Chapter

Congenital Adrenal Hyperplasia - The Main Effect of 21-Hydroxylase Deficiency

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Abstract

Congenital adrenal hyperplasia (CAH) consists of a group of autosomal recessive disorders resulting from enzymatic defects in steroidogenesis. More than 95% of CAH cases result from a deficiency of the 21-hydroxylase enzyme, which leads to cortisol deficiency, with or without aldosterone insufficiency, and also an excess of androgen. The clinical spectrum varies from milder symptoms to severe cases settled by the functional impairment of the corresponding pathogenic variant in the *CYP21A2* gene. The two major forms of CAH caused by 21-hydroxylase deficiency are the classical form and the non-classic, or late onset form. There are two subtypes of the classic form: salt wasting and simple virilized. Diagnosis is clinically confirmed by 17OH-progesterone measurements, although genotyping is now progressively assuming an essential role for characterising patients. Genotyping is sometimes challenging, due to the existence of the highly homologous *CYP21A1P* pseudogene. The 21-hydroxylase enzyme is encoded by the *CYP21A2* gene, where most of the pathogenic variants defects are due to meiotic recombination phenomena events between the *CYP21A2* and *CYP21A1P*. Complete gene analysis is recommended to obtain a correct diagnosis and a better understanding of the underlying mechanisms of the disease in patients with CAH, and is relevant for prognosis and for prescribing the appropriate type of genetic counselling.

Keywords: CAH, cortisol, aldosterone, androgens, 21-hydroxylase, *CYP21A2*

1. Introduction

Congenital adrenal hyperplasia (CAH) consists of a group of autosomal recessive disorders caused by a defective activity of one of the enzymes involved in the steroidogenic pathway on the zona fasciculata of adrenal cortex, leading to an impairment in cortisol synthesis by the adrenal gland [1–5]. From the several enzymes involved in the adrenal steroids pathway, the 21-hydroxylase (21-OH) enzyme is responsible for the majority of CAH cases [5, 6]. Less frequently, other rare deficits in other enzymes may occur, namely: 11β-hydroxylase (11β-OH), 17α-hydroxylase (17α-OH), 3β-hydroxisteroid dehydrogenase (3β-HSD), and StaR protein cholesterol side chain and cholesterol 20–22-desmolase. 21-OH, which is an enzyme belonging to

the cytochrome P450 enzyme group, is responsible for the conversion of progesterone into deoxycorticosterone and for the conversion of 17-hydroxyprogesterone (17-OHP) into 11-deoxycortisol [7, 8]. The impairment of this enzyme leads to the accumulation of precursor substrates for 21-hydroxylation, particularly 17-hydroxyprogesterone (17-OHP), which in turn causes elevated levels of androstenedione, testosterone, dihydrotestosterone, and other peripherally estrogens [4, 7–9]. Deficits in cortisol or aldosterone and the consequent excess of androgens caused by the accumulation of precursors may be responsible for distinct levels of enzyme activity, leading to different forms of the disease, ranging from severe, moderate, to mild. This monogenic autosomal disorder is transmitted in a recessive pattern, requiring two mutated alleles for the disease to occur [8]. The clinical manifestations are distinct and, in general, correspond to the enzymatic activity of the less-affected allele [6, 10, 11]. Approximately 95% of CAH cases are the result of an impairment of the 21-hydroxylase enzyme, which is one of the several enzymes involved in the androgens pathway. Pathogenic variants of the encoding *CYP21A2* gene may lead to a partial or complete loss of function and an impairment of cortisol and aldosterone synthesis. In turn, the depletion of cortisol concentration may inhibit the negative feedback loop, leading to a compensatory release of the corticotrophin release hormone (CRH) and the adrenocorticotropic hormone (ACTH) by the hypothalamus and the pituitary gland, respectively, and also an adrenal cortex hyperplasia.

The phenotypic spectrum of CAH is widely variable, and the major clinical manifestations of adrenal insufficiency caused by aldosterone and cortisol deficits and androgen excess constitute the main clinical concern, with morbidity and mortality becoming less common. Therapy with hormone replacement constitutes a considerable benefit for patients since early diagnosis, although precise and individualised treatment is required for better outcomes.

Genetic counselling is essential, especially because this disease affects many individuals of reproductive age [1–3], and for the elucidation of the consequences and complications for offspring. Molecular analysis of the gene responsible for 21-OH deficiency is a necessary tool for the confirmation of clinical diagnosis of the affected individual, which can also provide added valuable information for relatives or offspring at risk.

2. Clinical manifestations of CAH due to 21-OH deficiency

Based on distinct phenotypes, the disease was classified into two forms: the classic form, subdivided into the salt-wasting (SW) and simple virilising (SV) forms, and the non-classical or late-onset form (**Table 1**) [12]. In both forms, decreased cortisol synthesis causes increased secretion of ACTH, which in turn stimulates the adrenal gland to produce cortisol precursors, including androgens and their intermediates (DHEAS, androstenedione and testosterone). In this way, hyperplasia of the adrenal cortex is caused, as mentioned above [13].

