

DOUTORAMENTO

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Characterization of the Pathogenic Variants Landscape in 21-Hydroxylase Deficiency

Caracterização do Espetro das Variantes Patogénicas na Deficiência de
21-Hidroxilase

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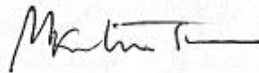
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“A Esperança não murcha, ela não cansa,
Também como ela não sucumbe a Crença,
Vão-se sonhos nas asas da Descrença,
Voltam sonhos nas asas da Esperança.”

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In memoriam

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- Marques CJ, Pignatelli D, **Carvalho B**, Barceló J, Almeida AC, Fernandes S, Witchel SF, Sousa M, Oliveira MJ, Freitas P, Fontoura M, Carvalho D, Barros A, Carvalho F. Mutational characterization of steroid 21-hydroxylase gene in Portuguese patients with congenital adrenal hyperplasia. *Experimental Clinical Endocrinology & Diabetes*. 2010;118(8):505-12. doi: 10.1055/s-0029-1237363.
- **Carvalho B**, Pereira M, Marques CJ, Carvalho D, Leão M, Oliveira JP, Barros A, Carvalho F. Comprehensive genetic analysis and structural characterization of *CYP21A2* mutations in CAH patients. *Experimental Clinical Endocrinology & Diabetes*. 2012;120(9):535-9. doi: 10.1055/s-0032-1323805.
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- Pignatelli D, **Carvalho B**, Palmeiro A, Barros A, Guerreiro SG, Macut D. The Complexities in Genotyping of Congenital Adrenal Hyperplasia: 21-Hydroxylase Deficiency. Corrigendum in *Frontiers Endocrinology (Lausanne)* 2020; 10: 432. Erratum for: *Front Endocrinol (Lausanne)*. 2019; 10:432. doi: 10.3389/fendo.2020.00113.
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- Silva RS[#], **Carvalho B[#]**, Pedro J, Castro-Correia C, Carvalho D, Carvalho F, Fontoura M. Differences in hormonal levels between heterozygous *CYP21A2* pathogenic variant carriers, non-carriers, and females with non-classic congenital hyperplasia. Archives of endocrinology and metabolism 2022; 28;66(2):168-175. doi: 10.20945/2359-3997000000437.

(**#Both authors contributed equally to this work**)

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Congenital adrenal hyperplasia concerns to a group of autosomal recessive disorders resulting from enzymatic defects in steroidogenesis. More than 95% of all cases of CAH are due to 21-hydroxylase deficiency, that conducts to a cortisol deficiency and androgen excess, with or without aldosterone deficit.

The clinical spectrum varies from mild to severe or life-threatening disorder, settled by the functional impairment of the corresponding pathogenic variant on *CYP21A2* gene. There are two major forms of the congenital adrenal hyperplasia due to 21-hydroxylase deficiency, the classical and the non-classic, or late onset. The classic form is the most severe and can be divided in two subtypes: salt wasting and simple virilizing. Most classic congenital adrenal hyperplasia cases due to 21 hydroxylase deficiency are salt wasting forms, that affect approximately 75% of patients and they may suffer potentially lethal adrenal insufficiency. Classic non-salt-wasting forms of CAH due to 21 hydroxylase deficiency manifest as genital ambiguity in affected females and signs of androgen excess at later childhood also in male patients.

Neonatal screening newborns programmes worldwide are of major importance in the establishment of epidemiology data based mainly on the classic form, with an overall incidence of about 1:10.000-1:15.000 cases. Neonatal screening is not the appropriate method to identify less severe forms, nevertheless non-classic congenital adrenal hyperplasia affects approximately 1 in 1.000 individuals.

The 21-hydroxylase enzyme is encoded by the *CYP21A2* gene on chromosome 6 placed within the human leukocyte antigen region. Hundreds of pathogenic variants have been described and a group of about 10 most common pathogenic variants accounting for approximately 90% of all pathogenic variants, some of them associated with the non-classic phenotype. The mild phenotype of non-classic congenital adrenal hyperplasia is conferred by pathogenic variants that retain 20 to 50% of enzyme activity.

