The Many Faces of Non-Classic Congenital Adrenal Hyperplasia

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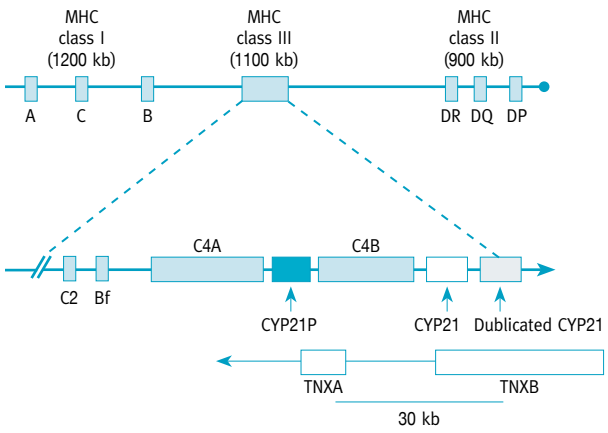
Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder and one of the most common inherited metabolic diseases [1]. It is characterized by impaired cortisol synthesis secondary to deficiency in one of the enzymes involved in adrenal steroidogenesis [1]. The disease was described for the first time in the mid-19th century [2] and its pathophysiology and molecular biology were elucidated in the second half of the 20th century [1,3]. Deficiency of 21-hydroxylase is responsible for more than 95% of cases. It is clinically divided into two groups and three main clinical presentations [1,3] depending on the type of mutation and the level of enzymatic activity. The classic and more severe forms lead to prenatal virilization in the female fetus, peripheral precocious puberty in boys, and salt wasting in 75% of patients [1,3]. The non-classic form presents with various degrees of postnatal virilization and might be asymptomatic [3-5]. This review focuses on the diagnosis and therapy for the non-classic form of 21-hydroxylase deficiency (NC21OHD) and highlights the controversial issues surrounding this disorder.

GENETICS

A deficiency of the steroid 21-hydroxylase enzyme is caused by mutations in the *CYP21A2* gene located on the short arm of chromosome 6 (6p21.3) within the human histocompatability complex [Figure 1]. The gene encodes for a cytochrome P450 type II enzyme composed of 495 amino acids. A non-functional pseudogene *CYP21A1* is located adjacent to the *CYP21A2* functional gene, and both consist of 10 exons with a 98% homology [1,3]. Most of the disease-causing mutations in *CYP21A2* are converted from the pseudogene. The proximity of the two genes creates a sensitive region leading to a chromosomal rearrangement in which deletions/mutations from the pseudogene are transferred to the functional gene. This proximity complicates the molecular analysis of subjects with NC21OHD, since the examination is intended to reveal mutations found only in the functional gene, thus indicating that the molecular analysis would need to be carried out in a genetic laboratory with extensive experience in the analysis of this gene [1,4,6-8].

More than 140 mutations have thus far been identified; however, only 10 of them are responsible for about 95% of the disease-causing alleles [3,6]. The mutations differ in prevalence and severity, depending on the ethnicity of the patients [4,6,8-9]. Previous studies that related mutations to the level of enzymatic activity [8,10,11] have shown that mild mutations, such as p.V281L and p.P30L, result in an enzyme with 20–50% of normal activity and a non-classic clinical phenotype. Severe mutations, such as p.I172N, result in 2–5% enzyme activity and a clinical presentation of the classic simple virilizing form. Intron 2 splice mutation may cause 0–2% activity, depending on the different

Figure 1. The MHC complex and *CYP21* genes



(Modified from: http://unswcah.tripod.com/genetics.htm. Entered 16.8.2016)
MHC = major histocompatibility complex

