

## Congenital adrenal hyperplasia: clinical symptoms and diagnostic methods

Rafał Podgórski<sup>1,2✉</sup>, David Aebisher<sup>3</sup>, Monika Stompor<sup>1,2</sup>, Dominika Podgórska<sup>4</sup> and Artur Mazur<sup>5</sup>

<sup>1</sup>Centre for Innovative Research in Medical and Natural Sciences, University of Rzeszów, Rzeszów, Poland; <sup>2</sup>Department of Biochemistry, Faculty of Medicine, University of Rzeszów, Rzeszów, Poland; <sup>3</sup>Department of Human Immunology, Faculty of Medicine, University of Rzeszów, Rzeszów, Poland; <sup>4</sup>Department of Rheumatology, Clinical Provincial Hospital No. II in Rzeszów, Rzeszów, Poland; <sup>5</sup>Department of Pediatrics, Pediatric Endocrinology and Diabetes, Medical Faculty, University of Rzeszów, Rzeszów, Poland

The aim of this paper is a straightforward presentation of the steroidogenesis process and the most common type of congenital adrenal hyperplasia (CAH) – 21-hydroxylase deficiency – as well as the analytical diagnostic methods that are used to recognize this disease. CAH is a family of common autosomal recessive disorders characterized by impaired adrenal cortisol biosynthesis with associated androgen excess due to a deficiency of one or more enzymes in the steroidogenesis process within the adrenal cortex. The most common and prototypical example of the CAH disorders group (90–95%) is caused by 21-hydroxylase deficiency. Less frequent types of CAH are 11 $\beta$ -hydroxylase deficiency (up to 8% of cases), 17 $\alpha$ -hydroxylase deficiency, 3 $\beta$ -hydroxysteroid dehydrogenase deficiency, P450 oxidoreductase deficiency and StAR deficiencies. In the 21-hydroxylase and 11 $\beta$ -hydroxylase deficiency, only adrenal steroidogenesis is affected, whereas a defect in 3 $\beta$ -hydroxysteroid dehydrogenase or 17 $\alpha$ -hydroxylase also involves gonadal steroid biosynthesis. Many countries have introduced newborn screening programs based on immunoassays measuring 17-hydroxyprogesterone from blood spots used for other neonatal screening tests which enable faster diagnosis and treatment of CAH. Currently, chromatographic techniques coupled with mass spectrometry are gaining popularity due to an increase in the reliability of the test results.

**Key words:** Congenital Adrenal Hyperplasia, CAH, steroidogenesis, 21-hydroxylase deficiency

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✉ e-mail: rpodgorski@ur.edu.pl

**Abbreviations:** 17-OHP, 17-hydroxyprogesterone; 21-OHD, 21-hydroxylase deficiency; ACTH, Adrenocorticotrophic hormone; CAH, Congenital adrenal hyperplasia; CRH, Corticotrophin hormone; CYP450, Cytochrome P450; DHEA, Dehydroepiandrosterone; GC-MS, Gas chromatography mass spectrometry; LC-MS/MS, Liquid chromatography tandem-mass spectrometry; HSD11 $\beta$ , 11 $\beta$ -hydroxysteroid dehydrogenase; HSD17 $\beta$ , 17 $\beta$ -hydroxysteroid dehydrogenase; HSD3 $\beta$ , 3 $\beta$ -hydroxysteroid dehydrogenase; HSDs, Steroid dehydrogenase; NBS, Newborn screening; NC CAH, Non-classical congenital adrenal hyperplasia; StAR, Steroidogenic acute regulatory protein; SV CAH, Simple virilizing congenital adrenal hyperplasia; SW CAH, Salt-wasting congenital adrenal hyperplasia

### INTRODUCTION

The first case of a patient with congenital adrenal hyperplasia (CAH) was described by the Italian physician

Luigi De Crecchio in 1865. He released the autopsy report of a 44-year-old man who died from an apparent Addisonian crisis that had external male genitals and internal female reproductive organs and significantly enlarged adrenal glands (New, 2011; Delle Piane *et al.*, 2015). After that, at least five different types of congenital adrenal hyperplasia associated with impaired function of enzymes involved in the synthesis of steroids have been reported. As mentioned above, 21-hydroxylase deficiency (21-OHD) is the most common type of CAH; less frequent are 11 $\beta$ -hydroxylase deficiency, 17 $\alpha$ -hydroxylase deficiency, P450 oxidoreductase deficiency and lipoid congenital adrenal hyperplasia (StAR deficiency) (Krone *et al.*, 2007; Kaur *et al.*, 2016) (Table 1). In all types of CAH, the most important result of dysfunction of one of the intermediate steroidogenesis stages is low cortisol production which is the major glucocorticoid hormone. Low blood cortisol levels stimulate secretion of corticotrophin (CRH) and adrenocorticotrophic hormone (ACTH) by the hypothalamus and pituitary, which indirectly (CRH) and directly (ACTH) induces secretory function of the adrenal cortex. Constantly elevated ACTH levels lead to adrenal hyperplasia, accumulation of steroid hormones precursors, and hyperandrogenism (Pang *et al.*, 1993; Speiser *et al.*, 2010).