Due to the autosomal recessive pattern of inheritance, the phenotype is typically correlated with the less affected allele. Genotype-phenotype correlation is well established for congenital adrenal hyperplasia due to 21-hydroxylase deficiency, but this correlation is weaker in the mild form of the disease. Although, there are several pitfalls in the genetic diagnosis of congenital adrenal hyperplasia individuals and some patients

remain undiagnosed due to an incomplete genetic screening of the *CYP21A2* gene or due to incorrect achievement of the gene responsible for the cortisol and aldosterone defect. The aim of this thesis was gathering several contributions of the last years, providing a state-of-the-art on the molecular analysis of pathogenic variants on *CYP21A2* gene responsible for multiples cases admitted for genetic analysis at the Genetics Unit of the Faculty of Medicine of Universidade do Porto.

Firstly, *CYP21A2* gene pathogenic variant profile was studied in a cohort of Portuguese congenital adrenal hyperplasia patients, by assaying for the 10 most frequent gene pathogenic variants. Pathogenic variants were detected in 91.7% of the patients and the frequency of alleles carrying two or more *CYP21A2* pathogenic variants was greater than in other populations. The non-classical variant p.Val282Leu is the most frequent pathogenic variants identified in our population (41.7%) followed by the deletions/conversions involving the promoter region of the *CYP21A2* gene. This study allowed to understand that our population exhibits specific genetic features with differences from other worldwide countries.

Additionally, in remaining cases without genetic diagnosis a whole genetic sequence analysis strategy was carry out. In those patients a better molecular diagnosis was achieved due to the identification of other pathogenic variants not found on targeted screening, as well as novel pathogenic variants.

After these observations, a completed gene study was implemented as routine, and a nationwide genotype characterization of the congenital adrenal hyperplasia patients from Portuguese pediatric endocrinology centers was started to establish their genotype–phenotype correlation. The global genotype–phenotype correlation obtained was higher than the worldwide average (92.4%) in an vast population of males diagnosed after a neonatal adrenal crisis. According to the previously published in a Portuguese cohort, the most prevalent genotype in this population was the homozygosity for the non-classical pathogenic variant, p.Val282Leu.

After these observations of major prevalence for p.Val282Leu non-classical pathogenic variant, we aimed to characterize a population of Portuguese females suspected of having non-classic form of disease by their clinical and biochemical criteria, *CYP21A2* molecular analysis and clinical risk of hyperandrogenism. This study showed that heterozygous carriers presented higher basal and post-cosyntropin 17-

hydroxyprogesterone levels than wild type individuals and lower basal and stimulated hormone levels than non-classical congenital adrenal hyperplasia patients. A substantial overlap between 17-hydroxyprogesterone levels among patients' groups was observed and therefore, normal basal and stimulated 17-hydroxyprogesterone responses do not exclude heterozygosity for *CYP21A2* pathogenic variants. In conclusion, the data which constitutes this thesis contributes for a better characterization of Portuguese congenital adrenal hyperplasia patients due to 21-hydroxylase deficiency.

A hiperplasia suprarrenal congénita faz parte de um grupo de doenças autossómicas recessivas que resultam de defeitos enzimáticos na esteroidogénese. Mais de 90% de todos os casos de hiperplasia suprarrenal congénita são devidos à deficiência na enzima 21-hidroxilase, que conduz a uma carência de cortisol, e excesso de androgénios com ou sem diminuição de aldosterona. O espectro clínico varia de ligeiro a grave e é determinado pelo nível de comprometimento funcional da variante patogénica no gene *CYP21A2*. Existem duas formas principais de hiperplasia suprarrenal congénita devido à deficiência de 21-hidroxilase, a clássica e a não clássica, ou de início tardio. A forma clássica é a mais grave e pode ser dividida em dois subtipos: perdedora de sal e virilizante simples. A maioria dos casos de hiperplasia suprarrenal congénita clássica por deficiência de 21 hidroxilase são formas perdedoras de sal, que afetam aproximadamente 75% dos doentes, e podem ser potencialmente letais. As formas da doença sem perda salina, devido à deficiência de 21 hidroxilase, manifestam ambiguidade genital no sexo feminino e sinais de excesso de androgénios na infância tardia, também em doentes do sexo masculino.

Os programas de rastreio neonatal de recém-nascidos são de grande importância no estabelecimento de dados epidemiológicos globais, mas são baseados principalmente na forma clássica, com incidência global de cerca de 1:10.000-1:15.000 casos. O rastreio neonatal não é o método de eleição para identificar formas menos graves, contudo a hiperplasia suprarrenal congénita não clássica afeta aproximadamente 1 em 1.000 indivíduos.