2.1 Salt-wasting (SW) form

The SW form of CAH is the most severe form of this disease, accounting for 75% of cases of the classic form of the disease [13]. This form is characterised by a complete absence of enzymatic activity, with consequent cortisol and aldosterone deficiency [14]. SW form patients present alterations in the body electrolyte balance

	Classic		Non- classic
	Salt Wasting	**Simple Virilizing**	
Age at diagnosis	NB till 6 months	Fem: NB till 2y; Male; 2 to 4y	Infancy till young adulthood
Genitalia	Fem: ambiguous Male: normal	Fem: ambiguous Male: normal	Fem: virilized Male: normal
Incidence	1:20000	1: 60000	1: 10000
Hormones			
Aldosterone	↓	N	N
Renin (PRA)	↑	N or ↑	N
Cortisol	↓	↓	N
18 OHP	> 50 ng/mL	25 to 50 ng/mL	5 to 25 ng/mL (after ACTH stimulation test)
Testosterone	↑	↑	Variable to ↑
Growth	-2 to -3SD	-1 to –2 SD	Probably N
21-hydroxylase activity (%)	0	<1–2	20 to 60
Typical pathogenic variants (*CYP21A2*)	Gene deletions and large conversions – 8 bp del; E6 cluster; p.Gln319Ter (Q318X); p.Arg357Trp (R356W)	p.Ile173Asn (I172N)	p.Pro31Leu (P30L); p.Val282Leu (V281L); p.Pro454Ser (P453S)

NB - Newborn; Fem – Female; PRA - Plasma Renin Activity.

Table 1.
Clinical characteristics, hormonal levels and genotype–phenotype correlation for the most common pathogenic variants, according to the percentage of enzyme activity.

due to the deficient production of mineralocorticoids. Appropriate therapy should be administered in a timely manner, as this form can range from severe forms to more discrete cases, with increased plasma renin activity [15]. In all cases, the decreased production of cortisol and aldosterone poses a threat to survival, with acute adrenal insufficiency occurring between the 1st and 3rd weeks of life [16]. Approximately 50% of salt wasting crisis occur between the age of 6 to 14 years old [15]. In the SV forms, external genitalia may also be affected, caused by a deficiency in cortisol and the consequent increase in adrenal androgens [9]. Affected males typically presented severe electrolyte imbalances within the first 2 weeks of life, however, in general, they do not show signs of excess androgen production, with only hyperpigmentation of the genital area occurring (**Table 1**) [17]. The excess of androgens produced during pregnancy in individuals with 21-OH cause the virilization of female foetuses, resulting in pseudohermaphroditism [4]. Prior to the onset of newborn screening programmess, affected females were more rapidly identified, on account of the simultaneous presence of genital ambiguity.

2.2 Simple Virilizing (SV) form

In the SV form of CAH there is a partial enzymatic deficit, which is manifested by a decrease in cortisol [18]. SV CAH patients present hypocortisolism and simple

Classic Salt-Wasting—severe form (0% enzymatic activity)
Failure to thrive
Cortisol deficiency
Mineralocorticoid deficiency
Hyponatremia
Hyperkalemia
High Plasma Renin Activity (or renin)
Hypovolemic shock
Excess androgen production, early in life
Virilization of external genitalia in females
Classic Simple Virilizing—intermediate severity form (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—mild form (20–50% enzymatic activity)
Between asymptomatic or with signs of androgen excess appearing later in life (acne; hirsutism; menstrual irregularities; anovulation; infertility)

Table 2.
Phenotypes of 21-hydroxylase deficiency.

virilisation. Furthermore, in the perinatal period, newborn female foetuses may present ambiguous genitalia without overt salt loss in affected females (**Table 2**) [19]. Virilization can be classified according to the Prader Stages scale, which ranges from 1 to 5, with Stage 5 being the most severe. Despite these external features, the female internal organs are not affected [4]. Male children may also present signs of early virilization, due to excess androgens, however, the undervaluation of signs of early virilization can delay the diagnosis of this disease in male individuals [4, 16].

2.3 Non-classical (NC) form

In the NC form of CAH due to 21-OH deficiency, the enzymatic impairment is partial and usually there is no cortisol deficiency [20]. This form is less severe and of late onset and manifests itself by signs of precocious puberty and gynaecological anomalies, which may occur either in late childhood or in early adulthood [14] (**Table 2**). Some affected women have no symptoms at all, and a considerable high number of men remain asymptomatic and only have a genetic diagnosis during segregation family analysis [20].

3. Prevalence in different ethnic populations

The overall incidence of classical CAH due 21-OH deficiency is 1:10,000–15,000 births, with a carriers' frequency of approximately 1:60–100 [3, 4, 10, 21]. Heterozygosity for 21-OH deficiency in the classical form is estimated to be 1:60 in non-Jewish Caucasians and 1:3 in Ashkenazy Jews [7, 8, 22]. The incidence is much lower in Afro-Americans, ranging from 1:25,000 to 1:42,000 in different studies [10, 11]. The most common form of CAH is the NC form, or the late onset form,

which affects between 0.1% and 1% of the overall population. The NC-CAH form presents non-specific features, oligomenorrhea, hirsutism and infertility, which causes an ascertainment bias which in turn leads to a diagnosis of a higher number of affected females. Affected males are mostly identified during family screening studies, since androgen excess manifestations are not commonly recognised. NC-CAH patients do not often oversecrete CRH and ACTH, although some patients might demonstrate an increase in glucocorticoids response to ACTH stimulation, reflecting a subtle adrenal hyperplasia [9, 14]. The increase of the secretion of androgens by adrenal glands without an overstimulation of ACTH can be justified by the alteration in kinetic enzymatic activity of CYP21A2 [22]. In addition, an increased level of androgens in NC-CAH may be the result of the peripheral conversion of precursors, or can be due to ovarian hypersecretion, as NC-CAH women frequently have polycystic ovaries.

