prepubertal (Tanner 1), active puberty (Tanner 2–4), and fully pubertal (Tanner 5). Body weight and height were measured to the nearest 0.1 kg and 0.1 cm, respectively, using a Harpenden stadiometer (Holtain Ltd, Crosswell, UK), and a balanced scale. Body mass index (BMI) was calculated. The height and weight of both parents were measured when possible as part of the clinic routine. The height, weight, and BMI-standard deviation score (SDS) were calculated and assessed according to the recommendations of the Centers for Disease Control and Prevention (19). In patients younger than 18 years, overweight was defined as a BMI within the 85–94th percentile for children of the same age, and obesity as a BMI $\geq$the 95th percentile. In older patients, overweight was defined as BMI 25–29.99, and obesity as BMI $\geq$30 (20). The prevalence of overweight and obesity at the last visit was calculated for the entire cohort. Data of patients in grades 11–20 years old ($n = 76$) were compared to the National Health and Nutrition Survey (21).

Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured in all subjects using the standardized automated Datascope Accutorr Plus (Soma Technology, Bloomfield, USA) on the right arm. SBP and DBP percentiles adjusted for sex, age, and height were calculated using the American Academy of Pediatrics guidelines (22). Patients were considered hypertensive if the average SBP and/or DBP was at or above the 95th percentile for sex, age, and height on three or more occasions.

Bone age was evaluated according to Greulich and Pyle (23), with the delta of bone age minus chronological age calculated accordingly.

The cohort was subdivided according to treatment status at the last visit: currently treated ($n = 76$), off-treatment (previously treated, $n = 27$), and untreated (never treated, $n = 11$), and again by gender.

Biochemical Analysis

The hormonal evaluation included 17-OHP and cortisol levels at presentation, baseline, and following intravenous administration

Abbreviations: ACTH, adrenocorticotropic hormone; BMI, body mass index; CAH, congenital adrenal hyperplasia; DBP, diastolic blood pressure; HDL, high-density lipoprotein; IDF, International Diabetes Federation; IR, insulin resistance; LDL, low density lipoprotein; NCCAH, non-classical congenital adrenal hyperplasia; 17-OHP, 17-hydroxyprogesterone; SBP, systolic blood pressure; TC, total cholesterol; TG, triglycerides.

of 0.25 mg Synacthen (Novartis, New York, NY, USA). In post-menarcheal girls, the test was conducted in the early follicular phase of the menstrual cycle. Follow-up basal androgen levels were measured every 6 months, or earlier when dose adjustment was required. All hormonal analyses were performed in the endocrine laboratory of our hospital with commercial kits; serum cortisol and 17-OHP were measured using the Coat-A-Count radioimmunoassay (Diagnostics Products Corporation, Los Angeles, CA, USA).

Annual fasting measurements of glucose, lipid profile, and liver function tests were performed as part of the routine standard of care. Patients were instructed to take the medication after blood samples were collected. Serum glucose was measured with the glucose oxidase colorimetric method (Hitachi 917 automated analyzer, Roche Diagnostics, Mannheim, Germany), and serum total cholesterol, triglycerides, and high-density lipoprotein (HDL)-cholesterol were measured with the enzymatic colorimetric method (Hitachi 904 automated analyzer, Roche Diagnostics). Low-density lipoprotein (LDL) was calculated according to the Friedewald formula: LDL, total cholesterol (TC)— HDL—triglycerides (TG)/5. The most recent data available were used for analysis. Fasting plasma TG concentrations of >110 mg/dl were considered elevated, and HDL <40 mg/dl was considered low (24). Additionally, the percentiles of HDL, LDL, TG, and age- and sex-adjusted TC were calculated relative to published normal values (25).

Extensive Metabolic Evaluation

A subgroup of 38 patients from the cohort underwent further assessment. The patients were consecutively and prospectively recruited during a routine visit to the clinic over 1 year and signed an informed consent form. The evaluation included measurement of waist and hip circumference, skin-fold thickness (iliac, scapular, and triceps), and body composition using a bioelectrical impedance analyzer (Tanita SC-331S, Tokyo, Japan). Measured values of fat mass were compared to the accepted normal range for age and sex (26, 27). Waist circumference was measured at the minimum circumference between the iliac crest and the rib cage, and hip circumference was measured at the maximum protuberance of the buttocks. The waist-to-hip ratio was calculated (28). Waist circumference percentiles were determined according to the estimated value for percentile regression for European and American children and adolescents (29). For female participants aged 11–20 years, mean and median waist and hip circumference, in addition to waist-to-hip ratio (as too few such measurements were available for male patients), were compared to the National Health and Nutrition Survey values for the same age group (21).

Insulin resistance (IR) was estimated using the homeostasis model assessment (HOMA) method (IR = insulin [μmol/mL] × glucose [mmol/L]/22.5) (30).

The consensus definition of the International Diabetes Federation (IDF) was used for the diagnosis of metabolic syndrome in children and adolescents. Patients were diagnosed with metabolic syndrome if they met at least 3 of the 5 criteria listed by the consensus (29).

The study was approved by the Institutional Review Board of Rabin Medical Center, Israel.

Statistical Analysis

Analyses were carried out using BMDP statistical software (University of California Press, Los Angeles, CA, USA) (31). Data were expressed as means and standard deviations for normally distributed variables and medians and interquartile ranges for variables with a skewed distribution. For continuous variables, groups were compared using analysis of variance. Discrete variables were compared using Pearson's chi-square test or Fisher's exact test, as appropriate. For variables that did not have a Gaussian distribution, and because of the extremely small numbers, the Mann–Whitney *U*-test was used to compare groups. A p value of ≤ 0.05 was considered significant.

RESULTS

The baseline characteristics of the study cohort are detailed in **Table 1**. Mean patient age at diagnosis was 7.9 ± 4.2 years (range, 5 months-18 years), 7.1 ± 4.4 years in girls and 8.4 ± 4.0 years in boys, and at the last follow-up, 17.1 ± 6.9 years. The mean duration of follow-up was 9.2 ± 6.6 years (median 8.25 years). At diagnosis, 54 patients (65% females) were prepubertal, 48 (92% females) were in active puberty, and 12 (all females) were fully pubertal. The main reason for referral, in 56 patients, was premature pubarche, either isolated or associated with signs of gonadarche. The remaining 58 patients were referred for hyperandrogenism in adolescence ($n = 16$), a family history of NCCAH ($n = 29$), clitoromegaly ($n = 5$), or short stature associated with advanced bone age ($n = 8$).