A enzima 21-hidroxilase é codificada pelo gene *CYP21A2*, localizado na região do antígeno leucocitário humano, no cromossoma 6. Centenas de variantes patogénicas estão descritas, mas existe um grupo de cerca de 10 variantes patogénicas mais comuns que correspondem a cerca de 95% de todas as variantes patogénicas, algumas delas associadas ao fenótipo não clássico. O fenótipo mais leve da hiperplasia suprarrenal congénita não clássica é conferido por variantes patogénicas responsáveis por 20 a 50% da atividade enzimática normal.

Devido ao padrão de transmissão autossómico recessivo, o fenótipo é estabelecido pelo alelo menos grave. A correlação genótipo-fenótipo está bem estabelecida nesta doença,

mas essa correlação é mais fraca nas formas mais leves da doença. No entanto, existem várias preocupações no diagnóstico genético destes doentes, pois alguns permanecem sem diagnóstico devido a uma análise genética incompleta do gene *CYP21A2* ou à incorreta atribuição do gene responsável pela carência em cortisol e aldosterona.

O objetivo desta tese foi contribuir para um melhor conhecimento do estado da arte na análise molecular de variantes patogénicas no gene *CYP21A2* de doentes admitidos para estudo genético no Serviço de Genética da Faculdade de Medicina da Universidade do Porto.

Inicialmente, foi estudado o perfil de variantes patogénicas do gene *CYP21A2* num grupo de doentes portugueses com hiperplasia suprarrenal congénita, analisando as 10 variantes patogénicas genéticas mais frequentes. As variantes patogénicas foram detetadas em 91,7% dos doentes e a frequência alélica nos portadores de duas ou mais variantes patogénicas no *CYP21A2* foi maior do que em outras populações. A variante patogénica, não clássica, p.Val282Leu foi mais frequentemente identificada nesta população (41,7%) seguida das deleções/conversões envolvendo a região promotora do gene *CYP21A2*. Este estudo mostrou que esta população apresenta características genéticas específicas e diferenças face a outros países. A existência de casos sem diagnóstico genético conduziu-nos ao estudo completo deste gene, começando num grupo de doentes com deficiência de 21-hidroxilase. A análise completa do gene permitiu identificar outras variantes em regiões não analisadas anteriormente, assim como a identificação de novas variantes patogénicas.

O estudo genético completo foi implementado na rotina e permitiu avançar com um estudo multicêntrico para a caracterização genotípica de doentes com hiperplasia suprarrenal congénita de centros de endocrinologia pediátrica portugueses, com o objetivo de estabelecer a correlação genótipo-fenótipo. A correlação genótipo-fenótipo global obtida foi superior à média mundial (92,4%), sobretudo devido à população de doentes masculinos diagnosticados após uma crise suprarrenal neonatal. De acordo com o previamente publicado no estudo anterior, o genótipo mais prevalente nesta população foi a homozigotia para a variante patogénica não clássica p.Val282Leu.

Após a demonstração desta elevada prevalência para a variante patogénica p.Val282Leu, foi efetuada a caracterização de uma população de doentes do sexo feminino com suspeita da forma não clássica da doença, pelos seus critérios clínicos e

bioquímicos, análise molecular do gene *CYP21A2* e suspeita clínica de hiperandrogenismo. Este estudo mostrou que portadoras heterozigóticas apresentaram níveis de 17-hidroxiprogesterona, basais e pós-estimulação com cosintropina (hormona adrenocorticotrópica sintética), mais elevados do que indivíduos sem variantes patogénicas e níveis hormonais basais e pós-estimulação mais baixos do que em doentes com forma não clássica. Uma sobreposição substancial entre os níveis de 17-hidroxiprogesterona entre os grupos de doentes foi observada e, portanto, as respostas basais normais e estimuladas de 17-hidroxiprogesterona não excluem a heterozigotia de variantes patogénicas no gene *CYP21A2*.

Em conclusão, os dados que constituem esta tese contribuem para uma melhor caracterização dos doentes portugueses com hiperplasia suprarrenal congénita, por deficiência de 21-hidroxilase.