### STEROIDOGENESIS

#### Synthesis of steroid hormones

Steroid hormones are produced in the adrenal cortex (glucocorticoids, mineralocorticoids), testis (strong androgens), ovary (estrogens), and some peripheral tissues (adipose tissue, the brain). One of the main organs, where the synthesis of steroid hormones occurs, is the adrenal cortex, which consists of 3 layers:

- **zona glomerulosa** – outwardly located, representing 15% of the organ weight; within it, the synthesis of the most important mineralocorticoid – **aldosterone** – takes place

- **zona fasciculata** – represents 75% of the cortex weight, situated between the zona glomerulosa and reticularis, and responsible for producing glucocorticoids such as **cortisol**

- **zona reticularis** – represents 10% of the gland weight and is located closest to the adrenal medulla; within it, there is the synthesis of adrenal androgens like dehydroepiandrosterone (DHEA) (Pang *et al.*, 1987; Yau *et al.*, 2016b).

Table 1. The most common steroidogenesis enzyme deficiencies inducing CAH.

Enzyme deficiency	F	Substrate	Product	A	M	G	References
21-Hydroxylase deficiency	90-95%	Progesterone, 17-OH-progesterone	Deoxycorticosterone, 11-deoxycortisol	↑	↓	↓	(Parsa & New, 2017)
11β-Hydroxylase deficiency	0.2- 8%	Deoxycorticosterone	Corticosterone	↑	↑	↓	(Bulsari & Falhammar, 2017)
17α-Hydroxylase deficiency	rare	Pregnenolone, Progesterone	17-OH- pregnenolon, 17-OH-progesteron	↓	↑	↓	(Miller 2012)
3β-Hydroxysteroid dehydrogenase deficiency	<5%	Pregnenolone, 17-OH-pregnenolone, DHEA	Progesterone, 17-OH-progesteron, Androstendione	↓	↓	↓	(Benkert <i>et al.</i> , 2015)
P450 oxidoreductase deficiency	rare	Pregnenolone, Progesterone	DHEA, 11-deoxycortisol	Antenatal↑ Postnatal ↓	↔	↓	(Arlt <i>et al.</i> , 2004)
StAR deficiency	rare	Cholesterol	Impaired transport of cholesterol to the inner side of the mitochondrial membrane	↓	↓	↓	(Miller & Auchus, 2011)

Abbreviations: **F** – frequency, **A** – androgens, **M** – mineralocorticoids, **G** – glucocorticoids

In the process of steroidogenesis (Fig. 1), two types of enzymes can be distinguished:

#### I – cytochrome P450 (CYP450)

#### II – steroid dehydrogenases (HSDs)

Cytochrome P450 is a generic term for a large group of oxidative enzymes that are composed of about 500 amino acids and contain a single heme group. When in reduced form and complexed with carbon monoxide, the enzyme absorbs electromagnetic radiation at 450 nm and for this reason, is termed P450 (Gonzalez, 1988). The human genome includes genes for 57 cytochrome P450 enzymes and they are named CYP genes (Lander *et al.*, 2001; Venter *et al.*, 2001). Most of the CYP450 is localized in the endoplasmic reticulum of liver cells, where nonspecific oxidation of endogenous toxins, drugs and xenobiotics takes place (Gonzalez, 1988).

In the process of steroidogenesis, 7 distinct cytochrome P450 enzymes are involved (Table 2). All P450 oxidases operate in correlation with the mitochondrial electron transfer system.

Electrons from NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) are transferred to CYP450 and reduce it:

**Type I P450** – reduced by adrenodoxin (a small iron-sulfur protein) with the participation of flavoenzyme – adrenodoxin reductase. Type I enzymes include P450<sub>scc</sub>, P450<sub>c11β</sub>, P450<sub>c11AS</sub>, and other enzymes involved in vitamin D and bile acid metabolism (Pang *et al.*, 1987; Gonzalez, 1988; Lander *et al.*, 2001; Venter *et al.*, 2001; Miller, 2005).

**Type II P450** – reduced with the involvement of a single 2-flavin protein termed P450 oxidoreductase (POR). Type II P450 enzymes include steroidogenic P450<sub>c17</sub>, P450<sub>c21</sub> and P450<sub>aro</sub> (Miller, 2005; Ghayee & Auchus, 2007; Miller & Auchus, 2011).