Glucocorticoid treatment was administered when clinically indicated. Treatment was initiated at a mean age of 8.6 ± 3.4 years; 81% of the cohort started treatment before age 10 years. At the last clinic visit, 76 patients were being treated with glucocorticoids (daily hydrocortisone or hydrocortisone-equivalent dose was 9.2 ± 4.5 mg/m^2), 27 had terminated treatment at a mean of 4.1 ± 0.9 years previously, and 11 had never been treated. The mean duration of steroid treatment was 7.3 ± 6.3 years for the glucocorticoid-treated group and 6.5 ± 5.2 years for the off-treatment group ($p = 0.5$).

Of the 11 patients who were never treated, 4 were diagnosed incidentally when evaluated for short stature, 4 were evaluated because of a family history of NCCAH, and 3 presented with mild premature pubarche without bone age advancement. This subgroup differed clinically from the rest of the cohort (Appendix I in **Supplementary Material**).

Overweight and Obesity

At the last clinic visit, the rate of overweight for the entire cohort was 21.9%, and of obesity, 11.4%, with no significant difference between patients who were older or younger than 20 years (**Table 2**). Rates of overweight and obesity for patients who were 11–20 years old at the last visit were comparable to the rates reported for the same age group in the general population. Rates of overweight were significantly lower in the patients than in their mothers ($p = 0.01$) and fathers ($p < 0.001$). The duration

TABLE 1 | Clinical characteristics at diagnosis of 114 children and adolescents with NCCAH.

Characteristics	All ($n = 114$)	Currently treated ($n = 76$)	Off-treatment ($n = 27$)	Untreated ($n = 11$)
Female: male, *n*	92:22	63:13	20:7	8:3
Age at diagnosis (years)	7.9 ± 4.2 (0.4–17.9)	8.2 ± 4.0 (0.7–17.9)	7.5 ± 3.8 (0.4–17.0)	7.8 ± 5.7 (1.2–17.5)
Age at treatment initiation (years)	8.6 ± 3.4	8.5± 3.3	9.1 ± 3.6	
1st year hydrocortisone* dose (mg/m^2)	10.6 ± 4.9	10.7 ± 4.9	10.2 ± 3.8	
Height-SDS	0.10 ± 1.22	0.14 ± 0.13	0.35 ± 0.89	−1.07 ± 1.62†
Weight-SDS	0.22 ± 1.23	0.27 ± 0.18	0.52 ± 0.83	−0.99 ± 1.93
BMI-SDS	0.35 ± 1.13	0.33 ± 1.09	0.62 ± 0.87	−0.21 ± 1.77
Δ Bone age- chronological age (years)	1.06 ± 1.26	1.29 ± 1.24	0.88 ± 1.29	0.00 ± 0.83§
Basal 17-OHP (nmol/l)	28.7 ± 27.6 (2.4–174)	33.8 ± 29.9‡ (3.2–174)	23.2 ± 23.7‡ (2.7–93.8)	10.5 ± 7.1§ (3.2–23.7)
Peak 17-OHP (nmol/l)	129.5 ± 96.8	143.7 ± 106.8$^{\ddagger *}$	105.6 ± 74.9‡	88.2 ± 52.4
Stimulated cortisol level (nmol/l)	567 ± 165	536 ± 132	641 ± 148	609 ± 172
Androstenedione (nmol/l)	4.1 ± 3.9	4.9 ± 4.4‡	3.0 ± 2.1‡	1.0 ± 0.7§
Paternal BMI	26.7 ± 3.9	26.8 ± 3.8	26.9 ± 4.5	26.2 ± 3.4
Paternal BMI-SDS	0.93 ± 0.95	0.95 ± 0.98	0.95 ± 0.97	0.87 ± 0.87
Maternal BMI	26.0 ± 5.1	25.8 ± 4.9	26.8 ± 5.6	23.1 ± 4.2
Maternal BMI-SDS	0.76 ± 0.86	0.75 ± 0.86	0.91 ± 0.81	0.16 ± 1.04

Data are shown as mean ± SD (range) but analyzed using the non-parametric Mann-Whitney U-test since the sample sizes are very small.
**If prednisolone was administered, the total dose of prednisolone was multiplied by 4 to yield an equivalent dose of hydrocortisone.*
$^{\dagger}P < 0.05$ for untreated vs. off-treatment patients.
$^{\ddagger}P < 0.05$ for treated vs. off-treatment.
$^{\S}P < 0.01$ for untreated vs. currently treated.
17-OHP, 17-hydroxyprogesterone; BMI, body mass index; NCCAH, non-classical congenital adrenal hyperplasia; SDS, standard deviation score.

of steroid treatment was inversely associated with BMI-SDS ($r = -0.20$, $P < 0.05$).

Cardiometabolic Risk Factors

None of the patients was classified as having the metabolic syndrome according to either the criteria of Ford (24) or the IDF consensus (27).

Systolic hypertension (SBP >95th percentile) was found in 12.2% of the cohort: in 10.5% of the currently treated group, in 18.5 % of the off-treatment group and in 9% of the untreated group ($p = 0.3$). SBP and SBP percentile were associated with BMI-SDS ($r = 0.275$, $p = 0.009$, and $r = 0.28$, $p < 0.02$, respectively). Among the 17 patients with systolic hypertension, only 4 had a BMI-SDS consistent with overweight or obesity. DBP above the 95th percentile was measured in 2.2% of the cohort: in 3.5% of the treated group and in none of the off-treatment group ($p = 0.49$). No association was found between DBP and BMI-SDS.

In a search for the explanation for the high rate of patients with systolic hypertension, we investigated a possible association of SBP and SBP percentile with the following variables: birthweight and levels of serum androstenedione, testosterone, and 17-OHP at diagnosis and at last visit. No association was found for any of these variables. This held true for DBP and DBP percentile as well.

Steroid treatment duration was associated with SBP ($r = 0.26$, $P < 0.05$) and DBP ($r = 0.31$, $P < 0.01$), but not with sex-, age-, and height-adjusted blood pressure percentiles (for SBP%: $r = 0.09$, $P =$ NS; for SDP%: $r = 0.15$, $p =$ NS).

TABLE 2 | Prevalence of overweight and obesity at last follow-up in children/adolescents with NCCAH by age at last visit (< or>20) compared to the Israel National Survey for ages 11–20 years and compared to mothers and fathers.