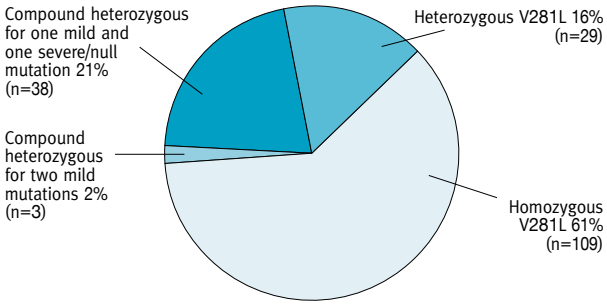
polymorphic splicing products [12]. Null mutations, such as gene deletions, p.Q318X, p.R356W, three cluster mutations in exon 6, and p.L307fs, result in an enzyme with 0% of normal activity and therefore cause the most severe salt-wasting form of CAH [6,8,10,11]. To further complicate the genetic analysis, the null mutation p.Q318X might be located on a duplicated *CYP21A2* gene [Figure 1], while there is, in fact, a normal functioning gene on the same chromosome. The carrier rate of the duplicated gene ranges between 7% and 80% in different populations [13]. The duplication can be detected by multiplex ligation-dependant probe amplification (MLPA), which is performed solely in specialized genetic laboratories [13,14].

Other very rare haplotypes are the combinations of the mild mutation p. V281L in tandem with rare severe mutations such as L307F (personal communication with S.I., January 2015). Consequently, if patients carry a severe mutation, their partners should undergo complete sequencing.

Of note, the phenotype of each patient reflects the less severely impaired allele. Phenotype-genotype correlation exists for approximately 90% of the cases [3,6-9]. The genotype of a subject with non-classic congenital adrenal hyperplasia (NCCAH) may be homozygous or compound heterozygous for two mild mutations or compound heterozygous for one mild and one severe/null mutation [5,16,17]. The common mild mutations are p.V281L, p.P30L, and p.P453S [1,5-8,16-18].

Most of the NC21OHD cases in Israel are homozygous p.V281L [5,17] [Figure 2]. However, about 20–30% are compound heterozygous to a mild/severe mutation, whereas in other countries it ranges between 50 and 70% of NCCAH patients [18]. The PCR method for *CYP21A2* molecular analysis performed in most genetic laboratories cannot differentiate between homozygosity to p.V281L and compound heterozygosity for p.V281L/deletion. Thus, if possible, parents' genotype should be analyzed or MLPA should be performed to detect deletion. If this is not possible, the human leukocyte antigen (HLA) typing can be performed to determine homozygosity of HLA-B14 because this allele is strongly linked with the *CYP21A2* gene carrying the V.281L mutation [4,16]. Despite the genetic homogenicity of NCCAH females in Israel, the clinical presentation is variable [19]. Therefore, we searched for other genetic variants in an attempt to explain such clinical diversity. Given that NC21HOD is a hyperandrogenic disorder, we tested the genetic polymorphism in the androgen receptor (AR) [20]. The AR gene, located on the X chromosome, is a key protein controlling cellular androgen sensitivity [21]. The length of CAG repeats of the AR gene is inversely correlated to activity of the human AR and affects the phenotype of several androgen-dependent disorders [22]. Indeed, in our study of 119 females with NC21OHD, we demonstrated that those with shorter CAG repeats in the AR presented earlier and had more frequent precocious pubarche and gonadotropin-dependent precocious puberty [20].

Figure 2. The genotype distribution among 179 Jewish patients with NC21OHD (authors' unpublished data)



Non-classic 21-hydroxylase deficiency is a common disorder presenting at different ages as either precocious pubarche, polycystic ovarian-like syndrome, or infertility

EPIDEMIOLOGY

Although the classic form of CAH occurs with an incidence of about 1:10,000–1:23,000 live births in the Caucasian population [1,9], NCCAH is much more frequent, with an incidence of 1:1000–2000 in the general population. Previous studies have reported a very high prevalence (1:27) of NC21OHD among Ashkenazi Jews living in New York, which was secondary to a high carrier rate (1:3) of the founder mutation p.V281L [23]. However, in a study conducted by Israel and colleagues [4], the p.V281L carrier rate was 1:10 in Ashkenazi Jews, 1:15 in Ethiopian Jews and 1:20 in Moroccan Jews. These carrier rates can be extrapolated to yield a prevalence of NC21OHD in 1:400 Ashkenazi Jews, 1:890 Ethiopian Jews and 1:1600 Moroccan Jews. These contradictory figures for the two locations might be secondary to intermarriage within the Ashkenazi community in New York.