Type I enzymes reside in the mitochondria, while type II reside in the smooth endoplasmic reticulum (Nelson *et al.*, 1993).

**Hydroxysteroid dehydrogenases and reductases** are an enzyme group with molecular weights between 35-45 kD that occur in one or more isoforms (each is en-

coded by a distinct gene). They lack a heme moiety and require nicotinamide adenine dinucleotide (phosphates) (NADH/NAD<sup>+</sup> or NADPH/NADP<sup>+</sup>) for their activity. Generally, HSDs catalyze redox reactions, regulate the ratio of hydroxy- and ketosteroids in cells and in plasma. Oxidative HSDs use NAD<sup>+</sup> as a cofactor and convert hydroxysteroids to ketosteroids, while reductive HSDs use NADPH to reduce ketosteroids to hydroxysteroids. Steroidogenic HSDs include 3α- and 3β-hydroxysteroid dehydrogenase (HSD3β2), two 11β- hydroxysteroid dehydrogenases (HSD11β1 and HSD11β2) and a series of 17β-hydroxysteroid dehydrogenases (HSD17β) (Penning, 1997; Agarwal & Auchus, 2005; Ghayee & Auchus, 2007).

#### Regulation of hormone secretion

The secretion of adrenal cortex hormones is always correlated with the *de novo* synthesis of steroids because unlike other secreting glands (e.g. pancreas), adrenal glands tend not to accumulate hormones stocks. This entails the necessity of an immediate response to the demand and production of the new hormones.

As shown in Fig. 1, the synthesis of steroid hormones is a multistep process that is regulated by adrenocorticotrophic hormone (ACTH) through:

- trophic action (as a result of long-term stimulation)
- physiological growth of adrenal cells (Ferguson, 1963; Ney *et al.*, 1967)
- cell mediators (cAMP) – they stimulate the expression of genes encoding enzymes involved in steroidogenesis (mainly CYP11A1 gene encoding P450<sub>scc</sub>) (Mesiano *et al.*, 1991)
- increasing the production and activation of StAR (steroidogenic acute regulatory protein) that is responsible for the transfer of free cholesterol from cytosol or outer mitochondrial membrane to the inner mitochondrial membrane. As a result, cholesterol and P450<sub>scc</sub> are brought into contact to initiate biosynthesis. This is the most important stage of the regulating process which limits the speed of the whole steroid biosynthesis (Pon *et al.*, 1986; Arakane *et al.*, 1997).