Parameter	*N*	Overweight (%)	Obese (%)
All patients	114	21.9	11.4
Comparison by age at last visit			
<20 years	76	23.6	10.5
>20 years	38	18.4	13.2
P value		0.3	0.4
Comparison with national survey (for age 11–20 years)			
Age 11–20 years, study	76	23.6	10.5
Age 11–20 years, national survey	3,443	26.5	15.1
P value		0.24	0.18
Comparison with parental values			
Mothers	100	36.8	13.6
P value		0.01	0.4
Fathers	86	52.5	14.5
P value		<0.001	0.3

NCCAH, non-classical congenital adrenal hyperplasia.

There was no significant association between treatment duration and levels of total and LDL-cholesterol, TG, fasting glucose, HOMA-IR, skin-fold thickness, and body fat mass (as percentiles). The dose at the last clinic visit was associated only with higher LDL-cholesterol ($r = 0.46$, $P < 0.05$).

Waist circumference was negatively correlated with HDL (−0.49, $P < 0.02$) but not with LDL, total cholesterol, or TG level. In the female adolescent patients, waist circumference and

TABLE 3 | Effects of glucocorticoid therapy duration and dose on cardiometabolic risk factors in patients with NCCAH, currently-treated and off- treatment ($N = 103$).

Risk factors	Therapy duration		1st year dose		Current dose	
	r	*P*	*r*	*P*	*r*	*P*
Current BMI-SDS	−0.202	<0.05	0.074	ns	0.033	ns
Treatment duration			0.359	<0.001	0.106	ns
SBP (mmHg)	0.262	<0.05	0.226	<0.05	−0.126	ns
SBP percentile	0.089	ns	0.241	<0.05	−0.14	ns
DBP (mmHg)	0.309	<0.01	0.29	<0.02	0.007	ns
DBP percentile	0.15	ns	0.257	<0.05	−0.07	ns
% fat by bioelectrical impedance	0.075	ns	0.200	ns	0.046	ns
Iliac skinfold (mm)	0.306	ns	0.15	ns	0.22	ns
Triceps skinfold (mm)	0.419	ns	0.17	ns	0.12	ns
Scapular skinfold (mm)	0.267	ns	0.12	ns	0.30	ns
Waist circumference (cm)	0.264	ns	−0.08	ns	0.33	ns
Hip circumference (cm)	0.542	<0.005	−0.21	<0.005	0.478	<0.05
Waist/hip ratio	−0.507	<0.005	0.295	<0.01	−0.37	ns
Total cholesterol	0.09	ns	−0.186	ns	0.289	ns
HDL	0.22	<0.01	−0.057	ns	0.009	ns
LDL	0.159	ns	−0.250	ns	0.46	<0.05
Triglycerides	−0.083	ns	−0.43	ns	−0.08	ns
Glucose	0.244	ns	0.168	ns	0.125	ns
Insulin	−0.32	ns	0.36	ns	−0.53	ns

DBP, diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NCCAH, nonclassical congenital adrenal hyperplasia; ns, not significant; SBP, systolic blood pressure.

waist/hip ratio were similar to values in the national survey: for girls 11–20 years old mean waist circumference was 73.5 cm (median 73.8) compared to 73.1 cm (median 73.8 cm) in the national survey ($P = 0.25$), and mean waist-to-hip ratio was 0.78 (median 0.79) compared to 0.77 (median 0.76) in the national survey ($P = 0.6$). Hip circumference was associated with glucocorticoid therapy duration ($r = 0.542$, $p < 0.005$) and current dosage ($r = 0.478$, $p < 0.05$). On stepwise regression analysis, only BMI-SDS and age were strongly associated with hip circumference (multiple $R^2 = 0.88$).

Effects of dose and duration of therapy are presented in **Table 3**.

Analysis by Glucocorticoid Treatment Status

There were no differences in clinical characteristics between the patients receiving treatment or those off treatment at the last follow-up visit (**Table 4**).

At the last clinic visit, the group receiving treatment had a tendency to higher mean fat mass than the untreated group (27.8 ± 6.8 vs. 23.0 ± 7.7% of body weight, $p = 0.06$) and a higher percentage of patients with fat mass (expressed in percent) above the accepted normal range for age and sex (38.8 vs. 7.1%, $p < 0.05$). They also had a tendency for higher weight-SDS and BMI-SDS. No significant differences were found between the treated and off-treatment patients in lipid profile, levels of glucose and insulin, or HOMA-IR (**Table 5**).

TABLE 4 | Clinical characteristics by treatment status at last follow-up in patients with NCCAH.

Characteristics	Currently treated (*N* = 76)	Off-treatment (*N* = 27)	Untreated (*N* = 11)
Age (years)	17.4 ± 7.1	18.7 ± 5.7	11.4 ± 5.7*
Tanner (1, 2–4, 5)	8, 14, 54	3, 1, 23	3, 5, 3
Follow-up duration	9.1 ± 6.5	11.2 ± 6.9	3.6 ± 2.6†
Treatment duration (years)	7.3 ± 6.3	6.5± 5.2	
Weight-SDS	0.39 ± 0.99	0.37 ± 0.92	−0.69 ± 1.75
Height-SDS	−0.44 ± 0.99	−0.36 ± 0.83	−0.57 ± 1.76
BMI-SDS	0.65 ± 0.89	0.64 ± 0.73	−0.37 ± 1.23*
SBP (mmHg)	113.3 ± 10.9	118.1 ± 9.3	108.2 ± 14.5
SBP percentile	61.2 ± 27.1	75.1 ± 18.0	48.7 ± 33.1
DBP (mmHg)	66.7 ± 9.5	69.8 ± 7.2	61.1 ± 6.9
DBP percentile	56.1 ± 26	66.2 ± 19.7	41.0 ± 21.8
17OHP (nmol/l)	22.0 ± 29.5	23.9 ± 19.1	10.2 ± 9.5
Androstenedione (nmol/l)	6.4 ± 4.1‡	12.1 ± 8.4‡	3.5 ± 4.5
Testosterone (nmol/l)	2.5 ± 4.8	2.5 ± 3.5	0.2 ± 0.5*

Data are mean ±SD unless otherwise specified.
**P < 0.05 for untreated vs. off-treatment and currently treated groups.*
†P < 0.01 for untreated vs. off-treatment and currently treated groups.
‡P < 0.05 for treated vs. off-treatment.
17-OHP, 17-hydroxyprogesterone; BMI, body mass index; DBP, diastolic blood pressure; NCCAH, nonclassical congenital adrenal hyperplasia; SBP, systolic blood pressure; SDS, standard deviation score.