ACTH	Adrenocorticotrophic hormone
ADO	Allele drop-out
AMH	Anti-müllerian hormone
AVP	Arginine vasopressin
CFF	Cell free fetal
CRH	Corticotropin releasing hormone
CAH	Congenital adrenal hyperplasia
CVS	Chorionic villus sampling
DHEA	Dehydroepiandrosterone
DOC	Deoxycorticosterone
EMQN	European Molecular Genetics Quality Network
GnRH	Gonadotropin-releasing hormone
hiSCs	Human induced steroidogenic cells
HSD3	3-β hydroxysteroid dehydrogenase
HLA	Human leukocyte antigen
HPA	Hypothalamic-pituitary-adrenal
LC-MS/MS	Liquid chromatography-tandem mass spectrometer
MLPA	Multiplex ligation dependent probe amplification
NC-CAH	Non-classic congenital adrenal hyperplasia
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction
PCR-SSCP	Polymerase chain reaction- single strand conformation polymorphism
PCR-SSP	Polymerase chain reaction-sequence specific primer
PSCs	Pluripotent stem cells
PGT	Preimplantation genetic testing
PND	Prenatal diagnosis
POR	P450 oxidoreductase
RFLP	Restriction fragment length polymorphism
SNP	Single nucleotide polymorphism

SV	Simple-virilizing
SW	Salt-wasting
USA	United States of America
11-KT	11-ketotestosterone
11-KA4	11-ketoandrostenedione
11-OH	11 β -hydroxylase
11-OHA4	11-hydroxyandrostenedione
11-OHD	11 β -hydroxylase deficiency
11-OHT	11-hydroxytestosterone
17-OHP	17-hydroxyprogesterone
21-DOC	21-deoxycortisol
21-OH	21-hydroxylase
21-OHD	21-hydroxylase deficiency
3β-HSD	3 β -hydroxysteroid dehydrogenase

I. INTRODUCTION

1.1. Congenital adrenal hyperplasia

Congenital Adrenal Hyperplasia (CAH) is a complex disease, that is mainly due to a defective steroidogenesis. The production of cortisol occurs in five major enzyme-mediated steps and CAH results from a deficiency in one of the several enzymes involved in the androgens biosynthesis pathway. Impaired cortisol synthesis leads to chronic increase of adrenocorticotrophic hormone (ACTH), through a negative feedback system, causing overstimulation of the adrenal cortex and resulting in hyperplasia and oversecretion of the precursors above the enzymatic defect.

1.1. Androgens biosynthesis pathway

1.1.1 The role of cortisol and aldosterone

Adrenal steroidogenesis occurs in 3 main pathways, responsible for the secretion of mineralocorticoids, glucocorticoids and sex hormones (Fig. 1).