Among hirsute women, the prevalence of CAH attains between 1% and 10% [17, 22, 23]. A clinical study based on ACTH-stimulated 17-OHP concentrations reported the incidence to be highest among Ashkenazi Jewish populations [24]. In addition, Ashkenazi Jews are reported to have the pathogenic variant p.Val282Leu with a high allelic frequency of approximately 63%, which is mostly associated with non-classic CAH. On the contrary, p.Val282Leu was not detected in Yupik-speaking, Eskimos of Western Alaska, Native Americans, East Indians and Asians. Interestingly, the Yupik Eskimos, represent an isolated geographic population and the splice site intronic variation c.293-13A/C > G in intron 2 is found to be represent a founder effect for this population [25].

Several studies regarding the genotyping of 21-OH deficiency have been presented over recent years. A large study conducted on the French CAH population showed a frequency of the most common pathogenic variants, for the classic form, of 30% of c.293-13A/C > G in intron 2, 25% for large rearrangements, 17% for p.Ile173Asn (I172N) in exon 4, and 7% of p.Gln319* (Q318X) in exon 8. For the NC form, the same study found a frequency of 55% for p.Val282Leu (V281L), 9% for c.293-13A/C > G, 8% for large rearrangements, 4% for p.Ile173Asn, and 3% for p.Gln319* [25]. In Brazil, three novel pathogenic variants were described, namely: an insertion of adenine 1003∧1004 insA in exon 4, a transition C > T in codon 408 (p.Arg408Cys), and a transition A > G in intron 2, IVS2 − 2A > G, which it is suggested is due a founder effect, as it had previously been found in the G424S pathogenic variant in the same population [26–28].

In Finland, multiple pathogenic variants have been analysed and found to have an independent founder effect, with each one being associated with a distinct haplotype, where some are identical to other European populations. This is probably due to remote immigration phenomena originating from the Scandinavian or the Baltic countries, as well as other specificities from that population, albeit with a more recent origin.

In Tunisia, the pathogenic variant p.Gln319* (Q318X) was found to be the most prevalent in that population (35.8%) [29].

A study of the population of Iran showed the contrary, demonstrating that the common deletion of 8 nt in exon 3 is the most frequent pathogenic variant of the *CYP21A2* gene (10%), followed by the c.293-13A/C > G, p.Ile173Asn, and the promotor deletion in the gene. Unlike other populations, p.Arg357Trp (R356W) in exon 8, was not found in Iran [30].

Gene rearrangements, such as the large 3 kb deletion, are frequent among the Anglo-Saxon population (28%). Furthermore, the frameshift pathogenic variant

p.Gln319* in exon 8 of *CYP21A2* gene was found in 16% of East Indians and among Croatians, the missense p.Arg356Trp variant is one of the most frequent pathogenic variants (14%) [29].

The pathogenic variant in exon 7, p.Val282Leu is often found in NC-CAH forms and represented 86% of all alleles studied [31, 32]. *De novo* rearrangements and deletions/conversions of *CYP21A2* gene represent 1% of the affected alleles. Several forms of the chimeric *CYP21A2/CYP21A1P* gene have been described in different populations [33, 34].

4. Differential diagnosis

4.1 Biochemical

The biochemical diagnosis of CAH is made using hormone assays. 17-hydroxyprogesterone (17-OHP) is the gold standard screening marker for this disease [16], however other markers can also be used, such as DHEA, cortisol, testosterone, aldosterone, and renin (or plasma renin activity). Individuals with the classic form of CAH present a marked increase in serum 17-OHP, with values greater than 100 ng/mL, without an increase in 11-deoxycortisol and with a less marked increase in basal dehydroepiandrosterone and testosterone [14]. In patients presenting a SW form, there is also an increase in plasma renin activity, and manifestations such as hyponatremia and hyperkalemia [19]. CAH carriers frequently present normal basal 17-OHP levels and diagnostic strategy involves stimulation with synthetic ACTH with subsequent hormone measurement after 60 minutes. A concentration of 17-OHP greater than 15 ng/mL is indicative of an impairment in 21-OH activity, however many carriers' individuals have only slightly increase hormone concentration after stimulation [7].