The prevalence of NCCAH in hyperandrogenic females ranges between 1 and 10% depending on the population studied [24].

PATHOPHYSIOLOGY

NC21OHD is a mild form of congenital adrenal hyperplasia characterized by a partial decrease in the activity of the 21-hydroxylase enzyme. This decrease results in slightly reduced cortisol production and, through a negative feedback mechanism, leads to mild overproduction of corticotropin, which stimulates adrenal overproduction of pre-block metabolites and hormones that are not influenced by the defective

enzyme [25]. The 21-hydroxylase enzyme is necessary for both mineralocorticoid and glucocorticoid production, but it is not involved in sex hormone production [1,3].

Since blood aldosterone concentration is 1000-fold lower than cortisol concentration, its production neccesitates only 1% of enzymatic activity. Therefore, mineralocorticoid deficiency is not a feature of NC21OHD [1].

The most characteristic biochemical abnormality in 21-hydroxylase deficiency is the elevation of 17-hydroxyprogesterone (17-OHP), the main substrate to 21 hydroxylase. The block causes a shift of 17OHP to the sex hormone axis and therefore increases production of androstenedione and testosterone, leading to clinical and laboratory hyperandrogenism. DHEA and DHEAS are not elevated in 21OHD [1,3].

CLINICAL PRESENTATION

NC21OHD is associated with different degrees of postnatal virilization, presenting throughout childhood, puberty and young adulthood [5,17-19]. It can be asymptomatic, the cryptic form of NCCAH, and diagnosed incidentally, often by family screening [5,26]. NCCAH is rarely diagnosed during infancy, with mild clitoromegaly or premature development of pubic hair alerting to its presence. The most frequent presenting signs during childhood are precocious adrenarche or pubarche with slightly accelerated growth and advanced bone age in both males and females and macrogenitalia in males [5,17,19]. The clinical presentation in females during puberty resembles polycystic ovary syndrome (PCOS), including hirsutism, acne and menstrual irregularities, such as oligomenorrhea, or primary or secondary amenorrhea. Presentation in females during adulthood is either infertility or PCOS-like syndrome [18,20,26]. We have previously shown that children with the mild/severe genotype present earlier than children with mild/mild genotype exhibiting more advanced height age and bone age and earlier puberty, but resulting in decreased adult height [5,17,19]. Young females with the mild/severe genotype have more menstrual irregularities than females with the mild/mild genotype [18]. The female-to-male ratio is about 4:1 in most series of subjects with NC21OHD [5,16,17], probably secondary to a referral bias since symptoms are much more prominent in females [5,19].

DIAGNOSIS OF NCCAH

First and foremost, the diagnosis of NCCAH is based on clinical presentation combined with biochemical tests. Elevated early morning basal 17OHP levels, androstenedione or testosterone in a symptomatic patient should raise the suspicion of NC21OHD. Basal 17OHP levels ranging from 6 to 300 nmol/L most likely indicates NCCAH, and basal 17OHP levels below 6 nmol/L almost certainly rules it out [1,7,27]. However, about 10% of NC21OHD subjects have basal 17OHP levels below 6 nmol/L [5,17]. Therefore, the definitive diagnosis is made by the 30 and 60 minute response of 17OHP to adrenocorticotropic hormone (ACTH) stimulation (the standard Synacthen test, 250 μg/m^2). 17OHP levels post-ACTH stimulation above 45 nmol/L most likely indicate NCCAH and those below 45 nmol/L almost certainly rule it out [7,27].