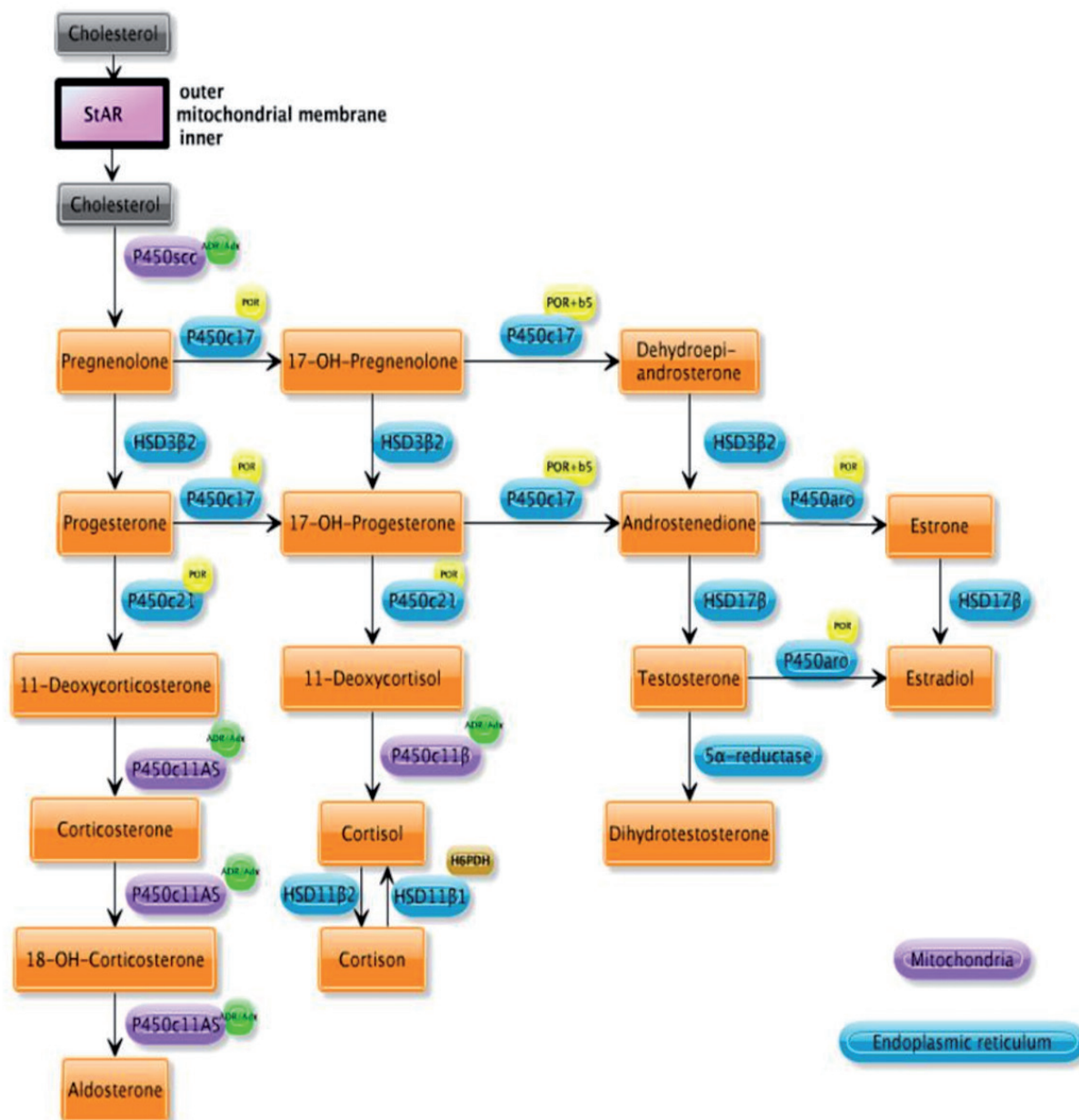


Figure 1. Pathway of human steroid biosynthesis (Krone *et al.*, 2010)

**Abbreviations:** ADR, adrenodoxin reductase; Adx, adrenodoxin; POR, P450 oxidoreductase; *b*<sub>5</sub>, cytochrome *b*<sub>5</sub>; H6PDH, hexose-6-phosphatedehydrogenase (cofactor); OH, hydroxyl; P450<sub>sccl</sub>, P450-side-chain-cleavage enzyme; HSD3β2, hydroxysteroid dehydrogenase type 2; P450c17, 17-α hydroxylase; P450c21, 21-hydroxylase; P450c11β, 11β-hydroxylase; P450c11AS, aldosterone synthase; HSD17β, 17β-hydroxysteroid dehydrogenase; CYP19A1, P450aro, P450 aromatase.

## CONGENITAL ADRENAL HYPERPLASIA (CAH)

As seen in Fig. 1, 21-hydroxylase deficiency impairs the conversion of 17-hydroxyprogesterone (17-OHP) to 11-deoxycortisol and progesterone to 11-deoxycorticosterone, which are both the key precursors in the synthesis pathway of cortisol and aldosterone. Accumulation of excessive 17-hydroxyprogesterone (before this point biosynthesis of steroid functions normally) amplifies the pathway of androgen production, wherein P450c21 is not involved. Depending on the severity of mutations and the loss of functionality of 21-hydroxylase, congenital adrenal hyperplasia can take two clinical forms:

a severe-classical and a mild non-classical (Trapp *et al.*, 2011).

### Classic Congenital Adrenal Hyperplasia

The **classical form** of CAH affects between 1:10 000 and 1:20 000 infants of the Caucasian race (Reisch & Kuhnle, 2014). However, there are communities where this frequency is much higher. For instance, for Yupik-speaking Eskimos, a small and closed ethnic group, the incidence was found to be 1:282 and in Brazilian patients 1:7 500 (Pang *et al.*, 1982, 1993). In contrast, considerably lower incidences were found in African-Americans and

**Table 2. Isoenzymes of cytochrome P450 involved in adrenal steroid hormone synthesis (Van der Kamp *et al.*, 2001; Miller 2005; Miller & Auchus, 2011)**

Enzyme	Symbol	Activity	Cellular localization	Adrenal localization	Gen
Cholesterol side-chain cleavage enzyme	P450scc (formerly 20,22-desmolase)	20 $\alpha$ -hydroxylase 22-hydroxylase, 20,22-lyase	Mitochondria	Each	CYP11A1
Steroid 11 $\beta$ -hydroxylase	P450c11 $\beta$	Steroid 11 $\beta$ -hydroxylase	Mitochondria	Fasciculata	CYP11B1
Aldosterone synthase	P450c11AS	11-hydroxylase, 18-hydroxylase, 18-hydroxydehydrogenase	Mitochondria	Glomerulosa	CYP11B2
Steroid 17 $\alpha$ -hydroxylase	P450c17	17-hydroxylase 17,20-lyase	Endoplasmic reticulum	Fasciculata Reticularis	CYP17A1
Steroid 21-hydroxylase	P450c21	21-hydroxylase	Endoplasmic reticulum	Each	CYP21A2
P450 oxidoreductase	POR	Transfer electrons from NADPH to cytochrome P450	Endoplasmic reticulum	Each	POR
P450 aromatase or estrogen synthetase	P450aro	Aromatization of androgens into estrogens	Endoplasmic reticulum	Gonads (granulosa cells)	CYP19A1

Native Americans – 1:42000 (Merke & Bornstein, 2005; Therrell & Adams, 2007).