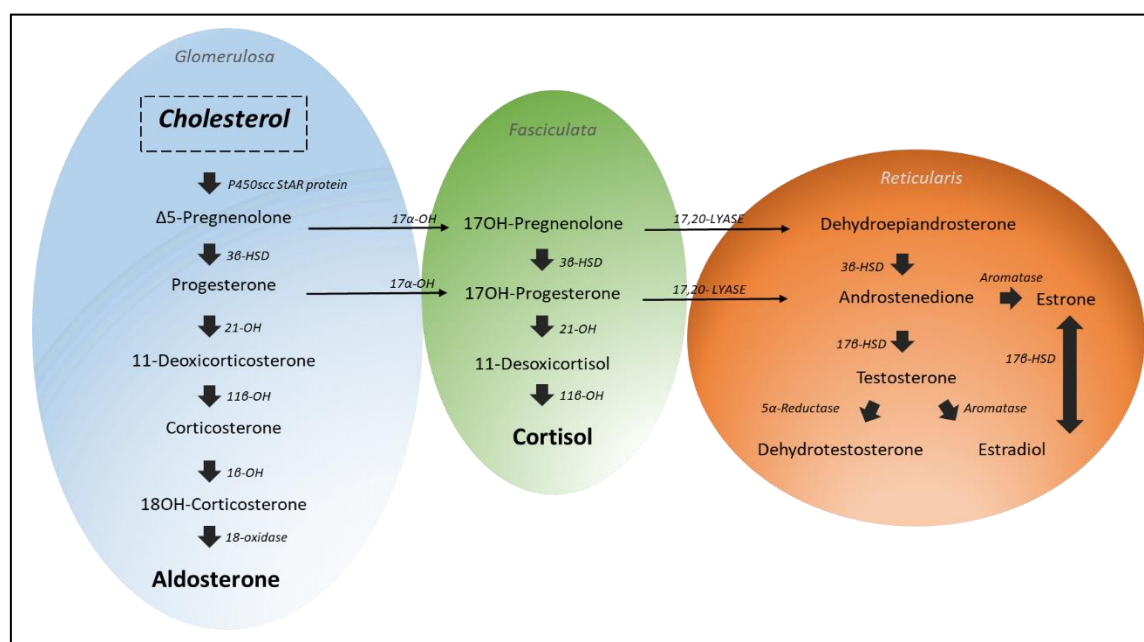


Figure 1 - Adrenal steroidogenesis: Main enzymes involved in cortisol production, mediated by steroid enzymes: cholesterol 20,22 desmolase; 17 hydroxylase (17-OH), 3 β -hydroxysteroid dehydrogenase (3 β -HSD), 21 hydroxylase (21-OH), 11 β hydroxylase (11-OH).

The defective steroidogenesis in CAH translates into a differential clinical spectrum in which severity and clinical features may depend on the enzymatic defect, on the enzyme residual activity and genotype.

The secretion activity on adrenal glands may occur in 3 distinct zones of the cortex *zona glomerulosa*, *zona fasciculata*, *zona reticularis* (Ganong, 1963; Guillemin and Schally, 1963). The impairment of both cortisol and aldosterone is regulated by hypothalamic-pituitary-adrenal (HPA) feedback system, mediated through the circulating level of plasmatic cortisol, by a negative feedback mechanism of cortisol on corticotropin releasing hormone (CRF) and ACTH secretion. For that, the different types of CAH that results in a reduced level of cortisol leads to an increase of ACTH production, leading to an overstimulation of adrenal products. The impairment caused by the enzyme deficiency leads to a build-up of proximal precursor molecules. Although ACTH is the major hormone controlling adrenocortical activity, numerous other hormones and biochemical factors influence the production of steroids from the adrenal cortex, with some minor molecular pathways that have related important actions. Aldosterone is not generally considered to be under the control of ACTH, although it can influence aldosterone, and this mineralocorticoid, produced in the *zona glomerulosa*, is part of the renin–angiotensin system, where angiotensin II is a potent stimulator of aldosterone in human and rat (Holt and Hanley, 2008). The gene induction profile of angiotensin II in both human and rat has been described by Nogueira and colleagues, in 2009.

The secretion of cortisol in the *zona fasciculata* of the adrenal cortex is controlled by hormones from the pituitary and hypothalamus. The anterior pituitary (adenohypophysis) is responsible for production and secretion of ACTH which is released into the blood stream and transported to the adrenal glands where it stimulates production and secretion of glucocorticoids from the *zona fasciculata* (Wild, 2013). The release of ACTH from the pituitary is stimulated by hypothalamic corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP), although several other hormones can also affect ACTH secretion. Cortisol is an important glucocorticoid hormone secreted by the adrenal gland that is responsible for a negative feedback inhibition, at both pituitary and hypothalamus level, to reduce ACTH release and regulate the axis.

There are several diseases that may be related with cortisol imbalance, such as Cushing syndrome that is associated with chronic exposure to excessive serum cortisol levels.

The excess of serum cortisol has several possible pathophysiologic origins: a pituitary tumor or hyperplasia leading to overproduction of ACTH by the pituitary gland, ectopic ACTH by neuroendocrine tumors, adrenocortical carcinoma or adenoma, or excessive administration of glucocorticoids (Feldman, 1995). Cushing's syndrome clinical manifestations are highly variable, mostly non-specific and they progress slowly, which makes the Cushing's diagnosis difficult when is based solely in clinical manifestations. Biochemical confirmation of Cushing's syndrome is generally accomplished by a complex analysis that includes the manipulation of the pituitary–adrenocortisol axis via low dose dexamethasone suppression testing. Beyond Cushing's syndrome, spontaneous hypoadrenocorticism (Addison's disease) is another example of adrenal insufficiency related disease, that is associated with low cortisol production by the adrenal cortex, most often caused by immune-mediated disease or drug therapy. The test of choice to diagnose Addison's disease is the ACTH stimulation test (Rasmuson et al., 1996).