Importantly, there is overlap between stimulated 17OHP among normal and heterozygous carriers. Therefore, while the diagnosis of NCCAH is biochemical, the diagnosis of a carrier is genetic. The molecular analysis of *CYP21A2* only confirms the diagnosis and is important for future genetic counseling [27]. In reproduction-aged females, measurement of the 17OHP and androgen levels as well as ACTH stimulation testing should be performed in the early follicular phase of the cycle. Of note, 17OHP is stress dependent; therefore, slightly elevated basal 17OHP levels are not evidence of NC21OHD and a supplementary ACTH stimulation test is required [1,5,7,27]. The ACTH stimulation test is also important for the evaluation of cortisol reserve. We have previously shown in a sample of 41 NCCAH subjects that about 50–70% of them had decreased cortisol response to the Synacten test [25]. Huerta et al. [28], who studied various hormonal responses to ACTH stimulation in 24 subjects with NCCAH, did not find a significant difference in the 60 minute stimulated cortisol levels compared to 37 healthy controls. However, there was a tendency for lower cortisol response in the NCCAH subjects, which did not reach significance perhaps due to the smaller sample size.

Since the genetic analysis of the gene encoding the 21-hydroxylase enzyme (*CYP21A2*) is complicated, the molecular analysis would need to be carried out in a genetic laboratory with extensive experience in the analysis of this gene

Programs for screening newborns are designed to detect the classic form of CAH, but they are not sufficiently sensitive for the non-classic form [29].

TREATMENT OF NCCAH

The treatment of symptomatic or hyperandrogenic subjects with NCCAH comprises low-dose glucocorticoids (GC) [17,19,25-27]. Asymptomatic subjects should not be treated. The therapeutic aim during childhood is to prevent early puberty, excessive bone age maturation and compromise of adult height. Our group had previously shown that early diagnosis and initiation of GC therapy might improve adult height [17,19]. During adolescence, the aim of therapy is to ameliorate hyperandrogenic symptoms, such as hirsutism, acne and menstrual irregularities [30]. The use of oral contraceptives can decrease the GC dose

in sexually active female adolescents, while the addition of anti-androgenic agents, such as spironolactone or cyproterone acetate, may offer an additional benefit in cases of severe acne or hirsutism [30]. Whether GC therapy improves fertility in young females with NCCAH is highly controversial [31-33].

The GC therapy goals are to decrease androgen levels (androstenedione and testosterone) to the upper normal levels, according to age and Tanner stage. In order to not overtreat, the 17OHP level should be kept slightly above the upper normal range [30]. The recommended GC doses are relatively low (mean 6–9 mg/m²/day) [17] to avoid long-term side effects of GC therapy. Although in the classic form of CAH the recommendation is to triple the glucocorticoid dose during stressful situations, administering a stress dose in the NCCAH form with slightly decreased cortisol response to ACTH stimulation [25] is controversial and should be implemented only in specific cases of symptomatic adrenal insufficiency during a concomitant illness.

NCCAH AND PREGNANCY

Elevated androgen and progesterone levels are risk factors for infertility, as shown in females with PCOS [34]. Two studies described an increased rate of first-trimester miscarriages in non-GC treated NCCAH women compared to treated ones [31,33], while a third study [32] showed a similar rate of miscarriages among treated and untreated pregnancies. However, a careful look at these studies, which found a decrease in miscarriages in women treated with GC, may show that the control group's miscarriage rate was much lower than expected and the timing of treatment could not necessarily have had an impact on the miscarriage rate. There is a need for prospective randomized control studies to resolve this controversy. Feldman et al. [31] have shown that time to conceive with GC therapy was shorter than time to conceive without GC therapy. If therapy during pregnancy is indicated, which is not the case in some patients, the aim of therapy is to normalize androgen and progesterone levels before pregnancy [35], and to normalize androgen levels according to the pregnancy norm, which are much higher than in non-pregnant females due to placental production [36] during pregnancy. The types of GC to be used during pregnancy are either hydrocortisone or prednisone. Dexamethasone should not be used routinely, since unlike the shorter-acting GCs it is not inactivated by placental 11β-hydroxysteroid dehydrogenase 2 and therefore crosses the placenta and might affect the fetus [27]. Little is known about fertility rates in men with NCCAH. We found that none of the 222 men who underwent fertility evaluation due to abnormal sperm parameters had NCCAH [37].