4.2 Molecular genetics

Molecular genetic testing of the *CYP21A2* gene is considered to be essential for the establishment of correlations between genotype and phenotype, as well as the confirmation of clinical and biochemical diagnosis, measuring the status of severity of the patients, distinguishing between severe and milder cases, and, very importantly, making the decision regarding the appropriate genetic counselling for at risk family members and their couples. The molecular diagnosis of CAH is based on the identification of point pathogenic variants and small deletions or insertions, which are mostly transferred from the pseudogene to the active gene [35]. The molecular analysis of the *CYP21A2* gene involves amplification with specific primers just for the *CYP21A2* gene, in order to ensure the targeting of the functional gene, instead of the inactive pseudogene [12, 36]. As *CYP21A2* is constituted by 10 exons and intronic regions of reduced size, it is possible to amplify the entire gene, enabling the possibility to analyse all exons and their intron-exon flanking regions. The most common strategy employed to achieve this specificity is to select a primer that is located at the site with a specific and unique gene sequence [8]. The presence of sequence variations can cause alterations in the sequence reading and interpretation and different forward or reverse primers have to be used to achieve a complete analysis, emphasising the complexity of CAH genomics.

4.2.1 Structure of the CYP21A2 gene

21-OH CAH deficiency is mainly caused by pathogenic variants in the *CYP21A2* gene, which is the gene that encodes the 21-OH enzyme [21]. *CYP21A2* is located in the Major Histocompatibility Complex (MHC) class III region, on the short arm of chromosome 6 (6p21.3), which is a region that displays a complex organisation of genes with high variability in gene size and copy numbers [37, 38] (**Figure 1**). Approximately 30 kb apart from the *CYP21A2* gene is a pseudogene – *CYP21A1P*. Both the *CYP21A2* gene and the *CYP21A1P* pseudogene share 98% homology between exons and 96% in introns, and both are constituted by 10 exons, spanning 3.1 kb [21, 39, 40].

Throughout evolution, the inactive *CYP21A1P* pseudogene has acquired multiple pathogenic variants, as well as small insertions or deletions and point pathogenic variants that prevent the synthesis of a normal functional protein. The high degree of homology between the gene and the pseudogene enables the occurrence of unequal pairing during meiosis between homologous chromosomes and sister chromatids [41]. Approximately 95% of pathogenic variants in the *CYP21A2* gene result from meiotic recombination events between the gene and the pseudogene [8], with approximately 75% of pathogenic variants resulting from conversion events of large sequences from the pseudogene to the gene or punctual conversion of single alterations (microconversion). Approximately 20% of the pathogenic variants found in this gene are the result of an unequal crossing-over during meiosis, leading to the occurrence of duplications and/or deletions. Large gene conversions and deletions may result in the *CYP21A1P/CYP21A2* chimeric genes, comprising around 20% of the pathogenic variants. The remaining 5% correspond to new pathogenic variants which are not related with recombination events and *de novo* pathogenic variants account for approximately 1–2% of cases [41]. Three other genes are placed adjacent and alternately to *CYP21A2* and *CYP21A1P,* namely the *RP1*, *C4*, *TNXB* genes, as well as two pseudogene – *RP2* and *TNXA* [42]. This configuration is designated by the RCCX module (RP-C4-CYP21-TNX) and it extends to approximately 30 kb, where the orientation from telomere to centromere is: *RP1-C4A-CYP21A1P-TNXA-RP2-C4B-CYP21A2-TNXB* (**Figure 1**) [42]. The *C4B* and *C4A* genes encode for the fourth component of serum complement [41] and the *TNXB* gene encodes for an extracellular matrix protein termed tenascin-X23. In turn, *RP1* encodes a DNA helicase nuclear protein [42]. The RCCX module organisation is usually bimodular, one with the active *CYP21A2* gene, and the other with the inactive pseudogene *CYP21A1P*, which is present in around 69% of the Caucasian population. Monomodular presentation is present in around 17% of the population, whereas trimodular haplotype occurs in almost 14% of cases [33, 42].

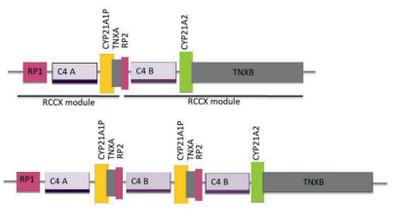

Figure 1.
RCCX modules: Bimodular haplotype (upper) and a three modular haplotype with two modules harbouring the CYP21A1P pseudogene and one the CYP21A2 gene.

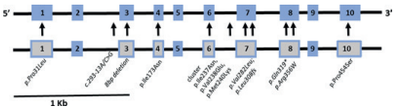

Figure 2.
CYP21A2 and CYP21A1P interconversion events responsible for the conversion of the most common pathogenic variants from the pseudogene to CYP21A2 gene.

The monomodular organisation is characterised by a deletion of 26 or 32 kb, depending on whether the *C4B* is a short or a long gene, without or with the HERV-K sequence. Several deletions have been described, giving rise to different chimera *CYP21A1P*/*CYP21A2* genes [34]. The most common deletion involves the 3′ end of *CYP21A1P*, the entire *C4B* gene, and the 5′ end of the *CYP21A2* gene, which produces a single non-functional chimeric gene with 5′ and 3′ ends of *CYP21A1P* and *CYP21A2*, respectively (**Figure 2**).