One of the most important enzymes in this pathway is 21-hydroxylase (21-OH), responsible for more than 90% of CAH cases (Speiser et al., 2018). When this enzyme is defective, consequently the function of 21-hydroxylating cytochrome P450 is deficient, creating a block in P450 cortisol production pathway. This one, catalyzes the conversion of progesterone to deoxycorticosterone (DOC) and 17-hydroxyprogesterone (17-OHP) to 11-deoxycortisol, responsible for the reduction or absence of important mineralocorticoid and glucocorticoid molecules, the aldosterone and cortisol, respectively. Due to this enzyme defect, an accumulation of 17-OHP, a precursor adjacent of the 21-hydroxylation step, is noted. This excess of 17-OHP is then shunted into the intact androgen pathway, where the 17,20-lyase enzyme converts the 17-OHP to Δ^4 -androstenedione, the major adrenal androgen. The 21-hydroxylase is the most important defective enzyme responsible for many of the cases and 17-OHP measurements (basal and/or ACTH stimulated) may guide the diagnosis of CAH due to 21-OH deficiency (New et al., 1983). Like the 21-hydroxylase deficiency, other rarer forms of CAH are diagnosed based only on hormonal testing. Biochemical screening is indicative of presence or absence of disease but may be not enough to inform about the carrier status or clinical severity. It is consensual that molecular analysis is mandatory for genotype achievement and essential to discriminate heterozygotes from the normal population and also useful for genetic counseling (New et al., 2013).

1.2 Defective enzymes

There are other less frequent forms of CAH due to defects in several other enzymes of the steroidogenesis biosynthesis that may be involved in low concentration of cortisol and aldosterone (Table 1). Those less frequent causes of CAH are accompanied of different clinical manifestations, some of them common to the 21-OH deficiency (OMIM 201910) and its prevalence is variable according to worldwide population (Hannah-Shmouni et al., 2009).

Table 1 - Defective enzymes involved in the steroids synthesis pathway.

Deficient Enzyme	% of CAH	Substrate	Product	Androgens	Mineralo-corticoid
Steroidogenic acute regulatory protein (StAR)	Unknown ¹	--	Mediates cholesterol transport through mitochondrial membrane	Deficiency ²	Deficiency ³
3β-hydroxysteroid-dehydrogenase (3β-HSD)	Unknown ¹	Pregnenolone, 17-OH pregnenolone, DHEA	Progesterone, 17-OHP, Δ^4 -androstenedione	Deficiency ²	Deficiency ³
17α-hydroxylase	Unknown ¹	Pregnenolone Progesterone	17-OH pregnenolone 17-OHP	Deficiency ²	Excess ⁴
21-hydroxylase	>90%	Progesterone 17-hydroxy progesterone	DOC 11-deoxycortisol	Excess ⁵	Deficiency ³
11β-hydroxylase	5%	DOC	Corticosterone	Excess ⁵	Excess ⁴

1- Unknown (rare disease); 2- Males undervirilized at birth; 3- Associated with SW; 4- Associated with hypertension; 5- Females virilized at birth, or later

i) 11 β -hydroxylase

After the 21-OH enzyme, claimed as the major responsible for CAH cases, the second most common form of CAH, that account for round 5% of the cases, is due to a deficiency in 11 β -hydroxylase (11-OH) enzyme (OMIM 202010) due to pathogenic variants in *CYP11B1* gene. This form of the disease affects approximately 1:100.000 of live births in general population (White et al., 1994), with a significant high prevalence in the Middle East and North Africa regions, where consanguinity is common. Clinically, the affected