There were no significant differences by sex in anthropometric, laboratory, and metabolic variables (data not shown) except for a higher body fat mass (expressed in percent) in female than male patients (26.9 ± 6.8 vs. 19.2 ± 8.9%, $p = 0.016$), which we assumed was physiologic (27).

At the time of evaluation, the mean age of the 56 patients referred for premature pubarche was 17 ± 6.3 years, and of those referred for other presentations, 17.3 ± 7.4 years ($p = 0.8$). There were also no significant differences between these subgroups in any of the anthropometric, laboratory, and metabolic variables (data not shown).

DISCUSSION

This study showed that adolescents diagnosed with NCCAH in childhood did not have an increased prevalence of obesity or overweight compared to the general population in our country. Furthermore, the rate of obesity was similar to the rate in their parents, and the rate of overweight was even lower than that in their parents.

TABLE 5 | Cardiometabolic risk factors by treatment status in patients with NCCAH who underwent extensive evaluation.

Risk factors	Treated ($n = 22$)	Off treatment ($n = 14$)	*P* value
Age (years)	15.7 ± 5.4	17.1 ± 5.7	0.5
% fat by bioelectrical impedance	27.8 ± 6.8	23.0 ± 7.7	0.06
BMI percentile	61.0 ± 29.4	59.7 ± 23.9	0.8
SBP (mmHg)	113.7 ± 11.4	117.8 ± 7.4	0.2
SBP percentile	61.4 ± 27.7	73.7 ± 17.5	0.2
DBP (mmHg)	68.0 ± 8.2	66.7 ± 6.3	0.6
DBP percentile	60.6 ± 25.9	54.8 ± 17.8	0.4
Iliac skinfold (mm)	16.1 ± 8.9	14.2 ± 7.0	0.5
Triceps skinfold (mm)	18.4 ± 8.7	14.1 ± 6.6	0.1
Scapular skinfold (mm)	13.4 ± 9.6	9.3 ± 6.0	0.2
Waist circumference (cm)	74.1 ± 13.2	67.4 ± 11.9	0.1
Waist circumference >50th percentile, *n* (%)	12 (54.5)	5 (35.7)	0.2
Hip circumference (cm)	85.7 ± 15.5	79.2 ± 18.1	0.28
Waist/hip ratio	0.88 ± 0.13	0.87 ± 0.09	0.8
Total cholesterol (mg/dl)	187.5 ± 48.8	191.4 ± 44.3	0.8
HDL (mg/dl)	59.9 ± 19.3	57.2 ± 16.5	0.6
LDL (mg/dl)	107.4 ± 33.2	106.5 ± 27.8	0.9
Triglycerides (mg/dl)	88.6 ± 31.8	118.3 ± 66.5	0.07
Fasting Glucose (mg/dl)	84.8 ± 14.3	82.4 ± 10.6	0.6
Fasting Insulin (micU/ml)	5.6 ± 6.1	3.9 ± 2.7	0.5
HOMA-IR	1.1 ± 1.2	0.8 ± 0.4	0.48

Data are mean ±SD unless otherwise indicated.
Untreated patients who underwent extensive evaluation (n = 2) were excluded due to small sample size.
HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment-insulin resistance; LDL, low-density lipoprotein.

These results are not in agreement with the US National Institutes of Health study wherein overall 35% of children with CAH were obese, with no differences between the classic and the non-classic groups (15). Others reported a higher than normal risk of obesity in children and adolescents with classical CAH (32). The higher BMI-SDS was associated with increased glucocorticoid dosage, parental obesity, and chronological age (32). A Swedish study comparing patients with 21-hydroxylase deficiency, presenting as either salt-wasting, simple virilizing, or NCCAH, to national population registers found that obesity was most pronounced in the patients with NCCAH, who also had increased cardiovascular morbidity (16). However, data on age at diagnosis and glucocorticoid duration and dose as well as other clinical details were missing from the national registers. The authors suggested that the mild phenotype of NCCAH was associated with delayed diagnosis and consequently prolonged androgen excess, which may precipitate cardiovascular morbidity.

In our study, BMI-SDS was not associated with either initial or current glucocorticoid dose. Similarly, in an earlier study of adolescent patients with CAH, there was no association of BMI-SDS with either glucocorticoid dosage or age (13). BMI-SDS at the last clinic visit was negatively correlated with the duration of glucocorticoid therapy. This finding may be explained by the regular follow-up of the treated patients, with tight surveillance of weight changes and diet, including dietary consultation that was provided as part of the clinic visits when indicated.

Patients still under treatment tended to have a higher body fat mass than off-treatment patients (with a similar male-to-female ratio in both groups), despite comparable BMI-SDS and weight-SDS. This finding could be due to the effect of glucocorticoid excess on body composition (33). Our data are in line with those of Nermoen et al. (34) who found higher fat mass in patients treated by steroids for classical CAH than controls despite a similar BMI-SDS. The higher fat mass in patients under treatment may contribute to insulin resistance. Indeed, insulin and HOMA-IR were higher in our treated group, but the difference was not statistically significant, and in both groups these values were all within the normal range.

The lower fat mass in the off-treatment patients may point to a waning of the glucocorticoid effect on body composition once therapy is discontinued; to the known effect of androgen on reducing body fat mass, as androstenedione was found to be higher in the off-treatment group; or both.

In our cohort the prevalence of systolic hypertension (12.2%) was higher than the 3.5% reported in children and adolescents (22), with no significant difference by treatment status. Data regarding blood pressure in NCCAH patients are scant and inconclusive: Finkielstain et al. found hypertension in 20% of the NCCAH patients (15), while no overt hypertension was found in a Polish study of 9 patients with NCCAH and 61 with classical CAH (35). Williams et al. (36) reported higher SBP values in 12 children with NCCAH than in controls, but they did not translate the numbers into percentiles adjusted for age, sex, and height, and did not report the rate of hypertension. By contrast, others noted that DBP-SDS was higher in a group of 9 adolescents with NCCAH than in healthy controls (37) and was associated with higher arterial intima-media thickness. The authors suggested that intermittent iatrogenic hypercortisolism may play an important role in the pathogenesis of artery alterations in CAH. In our study, a higher SBP percentile was associated with a higher drug dose in the first year of therapy, but not with the duration of therapy. This finding could reflect the severity of disease and possibly higher androgen levels. However, neither SBP nor DBP was associated with androgen levels at diagnosis, suggesting that the glucocorticoid therapy was responsible for the higher blood pressure, with a sustained effect probably due to residual visceral adiposity and/or insulin resistance. The role of glucocorticoids in systolic hypertension is supported by an earlier study comparing exercise performance between patients with CAH and patients receiving a similar dose of glucocorticoids for juvenile idiopathic arthritis (38). These results suggested that the treatment rather than the CAH itself was responsible for the enhanced SBP response and other abnormalities (38). Given that high DBP is a marker of cardiovascular risk (39), our finding of a normal DBP in both the treated and untreated patients with NCCAH is encouraging.