The classical form of CAH occurs in two phenotype forms: simple virilizing (SV) and salt-wasting (SW).

#### Salt-wasting Congenital Adrenal Hyperplasia

The **salt-wasting congenital adrenal hyperplasia** (SW) is the most severe type and accounts for 75% of all cases of classical CAH. SW appears when mutations in the gene CYP21A2 are so extensive that 21-hydroxylase loses nearly all its enzymatic activity (<2%) (Parsa & New, 2017). This leads to a life threatening impairment of the synthesis of not only cortisol but aldosterone as well. Aldosterone is responsible for the body's homeostatic sodium regulation. If left untreated, low levels of aldosterone leads to hypovolemia, hyponatremia, hyperkalemia, hyperreninemia, development disorders, weight loss, convulsions and finally death of a newborn during the 1–4 weeks after birth. Rapid diagnosis is crucial to increase the chance of the child survival. Subsequently, in many countries (for the first time in USA, in 1977), a national program of newborn screening for 21-OHD has been introduced (Pang *et al.*, 1977; Van der Kamp *et al.*, 2001).

Affected male newborns without signs of ambiguous genitalia, are particularly prone to the occurrence of associated salt adrenal crisis and death, a few days after discharge from the hospital. A similar situation occurs in adult patients that are treated with mineralocorticoid and abruptly stop the therapy. In boys, the main source of testosterone are testicles, not adrenal glands. For that reason, at the time of birth, they do not display ambiguous genitalia. The only external symptom pointing to irregularities is genital hyperpigmentation, and sometimes penis enlargement (Merke & Bornstein, 2005). Female infants with classic CAH typically have ambiguous genitalia at birth due to intrauterine exposure to high androgen concentrations. Characteristic symptoms include an enlarged clitoris, rugose and partly fused labia majora, a

common urogenital sinus in place of a separate urethra and vagina and even development of the male urethra. The internal female organs, the uterus, fallopian tubes, and ovaries are normal; fertility usually is preserved, but there is no development of the Wolffian duct (Speiser & White, 2003a). The occurrence of such obvious anomalies results in proper diagnosis and immediate treatment (Yau *et al.*, 2016b). The treatment is based on replacement therapy with glucocorticoids and mineralocorticoids (connected with periodic monitoring of renin and aldosterone levels).

Hydrocortisone is the glucocorticoid of choice during childhood (Pang *et al.*, 1977; Clayton *et al.*, 2002; Speiser & White, 2003b), whereas adults usually take longer-acting medicines like dexamethasone and prednisone, which can induce growth suppression in children. Mineralocorticoid replacement is achieved with fludrocortisone, and the dose is adjusted to maintain plasma renin activity in the mid-normal range (Rivkees & Crawford, 2000; Clayton *et al.*, 2002; Punthakee *et al.*, 2003).

If left untreated or improperly treated, postnatal hyperandrogenism causes progressive virilization, including premature development of pubic and axillary hair, acne, advanced skeletal age and somatic development, and centrally induced precocious puberty in boys and girls (Quintos *et al.*, 2001; New, 2003; Demirci & Witchel, 2008; Lin-Su *et al.*, 2011). One of the effects of CAH-induced precocious puberty is closing of the epiphyseal cartilage of bone during normal linear growth, which leads to the reduction in the patients' height, which is below the population mean.

Proper treatment affords the opportunity to improve the condition of patients with SW CAH, however, it does not change the need for glucocorticoid administration (Stoner *et al.*, 1986; Eugster *et al.*, 2001).

#### Simple virilizing Congenital Adrenal Hyperplasia

A small increase (1–2%) of the enzymatic activity of 21-hydroxylase, compared to SW CAH leads to the de-



velopment of SV CAH, which is responsible for the other 25% of classic CAH cases. Similarly to SW CAH, an accumulation of adrenal steroid precursors causes overproduction of androgens, which results in development of ambiguous genitalia of a various degree in girls. Aldosterone production is sufficient to prevent the onset of salt loss and adrenal crisis, so mineralocorticoids replacement therapy is not necessary (Parsa & New, 2017). Ambiguous genitalia thought to be caused by SW CAH are often finally diagnosed as SV CAH; regardless, patients are treated with both mineralocorticoids and glucocorticoids at the onset to prevent adrenal crisis. It should be noted that boys, who have not undergone screening tests, are often diagnosed with a delay of several years, after the manifestation of long-term hyperandrogenism symptoms.