female newborns are intensely virilized and both males and females display significantly advanced bone age and some also display hypertension. The main clinical features of 11-OHD, include virilization and low renin hypertension, are very similar with the classical form of CAH due to 21-hydroxylase deficiency (21-OHD) (Nimkarn and New, 2008). The steroid 11-desoxycortisol is not frequently assayed but is the best biochemical marker for diagnosing 11-OHD. Clinical manifestation of high blood pressure is usually not identified until later in childhood or in adolescence, although manifestations in a particular case of a child under 6 months have been reported (Mimouni et al., 1985). Although hypertension has been correlated with biochemical and clinical signs, virilization has not been well correlated. The literature points to cases of normotensive severely virilized females and by, opposite, mild virilized patients with severe hypertension leading to fatal vascular episodes (Globerman et al., 1988; Rosler et al., 1982). Other major complications, such as cardiomyopathy, long standing uncontrolled hypertension, retinal vein occlusion and blindness have been reported in patients manifesting deficiencies in 11OH (Chabre et al., 2000; Hague and Honour, 1983). A mild form of 11-OHD CAH, identical to NC form on CAH patients due to 21-OHD, has been reported. Unlike the common NC form of 21-OHD, this form of 17-OHD is rare and children have been diagnosed as normotensive and mild virilization precocious pubarche (Zachmann et al., 1983). Often, adult patients' manifest signs of hyperandrogenemia and women also manifest infertility (Joehrer et al., 1997; Peters et al., 2007). In summary, 11 β -hydroxylase deficiency patients seem to be protected from the symptoms associated with adrenal crisis, although they are subject to other manifestations, such as hypertension, due to salt retention, and ambiguous genitalia in females.

Several pathogenic variants have been reported as associated with this enzyme defect. A large international cohort of CAH patients with 11 β -hydroxylase deficiency were evaluated for 25 missense variants of *CYP11B1* by a computational modeling and revealed that specific modifications in the heme-binding or substrate-binding site of the enzyme, or alterations in its stability may have an important role in prediction of disease severity (Khattab et al., 2017). Despite a hormonal profile consistent with 11 β -OHD, pathogenic variants in the *CYP11B1* gene may not always be present. Based on the

literature available, there is not a consistent correlation between specific *CYP11B1* gene pathogenic variant and the clinical phenotype of 11 β -OHD (Marakaki et al., 2015).

ii) **3 β - hydroxysteroid dehydrogenase**

The 3 β -hydroxysteroid dehydrogenase deficiency (HSD3), (OMIM 201810), is a rare autosomal recessive disorder, with two isoforms of the 3 β -hydroxysteroid dehydrogenase enzyme (3 β -HSD): type 1 and type 2. Type 1, *HSD3B1*, is expressed in the placenta and peripheral tissues, while type 2, *HSD3B2*, is highly expressed in the adrenal glands and gonads and is responsible for conversion of the pregnenolone, 17-hydroxypregnenolone and dehydroepiandrosterone to progesterone, 17-OHP and androstenedione, respectively (Speiser et al., 2010). Like 17-OHD, both gonadal and adrenal hormone synthesis are affected. This enzyme assumes a very important role on the steroids pathway as is crucial for the synthesis of progesterone, the precursor of aldosterone, and 17-OHP, the precursor of cortisol in the adrenal cortex, as well as for several other androgens involved in the pathway: androstenedione, testosterone, and estrogen in the adrenal cortex and gonads (Lifton et al., 1992; Curnow et al., 1993). Clinically, CAH due to 3 β -HSD present distinct phenotypes in the classic form: simple virilizing (SV) characterized by insufficient cortisol synthesis and salt-wasting (SW) in the most severe form. Interestingly, both male and female children can be born with genital ambiguity, depending on the severity of the enzyme deficiency. Virilization of external genitalia in females is due to androgen effect from the peripheral conversion of circulating Δ 5 precursors to active Δ 4 steroid and in males, type 2 3 β -HSD deficiency in the gonads, results in incomplete virilization of the external genitalia. The non-classic form of 3 β -HSD remains a controversial discussion as the genetic cause has not been frequently described. This less severe form of the disease, characterized by hirsutism and menstrual irregularities in young adult females, was achieved based on hypersecretion of 17-hydroxypregnenolone after ACTH stimulation (Simard et al., 1995). Based on that, the non-classic diagnosis, or even the mild form, should not be considered unless the diagnostic laboratory parameters are at least six standard deviations above normal value. The genetic molecular study of *HSD3B2* gene, was first ascertained in a 46,XY karyotype patient, with 3 β -HSD type 2 deficiency, with ambiguous

genitalia, in whom a homozygous combined missense/frameshift variant on exon 4 of the gene was found (Lutfallah et al., 2002; Hannah-Shmouni et al., 2009).