Other than systolic hypertension, patients were not at higher risk of hyperlipidemia, impaired glucose metabolism, higher body fat mass, or change in body fat distribution. The waist circumference and waist-to-hip ratio in the adolescent female

patients with NCCAH were similar to values in the general population, as opposed to youth with classical CAH, who were found in previous studies to have increased adiposity and an increased waist-to-hip ratio (13, 38).

Most patients with NCCAH require therapy until completion of linear growth, at which point it can be discontinued. In our study, most of the currently untreated patients (27/38) had been previously treated with glucocorticoids. It remains unclear if the effects of past long-term steroid administration persist after therapy is discontinued. Longitudinal studies of these patients into adult life are needed.

The strength of the present study lies in the relatively long follow-up of a cohort attending a single tertiary medical center. Using this design, we were able to investigate a group of previously treated patients and compare them to patients still under treatment. This is also the first study to evaluate rates of overweight and obesity in pediatric patients with NCCAH compared to their parents. Parental measurements were taken at our institute to ensure accuracy, in contrast to studies based on self-reported data which is inherently biased because participants tend to under-report weight and over-report height (40). This comparison may also be superior to comparison to healthy controls, as the weight status of children has been shown to be associated with the parental status (41), possibly owing to genetic, environmental, and nutritional influences. Our data are further empowered by comparison to the local general population.

Limitations of the study include the relatively small size of the subgroup of patients who underwent the more extensive metabolic evaluation and the lack of a control group. The diagnosis was genetically confirmed in most but not all of the patients. Few data were available on inflammatory markers, considered to represent metabolic derangement. We did not evaluate other risk factors such as serum adiponectin, C-reactive protein, homocysteine levels, and arterial intima-media thickness, and we were unable to assess family history of cardiometabolic risk factors. Similar to previous reports (6, 7), there were more female than male patients in our study group, as male patients are often overlooked. Thus, although no gender difference was found in our cohort, we were cautious and chose not to present the results.

In summary, NCCAH diagnosed in childhood, whether treated or untreated, is not associated with an increased risk of overweight, obesity, or metabolic derangement except for a higher rate of systolic hypertension. Larger, longer-term studies are needed to confirm our results.

PRECIS

Pediatric patients with NCCAH do not appear to be at increased risk of overweight, obesity, or metabolic derangement, regardless of treatment, compared to the same age group in the general population.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript/**Supplementary Files**.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Declaration of Helsinki. The protocol was approved by the ethical committee of RMC.

AUTHOR CONTRIBUTIONS

LV contributed to the conception and design of the study, acquisition and interpretation of the data, prepared the first draft, and revised the manuscript. YL contributed to the acquisition and interpretation of the data and reviewed the manuscript critically for important intellectual content. MP, SS, and AT contributed to the acquisition of the data and reviewed the manuscript critically for important intellectual content. RB contributed to the conception and design of the study, acquisition and interpretation of the data, and revised the manuscript. Each author listed on the manuscript has seen and approved the submission of this version of the manuscript and takes full responsibility for the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00698/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Assisted Reproduction in Congenital Adrenal Hyperplasia

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Congenital Adrenal Hyperplasia (CAH) is a group of autosomal recessive disorders characterized by defects of adrenal steroidogenesis due to mutations in one of the following enzymes: 21-hydroxylase (21OH), 11β-hydroxylase (11βOH), 17α-hydroxylase (17OH; also known as 17, 20-lyase), 3β hydroxysteroid dehydrogenase type 2 (3βHSD2), steroidogenic acute regulatory protein (StAR), P450 cholesterol side-chain cleavage (P450scc), and P450 oxidoreductase (POR). More than 95% of congenital adrenal hyperplasia cases are due to mutations in CYP21A2, the gene encoding the adrenal steroid 21-hydroxylase enzyme (P450c21). This work focuses on this type of CAH given that it is the most frequent one. This disease is characterized by impaired cortisol and aldosterone production as well as androgen excess. A variant of the CAH is the non-classic type of CAH (NCCAH), usually asymptomatic before the 5th year of age, diagnosed during puberty especially in patients visiting a fertility clinic. NCCAH is characterized mainly by anovulatory cycles and/or high androgen concentrations. Both types of CAH are associated with infertility. Given that the incidence of NCCAH is greater than that of CAH, patients suffering from NCCAH are more often diagnosed for the first time in a fertility clinic. Thus, screening for NCCAH should always be considered. The causes of infertility in CAH patients are multi-factorial including virilization of external genitalia, altered psychosocial development, and hormonal disorders. The main challenges encountered in assisted reproduction are the androgen excess-associated anovulatory cycles as well as the increased circulating progesterone concentrations during the follicular phase which impact endometrial receptivity, tubal motility, and cervical thickness. Administration of sufficient substitution dose of glucocorticoids usually resolves these problems and leads not only to successful assisted reproduction treatment but also to spontaneous pregnancy. Patients with CAH should be followed by a multidisciplinary team including gynecologist, endocrinologist, and pediatrician.