The trimodular haplotype may exhibit two copies of the *CYP21A1P* gene and one copy of the *CYP21A2* gene, or two copies of the *CYP21A2* gene and one copy of the *CYP21A1P* pseudogene, the latter being described in patients who present both the p.Gln319* pathogenic variant and chimeric *CYP21A1P/CYP21A2* genes together [35, 36, 43]. The existence of different haplotype with simultaneous distinct copy number variation with a large number of sequence variants presents a challenge for the characterisation of *CYP21A2* alleles. Pathogenic variants have been described along the entire gene, in the coding region, as well as in the intron-exon boundaries and beyond, and also in the 5′ and 3′ untranslated region, which demands a careful full gene analysis.

CAH can also be caused by uniparental isodisomy events, although this phenomenon is less frequent [44]. To date, more than 1000 genetic variants have been reported, but only a quarter of them affect human health, with most of these resulting in classic CAH cases [45]. Several sequence variants have been described in the non-translated regions of the gene and even in the promoter sequence, many of them associated with NC-CAH [46]. 153 of the 230 genetic variants were demonstrated to be missense pathogenic variants [45] and these can result in all forms of the disease, whereas nonsense and frameshift pathogenic variants are prone to result in classical forms.

4.2.2 CYP21A2 gene pathogenic variants

The use of the screening approach for the detection of the ten most common pathogenic variants was the most-used practice among molecular genetic laboratories for several years, providing a molecular confirmation for the majority of cases. The most common pathogenic variants evaluated in classic and non-classic forms were responsible for almost 80% of the cases of CAH caused by 21-OH deficiency:

- **p.Pro31Leu (P30L or Pro30Leu):** This pathogenic variant has an enzyme activity of around 20–60% in cultured cells [45], however this enzyme activity is quickly lost when cells are lysed, pointing to a relative instability of this enzyme. p.Pro31Leu is associated with the mild form of disease and is found in one sixth of NC-alleles, although it can be present with a higher prevalence in specific ethnicities, such as the Japanese [47]. NC-CAH patients who carry this pathogenic variant may present more serious manifestations of androgen excess

than those patients who carry the most common pathogenic variants associated with NC forms, with p.Val282Leu being associated as having a dominant effect on patients [48].

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- **c.293-13A/C > G (IVS2–13A/C > G: A or C-G Pathogenic Variant in Intron 2):** This pathogenic variant is defined by a substitution of an adenine or cytosine nucleotide to a guanine, at 13 bp before the end of intron 2. This alteration is responsible for the alternative splicing of intron 2, which is characterised by a retention of 19 nucleotides, as well as usually the disruption of mRNA and a shift in the translation reading frame with a generation of a premature stop codon [49].

- **p.Gly111Valfs∗21 (Del 8 bp or G11018nt):** This pathogenic variant is characterised by a deletion of 8 nucleotide in exon 3, resulting in a frameshift variant with nil enzyme activity. Although this pathogenic variant is mainly associated with SW forms of CAH [50, 51].

- **p.Ile173Asn (I172N or Ile172Asn):** This pathogenic variant is marked by a nucleotide missense substitution that causes the shift of isoleucine by asparagine aminoacid. This alteration is associated with approximately 1% of normal activity and is mainly found in the SV forms of CAH, although it has also been described as being present in SW forms of CAH [51].

- **p.Ile237Asn, p.Val238Glu, p.Met239Lys (Cluster in Exon 6: I236N/V237E/M238K or Ile-Val-Met236–237-238-Asn-Glu-Lys):** The cluster of three missense pathogenic variants in the G helix supresses enzymatic activity, probably through interference with substrate binding [49].

- **p.Val282Leu (V281L or Val281Leu):** The p.Val282Leu occurs in the majority of patients with NC 21-hydroxylase deficiency form who carry the HLA haplotype B14; DR1, this association being consistent with a founder effect [52]. Overall, approximately 70% of all NC alleles carry the p.Val282Leu pathogenic variant [53]. This pathogenic variant results in an enzyme with 40–60% of normal activity when 17-OHP is the substrate, but with only 20% of normal activity for progesterone [54, 55].

- **p.Leu307PhefsTer5 (F306 + T or L306insT):** This nonsense pathogenic variant occurs due to an insertion of 1 thymine nucleotide in exon 7. This variant is often described as a conversion of exons 7 and 8 derived from pseudogene, particularly in the cases of Dutch patients [54].

- **p.Gln319* (Q318X or Gln318-Term):** This pathogenic variant is caused by a nucleotide substitution that shifts the CAG codon to a TAG codon, resulting in a premature stop of protein translation which in turn leads to a non-functional protein [56].

- **p.Arg357Trp (R356W or Arg356Trp):** This missense pathogenic variant is mostly associated with classic CAH forms and the enzyme activity is nil. This alteration is located in exon 8, in a region encoding the K helix of the enzyme, suggesting that interaction with cytochrome P450 reductase (POR) may be affected, although experimental studies are lacking [55].

- **p.Pro454Ser (P453S or Pro453Ser):** This missense pathogenic variant in exon 10 of the gene was initially described as being absent in the pseudogene and is associated with a 50–68% decrease of enzyme activity [57]. It occurs differently in several populations, which suggests that *CYP21A1P* may carry p.Pro454Ser as an occasional polymorphism and that this pathogenic variant is transferred to *CYP21A2* in the same way as the other pathogenic variants frequently cause 21-hydroxylase deficiency [32].