### Non-classic Congenital Adrenal Hyperplasia

Non-classical (NC CAH) is one of the most common autosomal recessive genetic disorders. It occurs when the enzymatic activity of 21-hydroxylase is impaired to a lesser extent (20–50% of the normal enzyme function). Cortisol and aldosterone levels are sufficient to sustain vital functions, hence hormone replacement therapy is not needed. However, there is no inhibition of ACTH secretion levels since cortisol levels are still too low and adrenal hyperplasia and hyperandrogenism eventually occur. NC CAH was diagnosed and described first in 1957 in a 26-year-old married woman who was regularly menstruating with properly developed genitalia, but with signs of hirsutism and acne (Decourt *et al.*, 1957; Speiser, 2009). At the time of birth, children have normal genitalia, and the level of 17-OH-progesterone is often normal unless a blood sample is collected in the early morning (i.e., before 8 AM). Additionally, for reasons that are as yet unclear, 17-hydroxyprogesterone levels may not be elevated in newborns with CAH (Azziz *et al.*, 1999). A diagnosis is often made after many years, mainly accidentally, during investigations of other diseases (Kohn *et al.*, 1982; Falhammar & Nordenström, 2015). According to the original clinical definition, the first symptoms should occur after 60 months of age. In a multicenter collaborative study on 220 women with NC CAH, only 11% were diagnosed before 10 years of age, while the majority (80%) were diagnosed between 10 and 40 years of age. Symptoms in children that indicate NC CAH are premature pubic hair, precocious puberty, acne, accelerated growth (however, final height is reduced), and advanced bone age (Temeck *et al.*, 1987; Ostlere *et al.*, 1998; Dacou-Voutetakis & Dracopoulou, 1999; Kashimada *et al.*, 2008; Knowles *et al.*, 2014; Voutilainen & Jääskeläinen, 2015). In 5–10% of all cases of premature pubic hair, patients are suffering from NC CAH (Balducci *et al.*, 1994; Dacou-Voutetakis & Dracopoulou, 1999). The most common symptoms for affected adults include hirsutism (60–78%), menstrual cycle disorders (55%), acne (33%) and decreased fertility (12%) (Moran *et al.*, 2000). These signs, as the results of elevated androgen levels, are more pronounced in women. In men, the disease is often asymptomatic or only with visible acne and/or a reduction of fertility (Falhammar & Nordenström, 2015).

Treatment is guaranteed only if the patient is symptomatic and desires treatment. Small dosages of glucocorticoids are required to control symptoms of androgen excess. Sometimes, unfortunately, this is not easy to achieve and higher doses are necessary in many cases resulting in overtreatment with an increased risk of long-term side effects (Falhammar *et al.*, 2007; Falhammar *et*

*al.*, 2013). For this reason, new generations of drugs displaying extended hydrocortisone release will be preferred in CAH therapy in the future (Verma *et al.*, 2010).

### DIAGNOSIS

The diagnosis of classical CAH may be based on a careful examination of the external appearance of newborns (mainly genitals), so physicians should be encouraged in this regard. It should be remembered that the symptoms of ambiguous reproductive organs in a child does not determine unequivocally the occurrence of congenital adrenal hyperplasia. The cause of such symptoms might be exposure of the fetus in the womb to elevated androgens levels, which may occur as a result of various factors (e.g. mother's illness). Before the final diagnosis, detailed studies should be carried out to determine the real cause of the disorder (Demirci & Witchel, 2008).

In countries where CAH screening tests have been introduced, diagnosis is based on the determination of 17-hydroxyprogesterone plasma levels, a precursor for a dysfunctional enzyme 21-hydroxylase (Barnes & Atherden, 1972). 17-OHP level is elevated at birth, quickly decreases in healthy newborns, but persists in affected children (Marumudi *et al.*, 2013). The 17-OHP level is measured in filter paper blood spots obtained by heel puncture preferably between 2 and 4 days after birth, along with other screening assays. Sample collection after 5–7 days of birth reduces the benefit of screening, because the adrenal crisis associated with loss of salt may have already occurred (Van der Kamp *et al.*, 2001). However, some reports indicate that an additional second newborn screening for congenital adrenal hyperplasia can identify about 30% of formerly unrecognized CAH cases (Therrell *et al.*, 1998; Chan *et al.*, 2013).