Keywords: infertility, IVF (*in vitro* fertilization), congenital adrenal hyperplasia (CAH), pregnancy, assisted reproduction (ART)

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INTRODUCTION

Congenital adrenal hyperplasia (CAH) is a group of seven autosomal recessive diseases. The genes responsible for congenital adrenal hyperplasia encode enzymes involved in cortisol biosynthesis. These enzymes are: 21-hydroxylase (21OH), 11β-hydroxylase (11βOH), 17α-hydroxylase (17OH; also known as 17, 20-lyase), 3β hydroxysteroid dehydrogenase type 2 (3βHSD2), steroidogenic acute regulatory protein (StAR), P450 cholesterol side-chain cleavage (P450scc), and P450 oxidoreductase (POR). Multiple hormonal imbalances occur and CAH manifests with a range of clinical and biochemical phenotypes, with or without alterations in glucocorticoid, mineralocorticoid, and sex steroid production. Congenital adrenal hyperplasia can be distinguished clinically in two forms, "classic" and "non-classic" (non-classic CAH; NCCAH) (1). Mutations in these enzymes result in reduced cortisol production, which leads in its turn to increased secretion of corticotrophin releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) causing adrenal cortex hyperplasia (2). As a result, precursor steroids accumulate before the point of enzymatic disruption shifting the biosynthetic pathway toward production of sex steroid hormones, more specifically adrenal androgens, which are found in excess. Approximately 90–95% of cases with CAH are attributed to 21OH deficiency (3). Clinical distinction to "classic" and "non-classic" forms depends on the severity of the clinical expression of this deficiency (3). "Classic" CAH can also be distinguished in two forms, "salt-wasting" and "simple virilizing." Seventy-five percent of cases with "classic" CAH represent the "salt-wasting" form presenting with cortisol and aldosterone deficiency. In "salt-wasting" CAH, the enzyme activity is completely silenced. In "simple virilizing" CAH, there is 1–2% enzyme activity with normal mineralocorticoid concentrations. In NCCAH, enzyme activity is satisfactory (20–50%) and thus, patients remain asymptomatic, or symptoms appear much later (this form is otherwise called "late-onset CAH") (3). Females with NCCAH are not virilized at birth (4). The incidence of "classic" CAH is 1:10000–1:20000 live births and that of NCCAH 1:1000 live births (3, 5). This classification is artificial because CAH has a wide and continuous range of clinical features depending on residual enzyme function (5).

Pregnancy rate is related to the clinical severity of the disease (6, 7). Severe infertility is associated with "salt-wasting" CAH. The low rates of fertility in women with "classic" CAH are also related to the decreased *libido* of these patients, with less chance of heterosexual relations and less desire to engage in family formation (8, 9). Many women with NCCAH present with mild symptoms and therefore remain undiagnosed. It is difficult to assess accurate infertility rates in NCCAH (8).

FERTILITY IN CAH

Fertility in "Classic" CAH

Fertility rates in women with "classic" CAH and especially those with "salt-wasting" CAH are significantly lower compared to those in the general population (6, 9–12). On the other hand, it is difficult to estimate these rates in women suffering from CAH because they do not usually seek pregnancy while studies include a small number of patients. These rates are improved when studies include only women with CAH who are actively trying to conceive which means women who undergo surgical and/or pharmaceutical therapy (8, 10). Indeed, in a study of 81 women with "salt-wasting" CAH evaluated since birth, only nine sought pregnancy, with eight of them conceiving in the end (8, 13). Independently of these women's infertility, pregnancies are usually normal and uneventful (11).

Fertility in NCCAH

"Non-classic" CAH is a frequent cause of infertility, often undiagnosed (14). Pregnancy rates in women with NCCAH who visit infertility clinics due to infertility or hyperandrogenemia, vary according to studies and range between 65 and 95% (8, 15–18). There is a significant phenotypic overlap between PCOS and NCCAH, often leading to misdiagnosis of patients who seek advice in fertility clinic. Patients with PCOS manifest hirsutism, hyperandrogenemia, variable degrees of insulin resistance, and anovulation (19). Because of the similarity of clinical features between the two conditions, it has been postulated that about 33% of patients diagnosed with PCOS actually suffer from NCCAH (20). Due to the different treatment of these two conditions and the possible incidence of NCCAH in the fetus during pregnancy, the correct differential diagnosis of patients with infertility is critical (20).

Etiology of Infertility in CAH

The etiology of infertility in patients with CAH is multifactorial, including ambiguous genitalia and their complications, excessive androgen secretion, adrenal progesterone hypersecretion, co-existence of PCOS, and various psychosocial factors (5–7, 11, 14, 20–22).

Female fetuses with "classic" CAH and adrenal androgen hypersecretion during endometrial life present with malformations of external genital organs such as presence of a urogenital sinus, labial fusion, and variable degrees of clitoral hypertrophy. These malformations render sexual intercourse unpleasant and sometimes prohibitive, reducing the chance of pregnancy. The possibility of sexual intercourse is related to introital width, vaginal length, and clitoral integrity. Internal genitalia remain intact (8). Exposure to increased androgens in endometrial life also affects psychosocial development of these patients. Women with "classic" CAH, experience gender-related disorders such as participation in games of masculine orientation as children and pursuit for men's occupations in adult life. An increased rate of homosexual and bisexual relationships among patients with CAH is reported. In addition, reduced *libido* and decreased desire for family formation are observed (8, 9, 20).

Chronic exposure to adrenal androgens causes disorders of the hypothalamic-pituitary-ovarian axis leading to hypersecretion of LH. In addition to increased androgen concentrations in peripheral blood, CAH is also associated with increased concentrations of progesterone. Progesterone secretion in these patients is continuous resulting to modification of GnRH pulsatility, prevention of normal endometrial development,

defective quality of cervical mucus, and decrease of tubal motility, resulting thus, in significant decrease in fertility (7, 8, 14, 20). Thirty to sixty-eight percent of women with "salt-wasting" CAH and 30–75% of women with "simple-virilizing" form manifest menstrual irregularities and anovulation (10).