Many other different pathogenic variants have been described, some of which are from wide families, while many others are private from particular families. Most of the pathogenic variants associated with CAH due to 21-OH deficiency are missense pathogenic variants. Their study requires functional studies to be correlated with a clinical form, although nonsense, frameshift, and rearrangement pathogenic variants are promptly deduced as being severe. Genotype–phenotype correlation data demonstrated that some variants were associated with the severe SW form, some with the SV form, and others with the NC form. It was also observed that some of these pathogenic variants confer different phenotypes, depending on whether they are isolated or are associated with another pathogenic variant, resulting in a synergistic effect [57]. Some sequence variants do not affect the protein production and are considered to be benign variants [57]. Some of these sequence variants are also present in *CYP21A1P* gene and can also be transferred by conversion events, although they do not affect the enzyme activity and have no effect on the clinical phenotype.

4.2.3 Targeted screening of the ten most common pathogenic variants

For several years, the study of *CYP21A2* gene was based on the evaluation of the ten most frequent pathogenic variants. Several different molecular methods and strategies have been described to cover just a limited number of pathogenic variants. Polymerase chain reaction (PCR), specific primer sequence (SSCP), restriction fragment length polymorphism (RFLP), and direct DNA Sanger sequencing were among the methods available for screening. The initial screening for the most frequent pathogenic variants included the search for large deletions or conversions events involving the promoter region of the *CYP21A2* gene (del/conv of promoter region), as well as pointing to the following pathogenic variants along the *CYP21A2* gene: exon 1, p.Pro31Leu; intron 2, which is a splicing pathogenic variant c.293–13A/C > G; exon 3, which is a 8 bp deletion (p.Gly111Valfs21); exon 4, p.Ile173Asn; exon 6, which is a cluster of three point pathogenic variants (p.Ile237Asn, p.Val238Glu, p.Met240Lys); exon 7, p.Val282Leu, and a thymine insertion (p.Leu307PhefsX5); exon 8, p.Gln319*, and p.Arg357Trp; and p.Pro454Ser in exon 10 [8].

The study of the large deletions/conversions involving the promoter region of the *CYP21A2* gene was achieved by a PCR-based experiment, followed by digestion with the *Taq I* restriction enzyme [8]. This method covers a large number of pathogenic variants found in CAH patients who are affected by 21-OH deficiency owing to alterations in *CYP21A2*, as the screening strategy fails to identify other pathogenic variants outside the target regions.

4.2.4 Complete gene analysis by sanger sequencing

Whole gene sequencing is nowadays the gold standard for genotyping the *CYP21A2* gene. The full gene analysis of *CYP21A2* by Sanger sequencing allows the detection of the above-described pathogenic variants, as well as the identification of new sequence variations. Specific gene amplification by PCR has dramatically improved the sensitivity of the various techniques available to detect *CYP21A2* pathogenic variants. However, the existence of a highly homologous *CYP21A1P* pseudogene which carries most of the pathogenic variants of interest has revealed the major difficulty in finding a strategy for the selective amplification of the *CYP21A2* gene, owing to the lack of specific primers. Indeed, locus-specific mutation primers and PCR conditions were revised and now enable the gene-specific amplification of *CYP21A2.* Gene sequencing usually analyses both the coding regions and the flanking intron-exon regions of the gene. *CYP21A2* whole genomic sequence may be performed by selecting the *CYP21A2* functional gene and amplification by PCR into two partially-overlapping fragments, with 1517 and 2214 base-pairs (bp), avoiding the co-amplification of the pseudogene *CYP21A1P* [58]. After the selective amplification of the targeted genes and their subsequent purification, the PCR products are sequenced with internal primers that cover the entire *CYP21A2* gene [59].

4.2.5 Large rearrangements

The molecular approach for the identification of large rearrangements is distinct from that used for the analysis of sequence pathogenic variants.

A diversity of methods is available for the detection of large rearrangements in the exonic and/or intronic regions of *CYP21A2* gene and also in the promoter and contiguous regions, such as for neighbours' genes (e.g., *C4B* gene). The southern blot technique has been used as a gold standard for many years in the study of large deletions in the *CYP21A2* gene, however this technique has now been substituted by MLPA (Multiplex Ligation-dependent Probe Amplification). The MLPA experimental technique enables the detection of large rearrangements, where several gene sequences can be simultaneously analysed. The detection of known deletions and duplications in the *CYP21A2* gene and in the *CYP21A1P* pseudogene is currently achieved by using this method, which analyses the variations in the copy number of several exons of the gene, and also identifies sequence variations, as well as contiguous genes and control sequences.

This semi-quantitative method is a simple, reliable, and highly sensitive approach for detecting copy number variations in genomic sequences based on the hybridisation of probes to locus-specific targets and the amplification of the effectively-matched targets. The products of amplification are then separated by capillary electrophoresis and comparison is made between a peak-area pattern obtained from reference and the tested samples enable the determination of which probes/locus have aberrant copy numbers [60, 61].