Medical societies in countries where NBS programs have not been implemented should strongly encourage national institutions and governments to introduce this screening test to their healthcare programs. Undoubtedly, screening markedly reduces the time required for the diagnosis of infants with CAH. The main putative benefit of early diagnosis is reduced morbidity and mortality, particularly among babies with the salt-wasting form of this disease. Newborn screening for CAH has been shown to be cost-effective when its cost is compared with the lifetime tax contribution of a productive citizen; the direct cost analysis for one specimen is only \$2.10 (Pang *et al.*, 1988; Chan *et al.*, 2013).

Three assay techniques are utilized for initial screening: radio-immunoassay (USA), enzyme-linked immunosorbent assay (Japan), and time-resolved fluoro-immunoassay (Europe). These tests allow one to clearly detect SW CAH. SV CAH is less likely to be detected while NC CAH is not recognized for the most part (slight increase of 17-OHP). It should be noted that a large number of false positives (approx. 0.5%), as a result of cross-reactivity of steroid conjugates and insufficient antibody specificity, is a cause of anxiety in parents (Gurian *et al.*, 2006; Trapp *et al.*, 2011). This risk increases in premature babies, sick and stressed children with low birth weight or term newborns tested earlier than 36 h after the delivery (Lee *et al.*, 1989; Wong *et al.*, 1992). On the other hand, glucocorticoid therapy during pregnancy can result in false negative results (King *et al.*, 2001; Gate-lais *et al.*, 2004). A positive result should be confirmed by a second, more advanced analytical method such as high performance liquid chromatography tandem-mass spectrometry (LC-MS/MS) or gas chromatography mass

spectrometry (GC-MS) (Shackleton, 1986; Monostori *et al.*, 2015). Chromatographic methods allow simultaneous measurement of several analytes in the sample and determination of the precursor/product ratio (e.g. 17-OHP + deoxycortisol/cortisol). This significantly reduces the possibility of a false positive test result particularly in premature infants and neonates under stress. Despite the fact that chromatographic techniques provide more reliable results, they are still used as a second-tier test because they are more expensive, time-consuming, demand technical expertise and require specialized knowledge; currently, these factors prevent the widespread use of chromatography in the field of CAH diagnosis (Kao *et al.*, 2001; White, 2009).

The gold standard for distinguishing 21-hydroxylase deficiency from other enzyme defects is the ACTH (cosyntropin) stimulation test, performed by injecting a bolus of cosyntropin and measuring baseline and stimulated (after 60 minutes) serum levels of 17-hydroxyprogesterone and often androstenedione as well. The level of target metabolites before stimulation does not differ from the norm, however, they increase significantly afterwards indicating the final diagnosis (New *et al.*, 1983). Children showing clinical evidence of androgen excess like oily skin, premature pubic and axillary hair growth and rapid somatic growth should undergo morning baseline 17-OHP testing. 17-OHP values >200–1000 ng/dl should be followed with a stimulated cosyntropin test measuring levels of 17-OHP and androstenedione at baseline and at 60 minutes (Parsa & New, 2017).

In order to diagnose patients with NC CAH, which present nonspecific symptoms of NCAH including hirsutism, irregular menses, chronic anovulation, acne, and infertility it is necessary to subject the patient to an ACTH stimulation test, which relies on the measurement of 17-OHP and androstenedione 60 minutes before and after administration of ACTH. The level of these metabolites before stimulation does not differ from the norm, however, they may increase significantly allowing diagnosis. Due to similar clinical features, it may be difficult to distinguish NC CAH patients from women suffering from polycystic ovary syndrome. The first group tends to have higher 17-OHP and progesterone concentrations than women in the second group, which demonstrate insulin resistance, obesity, polycystic ovary morphology, and elevated LH/FSH ratios. Men with NC CAH are typically identified through family studies (Witchel, 2017).

Other diagnostic procedures in patients affected by CAH can be broadened to include genetic tests that clearly confirm the nature of disorders (especially in patients with NC CAH) and determine the degree of mutation and disease severity. In most cases, phenotypic disease severity may be predicted from genotypic findings. This correlation can provide a significant guide for short- and long-term treatment of patients. Use of genetic testing is also helpful in prenatal counseling of mothers affected by NC CAH planning to have children. It should be noted that molecular genetic diagnosis is more complicated for 21-OHD than for many other monogenic disorders due to the high variability of the genomic region. Most CYP21A2 gene mutations encoding 21-hydroxylase arise from an exchange or conversion of genetic material at the time of meiotic recombination between CYP21A2 and inactive pseudogene CYP21A1P (98% homologous with CYP21A2). Both are located on the short arm of chromosome 6 (6p21.3). Another 20% of CAH cases are caused by unequal crossing-over during meiosis, resulting in the deletion of a 30-kb gene segment. The remaining part of the affected alleles con-

sists of the spontaneous mutations not carried by either parent. To date, more than 300 CYP21A2 mutations have been discovered, and about 10 common mutations account for approximately 90% of cases (Bronstad *et al.*, 2014; Falhammar *et al.*, 2015; Choi *et al.*, 2016). Genetic analysis of 1507 families, that had at least one member affected by CAH showed frequencies of this common allelic mutations as follows: V281L (23.9%), I2G (22.9%), 30-kb deletion or genomic rearrangement/conversion fusing CYP21 with CYP21P (20.0%), I172N (8.2%), R356W (3.6%), Q318X (3.5%), P30L (2.6%), and exon 6 cluster mutation (I236N, V237E, M239K) (2.1%) (New *et al.*, 2013).