Low-dose glucocorticoid therapy is indicated solely in symptomatic hyperandrogentic patients and the need for treatment can be changed during the their lifetime

GENETIC COUNSELING

The carrier rates of *CYP21A2* gene mutations in the general population are approximately 1:60 (1.6%) for the severe form and about 1:10–20 (6–10%) for the mild form [1,4]. About 50–70% of women worldwide with biochemically proven NCCAH are compound heterozygous for one mild and one severe mutation [18]. Due to the high carrier rate of the mild mutation p.V281L, 60–70% of the affected women in Israel are p.V281L homozygous [4,5,17,20]. A person with NCCAH will have a risk of about 1:1000 (0.1%) (1:60 x 0.5 x 0.25) of having a child with classic CAH and a 1:20 risk (5%) (1:10 x 1 x 0.5) of having a child with NCCAH [1,4,32]. Therefore, the partner of a subject who carries a severe mutation must undergo molecular analysis of the *CYP21A2* gene to rule out a severe mutation. If the partner is also a carrier of a severe mutation, the risk for a fetus with classic CAH is 25% and the risk for a girl with ambiguous genitalia is 12.5%. Therefore, this couple should undergo prenatal counseling to understand the risk of having a child with the classic form [14,38,39]. Of interest, a retrospective analysis of two studies on children born to NCCAH women found that the prevalence of classic and NCCAH was much higher (i.e., 1–1.5% and 14.2–24%, respectively) [32,33]. In addition, a pilot study by our group yielded similar results: of 120 pregnancies in 59 women with NCCAH, the prevalence of offspring with NCCAH was fivefold higher than expected for the Jewish population in Israel, and the prevalence of classic CAH fourfold higher than expected [unpublished data]. The high prevalence of offspring with NC21OHD might be due to a high frequency of paternal homozygosity for p.V281L in the Jewish population [4].

PRENATAL COUNSELING

Prenatal counseling has enormous significance for couples at high risk of having a child with the classic form of CAH. Due to the current therapeutic regimens and follow-up, children with the classic form of CAH grow to be healthy and normal functioning adults. Infants with the classic form need to take three medications (GC, mineralocorticoid, salt), with the GC being divided into three doses per day. They also have to be closely followed in a pediatric endocrinology clinic to undergo clinical and laboratory evaluations for the purpose of adjusting the medication doses. Females with the classic form are born with virilized external genitalia and urogenital sinus with normal ovaries and internal female structures. They will require corrective surgery during the first year of life, and they might need additional surgical interventions during adolescence [1,27]. The parents need to know the significance of having a child who will need to be

carefully monitored and to follow a strict medication regimen. They are offered various options:

- Carry the spontaneous and normal pregnancy to term with the chance of having an infant with classic CAH
- Continue the spontaneous pregnancy and undergo chorionic villus sampling at week 10–12 for karyotype and molecular analysis of *CYP21A2* and then decide about the continuation of pregnancy based on the results
- Continue the spontaneous pregnancy and decide whether to initiate high-dose dexamethasone therapy to prevent female fetus virilization, test the Y chromosome in the maternal blood at week 8 of pregnancy and stop dexamethasone should testing be positive for the Y chromosome [14]
- Undergo pre-gestational diagnosis (PGD) when pregnancy is achieved with in vitro fertilization [27]