Another eligible method for detecting copy number variations (CNV) is the quantitative real time PCR, which evaluates the progression of a PCR reaction in real time and simultaneously quantifies the amount of product amplified. This method is based on the detection of the fluorescence produced by a reporter molecule, which increases as the reaction proceeds, and is characterised by a quencher of fluorescence at the opposite end. The proximity of the reporter to the quencher prevents the detection of its fluorescence. During a real-time reaction, in each cycle the probe hybridises and elongates and the reporter that produces fluorescence that is captured is then released which is subsequently measured for each cycle. The increment of fluorescence is proportional to the increase of product, which is quantified to evaluate gene copy number variations through the co-amplification of a control gene [38].

5. CAH genotype: Phenotype correlation

Several studies have reported high levels of concordance between genotype and phenotype in patients with CAH, however genotype–phenotype correlation is weaker in less severe forms of the disease [42]. As described above, these pathogenic variants are almost entirely correlated with the clinical severity and are well characterised and are associated with distinct clinical forms of the disease. Accordingly, both the classic and non-classical forms of the disease are associated with different genotypes [21]. *In vitro* studies of the expression of the CYP21A2 protein enable the determination of the rate of enzymatic activity that is associated with each pathogenic variant. The majority of CAH patients due to 21-OH deficiency are characterised by compound heterozygotes and there are severe phenotypes of classic forms, which must present two severe pathogenic variants and have no mild pathogenic variants [10, 16, 21]. Alongside there is a genotype of a NC form which has either moderate pathogenic variants in both alleles or one severe mutation and one moderate mutation [38]. The mild pathogenic variant allows for the synthesis of 21-hydroxylase enzyme up to 50% of normal activity, although the severe pathogenic variant does not contribute to any synthesis (**Table 3**).

Variant		**% enzyme active**	**Phenotype**
Severe	Large gene deletions and conversions	0%	Classic SW-CAH
	8 bp del		
	E6 cluster		
	p.Gln319Ter (Q318X)		
	p.Arg357Trp (R356W)		
Intermediate	p.Ile173Asn (I172N)	<1–2%	Classic SV-CAH
Mild	p.Pro31Leu (P30L)	20–60%	Non-Classic CAH
	p.Val282Leu (V281L)		
	p.Pro454Ser (P453S)		

SW: Salt-wasting; SV: Simple virilizing.

Table 3.
Genotype–phenotype correlation for the most common pathogenic variants, according to the percentage of enzyme activity.

Patients with the classic form of CAH usually present severe pathogenic variants in both alleles, presenting nil or less than 5% of 21-OH enzymatic activity [23]. The well-known intronic splice site pathogenic variant in intron 2, which consists of the substitution of an A/C nucleotides by a guanine, constitutes a serious alteration, since it causes the occurrence of alternative splicing and an abnormal protein [21]. Many patients present this pathogenic variant in homozygosity with low levels, or even a complete absence of aldosterone, which is typical of the SV form. In turn, patients with the SV form often present only 1–2% of enzyme normal activity, which, despite being low, is sufficient for the synthesis of aldosterone (**Table 3**) [38].

The clinical spectrum of CAH varies widely between the different forms of the disease, however clinical presentation can be particularly diverse in less severe forms. The phenotype is usually predicted by the less severely-affected allele, although the existence of a more severe pathogenic variant in a second allele, rather an intermediate pathogenic variant, can develop into a more severe phenotype [25]. Compound heterozygous for a mild and a severe pathogenic variant has been reported in NC-CAH patients, showing higher basal and stimulated levels of 17-OHP and hyperandrogenic signs when compared with both mild pathogenic variants [6, 62]. Furthermore, CAH carriers are also characterised by higher than normal 17-OHP levels after ACTH stimulation, albeit not as high as the levels registered in CAH patients.

Although there is a relatively high concordance between genotype and phenotype, there are several examples of variability, particularly for moderately affected patients [63]. Both the pathogenic variants designated as c.293-13A/C > G (IVS2–13) and p.Ile173Asn (I172N) result in variable degrees of 21-hydroxylase activity. Those patients who would generally be expected to be SV cases can sometimes be SW, while others can have a NC-CAH-like phenotype [52, 58]. Another example is the p.Pro31Leu (P30L) in exon 1 of the *CYP21A2* gene, which is usually associated with the NC-CAH form, but is also often present in the SV-CAH phenotypes [63]. The lack of correlation between genotype and phenotype is probably the result of an incomplete gene sequencing, particularly in cases where the whole gene sequencing was not performed. The impairment of 21-OH deficiency is responsible for three major types of the disease, representing a continuum spectrum of clinical severity [22].

Familial segregation studies are recommended for a correct analysis of the parental segregation of the pathogenic variants in order to obtain a better understanding of the disease. Pathogenic variants may be placed in opposite alleles (*trans* configuration) or in the same allele (*cis* configuration). In the case of the latter, the individual is not clinically affected. A possible pitfall of PCR-based strategies is that the interpretation of the diagnosis can be complicated, due to the failure to amplify one haplotype, which results in misdiagnosis. The evaluation of flanking microsatellite markers in all family members can minimise this issue and the establishment of a familial segregation pattern is recommended for individuals with homozygous or hemizygous genotypes (i.e., those who have a pathogenic variant in one chromosome and a deletion in the other one).