It should also be mentioned that the possibility of a prenatal diagnosis of CAH exists. Performing chorionic villus sampling (9–11 week of pregnancy) or amniocentesis (15–20) followed by genetic testing enables early detection of 21-OHD and prescribed therapy before birth. In children with increased probability of CAH (family risk), treatment should be introduced before the 9th week of gestation (dexamethasone) which effectively lowers the excessive adrenal androgens preventing the masculinization of female external genitalia. The findings of a genetic test between 9–11 weeks of gestation determine further patient management. The treatment is discontinued when the fetus is male or an unaffected female. Otherwise, the treatment is continued to term in three divided doses starting as soon as pregnancy is confirmed and no later than 9 weeks after the last menstrual period with dexamethasone at a dose of 20 mg/kg/day based on the maternal pre-pregnancy bodyweight (Carlson *et al.*, 1999; New *et al.*, 2001; Nimkarn & New, 2009). The procedures of amniocentesis and chorionic villus sampling (CVS) are risky. Studies have shown that these invasive procedures cause 0.1% (amniocentesis) and 0.2% (CVS) of the fetal loss (Evans & Wapner, 2005; Akolekar *et al.*, 2015). Complications of CVS and amniocentesis include talipes (clubfoot), hemangioma, infection, amniotic fluid leakage, or limb reduction defects (seen in CVS) (Lo *et al.*, 1998; Bauland *et al.*, 2012). Due to this risk, noninvasive techniques were introduced in 2011 that are very promising for extraction of fetal cell-free DNA from maternal blood. The biggest advantage of this test is that it can be done in the 6th week of gestation, allowing early diagnosis, before the onset of genital organogenesis which begins at approximately 9 week with avoidance of unnecessary treatments (American College of Obstetricians and Gynecologists Committee on Genetics, 2012; Devers *et al.*, 2013; Benn *et al.*, 2013). New *et al.*, have reported that target capture sequencing of a 6 Mb genomic region flanking CYP21A2 facilitated noninvasive genotype analysis of fetus with CAH using relative haplotype dosage analysis. Further research have shown that haplotype-based approach has an accuracy of 96.41% for the inferred maternal alleles and an accuracy of 97.81% for the inferred paternal alleles (New *et al.*, 2014; Ma *et al.*, 2014; Ma *et al.*, 2017).

Because the genotype can be correlated to the phenotype in CAH, genetic counseling helps to make the decision on prenatal dexamethasone treatment to avoid genital ambiguity in affected female fetuses. Furthermore, postnatal complications of adrenal insufficiency can be avoided by initiation of glucocorticoid and mineralocorticoid replacement at birth, especially in male newborn. However, based on current American College of Obstetricians and Gynecologist's guidelines which states that "Cell free fetal DNA does not replace the accuracy and diagnostic precision of prenatal diagnosis with CVS or amniocentesis, which remain an option for women,"

noninvasive prenatal diagnosis has not yet been established as a standard of care (Yau *et al.*, 2016a).

## CONCLUSIONS

CAH is a widespread autosomal recessive genetic disorder that can appear in childhood, adolescence or adulthood. Early diagnosis of SW CAH is a key factor in saving a newborn's life, and an early diagnosis of NC CAH can significantly improve the quality of life and reduce the severity of ailments. The typical nonspecific symptoms of hirsutism, oligomenorrhea, infertility, acne, and premature pubic hair often leads to improper recognition of NC CAH. The 17-OHP level measurement is used in screening tests and in the diagnosis of 21-OHD. More sensitive and selective analytical techniques such as LC-MS/MS or GC-MS allow targeted steroid hormone analysis and are used to confirm the results. Due to high variability in 17-OHP levels among the patients, the identification of mutations within CYP21A2 is important to recognize the degree of mutation and the severity of the disease. Molecular genetic analysis enables confirmation of the diagnosis, providing genetic counseling and predicting treatment progress. The therapy should be individualized, adjusted and tailored to minimize the symptoms during the achievement of normal sexual development, fertility, height and generally a better quality of life (Speiser *et al.*, 1992; Wedell *et al.*, 1994; Krone *et al.*, 2000; Witchel 2010).

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