MANAGEMENT OF INFERTILITY IN WOMEN WITH CAH

Treatment of CAH

Women with "classic" CAH have ambiguous external genitalia. Female embryos are exposed to adrenal androgens from the 7th week of pregnancy resulting in clitoral enlargement, fusion, and scrotalization of the labial folds, and rostral position of the urethral/vaginal perineal orifice, placing the phallus in male position while the internal female reproductive organs are developing normally. These changes are classified according to the five Prader stages (14). In case of patients with "classic" CAH and ambiguous genitalia, the possibility of surgical rehabilitation should be considered (23). It includes clitoroplasty, vaginoplasty, and labiaplasty and aims at removing redundant erectile tissue, preserving the sexually sensitive gland clitoris, and providing a normal vaginal orifice that functions adequately for menstruation, intromission, and birthing. In addition these interventions protect from recurrent urinary tract infections which result from pooling of urine in the vagina or urogenital sinus (10, 14, 23, 24). The main complications of surgical interventions include urinary incontinence, clitoral pain, painful intercourse and inadequate introitus, vaginal stenosis, and anorgasmia. These complications lead to decreased intercourse frequency. Secondarily, they have been observed strictures, fibrosis and scarring, fistulas, and recurrent urinary infections (10, 14, 24). Glucocorticoid therapy is the pharmaceutical treatment of choice, both for patients with "classic" CAH and for those with NCCAH, with addition of mineralocorticoid to patients with "salt-wasting" CAH (9α-fludrohydrocortisone acetate) (1–3, 9, 23). Glucocorticoids substitute the deficient endogenous cortisol synthesis and thus, CRH and ACTH hypersecretion is reduced, leading to decreased adrenal androgens secretion (25). Subsequently, progesterone levels are reduced and normal ovulation, endometrial proliferation and implantation ensue (5, 12). In "classic" CAH, to control overnight HPA-driven increase of adrenal androgens, a variety of glucocorticoid treatment regimens have been used. Treatment with hydrocortisone administration in three equal doses (starting at 8.00 am) seems to be the most appropriate. Many specialists used to administer prednisolone in adult patients because of the more convenient dosage regimen. However, this treatment is gradually abandoned because it is accompanied by side effects, such as obesity, insulin resistance, bone loss, hypertension, and dermal atrophy (25). Combination therapies employing glucocorticoids for adrenal replacement and androgen suppression (even 2 different glucocorticoids) as well as anti-androgens and androgen biosynthesis inhibitors for treatment of hyperandrogenism might be useful for treatment optimization and minimization of side effects. Treatment regimens and goals should be individualized, while these targets might be modified throughout patient's life. Laboratory data for adults with 21OH deficiency are useful as markers, but they are eventually less important than clinical evaluation. They can be improved by incorporating steroid profiling by mass spectrometry (26). When necessary, low doses of glucocorticoids may be used in patients with NCCAH (1). In these patients, when signs of hyperandrogenemia manifest, treatment is sometimes successful only with oral contraceptives alone or with spironolactone (1, 23).

Although most patients will become ovulatory with the routine dose of hydrocortisone, some will require greater doses for suppression of progesterone of adrenal origin. In patients who do not achieve pregnancy, progesterone plasma concentrations should be measured in the follicular phase of the menstrual cycle. In most cases adequate suppression of 17-hydroxyprogesterone results in adequate peripheral concentrations of adrenal-derived progesterone (although in this case one might not avoid exogenous hypercortisolism) (9, 20). Of note, greater doses of glucocorticoids are required when the therapeutic aim is the reduction of androgen concentrations, as compared to replacement doses required only for substitution of hormonal deficiency. For women who attempt to conceive when in glucocorticoid treatment, hydrocortisone, which is inactivated by the placenta, is employed. This treatment continues during pregnancy (1). Unilateral or bilateral adrenalectomy has been used as last resort for patients who do not respond to other treatments, especially those with "salt-wasting" CAH and large adrenal myelolipomas (most commonly developing in poorly controlled "classic" CAH) as well as in persistent hyperandrogenemia (3, 6), but it is not recommended because of its life-time invalidating risks (6, 8, 20, 27).

Ovulation Induction in CAH

For those patients who cannot achieve ovulation despite adequate treatment and reduction of progesterone and androgen concentrations, gonadotropins or clomiphene may be useful to induce ovulation (20). *In vitro* fertilization (IVF) can be another treatment option for those, who fail to achieve pregnancy with these therapeutic means (8). By the time ovarian stimulation is achieved, the possibility to freeze all embryos and transfer them to a subsequent cycle should be considered in an effort to avoid the IVF protocol-induced increased progesterone concentrations. In cases where both parents are carriers of a CAH mutation or one parent is affected by CAH and the other is a carrier, there is an increased risk that the fetus will be affected from CAH. In this case it is essential to perform pre-implantation genetic diagnosis (PGD) (6, 8).

PREGNANCY IN CAH CARRIERS AND IN CAH PATIENTS

Prenatal diagnosis of CAH in the embryo or fetus can be done by performing chorionic villus sampling (9–11th week of pregnancy) or amniocentesis (15–20th week of pregnancy)

followed by genetic testing (28). Specific probes for 21-hydroxylase mutations allow direct and rapid identification of known mutations through the use of polymerase chain reaction (i.e., allele specific). Panels of oligonucleotide probes, currently available for use in prenatal diagnosis, are expected to identify well more than 95% of current 21-hydroxylase mutations (4). In embryos belonging to a high-risk group for CAH, prenatal therapy to prevent virilization of external genitalia of a female embryo affected with CAH should be regarded as experimental. Recent studies address four areas of concern when dexamethasone is used as treatment: potential teratogenicity (cleft lip with/without cleft palate), reduced birth weight, potentially brain/behavior problems such as verbal working memory, reduced self-perception of scholastic competence and increased self-rated social anxiety, and potential long-term effects (insulin resistance) (27). There are no recommended specific treatment protocols and prenatal treatment should be obtained only through approved clinical protocols or trials (27). In embryos with increased probability of CAH (because of family risk), treatment with glucocorticoids should be introduced before the 9th week of pregnancy which effectively lowers excessive adrenal androgens amounts and thus, prevents masculinization of female external genitalia. The results from the villocentesis or amniocentesis will determine further patient's management. Treatment is discontinued when the fetus is male or unaffected female. Otherwise, it is continued until term in three divided doses based on maternal pre-pregnancy bodyweight (28). Concerns arise regarding the unnecessary corticoid treatment of pregnant women in case of male and unaffected female fetuses. Therefore, it is important to identify female affected fetuses before 9 weeks of pregnancy (4). Non-invasive techniques introduced in 2011 are based on extraction of fetal cell-free DNA (cfDNA) from maternal blood (28). This may become the new standard diagnostic approach in the future (4). The advantage of this test is that it can be done at the 6th week of pregnancy, allowing early diagnosis before the onset of genital organogenesis (9th week of pregnancy) and by that unnecessary treatments would be avoided (23, 28).