PRENATAL DEXAMETHASONE THERAPY

One of the most controversial issues in the field of CAH is the question whether to treat mothers at risk of having a fetus with classic CAH [14,27,39,40] with high-dose dexamethasone during pregnancy. This therapy was first described during the early 1980s and 1990s [38] and proved to be effective in preventing female fetus genital virilization [40]. By crossing the placenta, dexamethasone suppresses the fetal hypothalamic-pituitary-adrenal axis and prevents fetal overproduction of adrenal androgens during the time of genital differentiation (6–12 weeks gestation). The treatment is given at a dose of 20 µg/kg of pre-pregnancy weight divided into three doses, and it has to be administered as soon as the woman knows she is pregnant and not later than week 8 of gestation [14]. This regimen results in a 60-fold GC level compared to a normal fetus [27].

Animal studies have shown that prenatal dexamethasone altered postnatal renal structures and function and produced hypertension in rodents. In addition, high-dose dexamethasone given to pregnant rhesus monkeys disrupted the development of hippocampal neurones in their fetuses [27]. Furthermore, high-dose GC given in early pregnancy due to various pathological conditions of the mother caused increased rates of cleft lip and cleft palate [27]. However, prenatal dexamethasone treatment for mothers with a risk of a classic CAH offspring was not associated with increased intrauterine growth retardation, preterm birth, small-for-gestational-age birth, or congenital malformations [14,40]. The weight of prenatal dexamethasone-treated neonates is about 0.5 kg less than the mean in the regular population [27,40].

Although this medical treatment protocol has been in clinical use for three decades, we did not find any publications of large studies on its long-term consequences. The results of psycho-neurocognitive tests among these children are conflicting [27]. No differences have been observed in psychopathology, behavioral problems or adaptive functioning compared to controls in an American group [27,39], while there was more shyness and poorer verbal working memory, mainly in unaffected subjects who were treated only in the first trimester in a Swedish group [27]. Maternal side effects are Cushing-like syndrome, with a slightly elevated rate of gestational diabetes, striae and edema [40].

Endocrinologists who favor prenatal dexamethasone treatment [14,39] must consider the emotional distress associated with the birth of a child with ambiguous genitalia, and the need for repeated reconstructive surgeries. The endocrinologists who oppose this therapy [27] voiced their concern that seven of eight fetuses are unnecessarily treated in order to prevent virilization in only one of them and the essentially unknown long-term side effects on the child associated with the use of high-dose dexamethasone during pregnancy. However, today we can test for the Y chromosome in week 8 of pregnancy [14,39] and therapy can be stopped immediately if the results are positive. Furthermore, detecting the disease-causing mutations can be done at the beginning of pregnancy in cell-free fetal DNA in the maternal plasma [39]. That test is still experimental, but with the advance of molecular genetic analysis methods, it will probably become common practice [39]. This controversy led to the Endocrine Society guidelines issued in 2010 [27], which stated that prenatal GC therapy is not considered the standard of care and should be implemented only in the framework of an experimental research procedure in centers capable of collecting data over time and under institutional review board approval with the purpose of helping us understand the long-term significance of this treatment. The U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) classified dexamethasone administration during pregnancy as a category B drug, meaning that its safety in pregnancy is unknown, and therefore the administration of dexamethasone for prenatal CAH is designated as off-label use [27]. Finally, the decision about initiating treatment should be based on the values and preferences of the parents and should require their fully informed consent [27,40].

CONCLUSION

NCCAH is a very mild form of adrenal hyperplasia in which the hyperandrogenism is easy to control. It should be noted, however, that it is an evolving disorder and NCCAH females should therefore be followed to enable normal fertility in the future in those who develop significant hyperandrogenism or secondary PCO.

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"When an individual is protesting society's refusal to acknowledge his dignity as a human being, his very act of protest confers dignity on him"

Bayard Rustin(1912–1987), an American leader in social movements for civil rights, socialism, nonviolence, and gay rights