6. Prenatal diagnosis and treatment

The objective of prenatal diagnosis and treatment of CAH is to avoid genital ambiguity in females and prevent precocious puberty in males, as well as to minimise the psychological and physical aspects related with manifestations of an excess of

androgen [39]. Prenatal diagnosis is also of major importance for families at risk of having children with the classic form of CAH, due to the determination of previous carrier status, or due to previous CAH-affected children [39]. Different methods of prenatal detection have been used for several years, such as measurements of 17-OHP in amniotic fluid, or chorionic villus sampling and the molecular genetic analysis of pathogenic variants through DNA that is extracted from chorionic villus cells or amniocytes [27]. These procedures can be informative for fetal gender and carrier/affected status and they can be carried out late on during the first trimester through to the second trimester by the 10th week of gestation for chorionic villus sampling, or around the 16th–17th week in the cases of amniocentesis [39]. The prenatal treatment of this disease involves the administration of dexamethasone to the pregnant woman, starting from 6th to the 7th week of gestation, in order to minimise the effects of genital virilization in affected female foetuses [64]. Dexamethasone is a very powerful glucocorticoid with weak mineralocorticoid activity and is still considered to be a controversial therapy in the prenatal context, as the effectiveness of this treatment is approximately 75%, and the adverse effects for both pregnant woman and the fetus in the long term are not yet fully known [21, 64]. Postnatal treatment can be administered chronically or acutely during a salt wasting crisis and in both cases high amounts of sodium chloride (NaCl) need to be infused, as well as hydrocortisone in an acute case [15]. A careful assessment of the efficiency and suitability of glucocorticoid therapy is carried out by the monitorisation of the urinary excretion of 17-ketosteroids, pregnanetriol, and also plasma levels of 17-OHP [15].

7. New clues at genotyping

A promising aspect that can result from genotyping is prevention of CAH. *CYP21A2* genotyping is strongly recommended in couples with a personal or familial history of CAH who are trying to conceive, in order to obtain a genetic diagnosis of clinically-confirmed cases and prescribe suitable genetic counselling. Although there is a strong correlation between genotype and phenotype, occasionally the genotype interpretation is rather difficult to make due to genetic complexity. In the case of a couple where one of the partners has CAH, the risk of giving birth to an offspring who is affected is dependent on the partner's genotype. If a CAH-homozygous patient with p.Val282Leu has a partner who is a carrier of a CAH pathogenic variant, then the risk of having a child with NC-CAH is 50%. The probability of an individual from the general population being a carrier of a severe pathogenic variant is 1:60 (approximately 1.7%) [22, 24] and the probability of a NC-CAH patient being a carrier of a mild pathogenic variant is approximately 60%, which occurs in about two thirds of cases. In such a case, the risk of having a child with a classical form of CAH is expected to be just 1:600 [62]. Nevertheless, it is understood that the real frequency would be higher, due to the higher carrier frequency in particular ethnic groups.

Therefore, the genotyping of both parents should be a mandatory procedure as part of a prenatal study protocol for couples in which one has CAH [65]. Prenatal diagnosis using nucleic acids circulating in maternal blood (cff-DNA: cell free-fetal DNA) enables gender determination during early pregnancy. By being able to identify the *SRY* gene sequences in maternal circulation, male foetuses do not need to continue to be exposed to prenatal treatment with glucocorticoids, contrary to female foetuses, in which the prevention of genital ambiguity is a concern. Besides determining

gender, an analysis of the *CYP21A2* gene by using the most recent massive sequencing technologies for fetal fraction present in maternal blood should be used, however this procedure is very complex and it is subject to a relatively large rate of false positive and negative results.

Traditional invasive methods still provide more confident results, albeit these are usually only performed rather late during pregnancy, which thus compromises decision making regarding the administration of dexamethasone-suppressive treatment during pregnancy when genetic severe forms are identified [66]. Furthermore, Preimplantation Genetic Testing (PGT) in conjunction with *in vitro* fertilisation (IVF) techniques is already a reality, which is designed to avoid the transmission of various diseases. Embryos are genetically analysed by using molecular genotyping and haplotyping techniques and only genetically transferable embryos (i.e., without the disease) are transferred to the woman's uterus, which accordingly stops the transmission of a disease within a family [67].

8. Conclusions

As congenital adrenal hyperplasia is a common autosomal recessive disorder, its incidence in a carrier is higher, particularly in specific populations. The typical symptoms are manifested during the neonatal period, childhood, adolescence, and adulthood, and may lead to an ascertainment bias in favour of the identification of affected females. This is particularly the cases in NC-CAH patients, where the use of 17-OHP measurements tests are not so precise and it is essential to carry out molecular diagnostic studies at *CYP21A2* locus in addition. Genotype–phenotype correlations are of major importance for contributing to an integrated analysis, where specific treatment and genetic counselling also needs to be personalised.

Nomenclature

Nomenclature of *CYP21A2* variants is in accordance with the CYP21A2–002 ensembl transcript, namelly ENST00000418967.6 [NM_000500 from National Center for Biotechnology Information].

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