Spontaneous abortion rates appear to be greater, as compared to healthy pregnant women, in patients with CAH, as well as in patients with NCCAH who were not treated with glucocorticoids. These rates are normalized after glucocorticoid treatment (5, 8, 9). Pregnancies of women already diagnosed with CAH seem to be normal and uneventful (9). Genetic counseling is essential (5). Monitoring of pregnancy should be performed by a specialized team, which should include obstetrician, pediatrician and endocrinologist (5, 12). Symptoms of fatigue, nausea, and vomiting are common in pregnancy and overlap those of adrenal insufficiency. Overtreatment with hydrocortisone can lead to fluid retention, excessive weight gain and hypertension. Mothers should be evaluated for signs of adrenal insufficiency in pregnancy (i.e., postural hypotension) and stress dose steroids should be administered during labor. In the second and third trimester of pregnancy, the dose of hydrocortisone may need to be increased by 25–40%, although there is no consensus on this (5, 6, 8, 27). No dose adjustment of hydrocortisone is required in the early stages of pregnancy (9). Dose control of treatment with hydrocortisone during pregnancy should not be performed with plasma renin activity levels as they increase normally during pregnancy, but with testosterone and androstenedione concentrations (5, 6). Pregnant women with CAH appear to be at greater risk for developing gestational diabetes mellitus. The incidence of pre-eclampsia and premature delivery does not seem to change (5, 7, 9). Finally, cesarean section is preferable, especially for women who have prior genital reconstructive surgery, although vaginal deliveries have also been reported (5, 8, 9).

Babies from mothers with CAH and NCCAH have an increased risk to be small for gestational age (SGA) babies, especially when parents suffer from NCCAH. The long-term follow-up of the offspring has shown normal physical and intellectual development although these children might show deranged renal (particularly evident in females below the age of 5) and liver biochemistry (9, 29).

CASE REPORTS

In the literature there are a few cases of severe CAH which needed to undergo IVF. Albarel et al. reported a patient with StAR deficiency, homozygous for 1 bp delection in the StAR gene (719del). The patient, after missing ovarian response to clomiphene, underwent IVF with a long agonist protocol with 300 units menotropin per day. The procedure resulted in pregnancy with delivery of a normal female child (weight: 3.150 kg) at 40 weeks of gestation (30).

Bianchi et al. reported a 26 years old patient with CAH, associated with 17OH deficiency, a rare defect of steroid biosynthesis characterized by inability to synthesize cortisol, androgens or estrogens, complete absence of follicular maturation, hypergonadotropic hypogonadism, primary amenorrhea, and hypertension. The defect was due to a compound heterozygous mutation (p.W406R/P428L) in the *CYP17A1* gene. The patient underwent IVF with a long agonist protocol receiving 112.5 I.U. recombinant FSH per day. Four mature oocytes were retrieved and 3 blastocysts were obtained. Two of them were transferred and pregnancy was achieved. Pregnancy was complicated by pre-eclampsia, gestational diabetes (requiring insulin administration), cholestasis gravidarum (requiring ursacol administration), and cellulitis of the lower right extremity. At 30 weeks and 4 days, an emergency cesarean section was performed due to acute fetal distress. A true umbilical knot was identified, and a live normal male newborn was delivered (weight: 1,945 g; length: 43.5 cm) (31).

Neuwinger et al. also treated a 28 year female with 17OH deficiency. Because the ovaries of these patients contained numerous primordial follicles, the authors hypothesized that the absence of spontaneous follicular maturation could be due to a lack of aromatizable substrate. To provide this substrate, testosterone was administered either by intra-ovarian injection or by vaginal administration. Ovarian stimulation was performed with human urinary gonadotropins. Follicular maturation and ovulation were induced with this treatment, as

confirmed by ultrasonography, measurement of LH, estradiol and progesterone serum concentrations and finally, aspiration of oocytes from the mature follicles. Fertilization of these oocytes *in vitro*, however, did not succeed (32).

Ben-Nun et al. reported the first viable pregnancy in a woman with 17OH deficiency in which embryos produced with donated oocytes were transferred to the uterus. At the fifth embryo transfer attempt, the treatment resulted in a twin pregnancy which was further complicated with severe pre-eclampsia, hemolysis, elevated liver enzymes, low platelet (HELLP) syndrome, and premature delivery. One newborn died minutes after delivery, whereas the other was kept for several weeks at the neonatal intensive care unit and discharged without apparent disabilities (33).

DISCUSSION

As previously described, the clinical presentation of "classic" CAH and NCCAH is in direct correlation with the genomic and biochemical background of the disease. Therefore, it is important to emphasize that treatment should be individualized. Moreover, it should be a matter of collaboration between health-providers of many disciplines.

Managing patients with "classic" CAH is challenging. These patients, in addition to treatment with glucocorticoids and mineralocorticoids depending on the form of the disease, might need surgical treatment of ambiguous genitalia. The right moment to operate these patients is a field of controversy. In the past, the decision on surgery was taken on the basis of appearance of external genitalia and the possibility of conception. However, in the past two decades it is preferred that surgery is postponed so that the patients gives his/her informed consent (1). Because of this controversy over gender behavior, gender identity, surgical outcome, and long-term sexual function, it is imperative to consider all therapeutic options on an individual basis (23).

In an infertility clinic, health professionals are much more frequently confronted with patients with NCCAH compared to patients with "classic" CAH because the former represent a larger undiagnosed population while the latter are identified early in infancy (20). Many patients with NCCAH are undiagnosed due to the mild symptoms that lead them to seek medical advice only in case of infertility (8). Moreover, there is a phenotypic overlap between NCCAH and PCOS (20). The distinction is made as women with NCCAH manifest greater concentrations of 17-hydroxyprogesterone and progesterone than women with PCOS, who present insulin resistance, obesity, polycystic ovary morphology, and increased LH/FSH ratios (28).

The main problems in NCCAH women are the increased progesterone concentrations which alter endometrial receptivity and tubal motility and lead to ovulation disorders. Appropriate therapy usually leads to regular menses and spontaneous pregnancies. Sometimes ovulation induction regimens (i.e., clomiphene) can be used as well as IVF techniques in case of insufficient ovarian function and pregnancy is not achieved.

CONCLUSION

The treatment of infertility in CAH patients is a major challenge. Hydrocortisone is at the time being the gold standard treatment which restores ovarian function, ovulation, and endometrial receptivity. Performing PGD should be taken into consideration in cases where both parents are affected. Pregnancy should be followed by an expert team in a tertiary hospital in case of suspected affected fetus with CAH. Finally, patients with CAH should be followed by a multidisciplinary team including gynecologist, endocrinologist, and pediatrician.

AUTHOR CONTRIBUTIONS

AC and ES: literature review. RV and MP: manuscript. GM: revision.

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