Moreover, several studies on the *HSD3B2* gene may provide insight to the pathophysiology and clinical aspects of the disease and may provide prognostic information on disease severity. In summary, HSD3 deficiency leads to ambiguous genitalia in males and females and in both genders, it can carry to salt-wasting manifestations (Mermejo et al., 2005).

iii) **17 α -hydroxylase**

The block of 17 α -hydroxylase (17-OH) on steroidogenesis pathway is responsible for around 1% of the cases of CAH. The 17-OH deficiency (OMIM 202110) result from pathogenic variants on the gene *CYP17A1* responsible for encoding this enzyme. The 17-OH is a microsomal P450 type II enzyme essential on the catalyzation of two different enzymatic reactions: the 17- α hydroxylation of pregnenolone and progesterone, and the conversion of 17-hydroxypregnenolone to dehydroepiandrosterone and, with less importance but also with some efficiency, the conversion of 17-OHP to androstenedione through the 17,20 lyase reaction (Figure 1). The impairment of 17-OHP enzyme results in deficiency of glucocorticoid and sex steroid hormones and impairs both adrenal and gonadal function (Goldsmith et al., 1967). Consequently, the accumulation of mineralocorticoids precursors such as, corticosterone and DOC, lead to a compensatory activity of glucocorticoid and mineralocorticoid, leading to hypertension and hypokalemia conditions in those patients, together with hypogonadism. Genital ambiguity is one of the major classic manifestations of 17-OH deficiency. Individuals, phenotypically females, with karyotype 46,XX or 46,XY, may present hypertension and absence of secondary sexual characteristics. Less severe form, with partial deficiency in 17-OH, may affect patients with a 46,XY karyotype and undervirilized, with a previous history, in infancy, of ambiguous genitalia. Summing up, 17 α -hydroxylase deficiency results in ambiguous external genitalia in males and in females, in absence of pubertal development or menstrual cycles (amenorrhea). Rarely, isolated 17,20 lyase deficiency, that results in amino acid substitutions located within the area of the CYP17A1 molecule that interacts with the electron-donating redox partner, P450 oxidoreductase (POR)

specifically for the conversion of 17-pregnenolone to dehydroepiandrosterone (DHEA) manifests with an impaired sex steroid biosynthesis (Krone et al., 2007). Isolated 17,20 lyase deficiency is not considered a type of CAH.

The *CYP17A1* gene, located on chromosome 10 (10q24.3) harbors 8 exons encoding a 508-amino-acid protein (Yanase et al., 1991). More than 70 pathogenic variants have been reported in *CYP17A1* gene. However, certain ethnicities are at higher risk of 17-OHD, including Canadian Mennonites and Dutch Frieslanders, Japanese, East Asians, and the Brazilian population, where more than 80% of mutant alleles are due to two pathogenic variants, suggesting a founder effect (Imai et al., 1992; Miura et al., 1996; Costa-Santos et al., 2004).

iv) P450 oxidoreductase

The P450 oxidoreductase (POR) deficiency (OMIM 613571) is a rare variant of CAH with unknown incidence, characterized by adrenal insufficiency in most patients (Krone et al., 2012). This enzyme deficiency seems to result of an apparent combination of deficiencies in both, *CYP17A1* and *CYP21A2* genes (Fluck et al., 2004). The *POR* gene, located on the long arm of chromosome 7 (7q11.2), consisting of 15 translated exons spanning a region of approximately 32.9kb, behaves as an electron donor enzyme for *CYP17A1*, *CYP19A1* (aromatase) and *CYP21A2* genes. When 21-hydroxylation cytochrome 450 is not adequate, the cortisol pathway is blocked and lead to an accumulation of 17-OHP. The excess of 17-OHP is shunted into the intact androgen pathway where the 17,20-lyase enzyme converts the 17-OHP to Δ^4 -androstenedione, which is converted into androgens. Since the mineralocorticoid pathway requires minimal 21-OH activity, SW form, caused by mineralocorticoid deficiency, is a feature of the most severe form of the disease. The absence of steroid production impairs the negative feed-back control of ACTH secretion leading to adrenal hyperplasia. Clinical manifestations range from clinically insignificant to life threatening, including sexual ambiguity on both sexes: male infants with severe undervirilization (including small penis and undescended testes) and female children presenting severe virilization (vaginal atresia, fused labia minora, hypoplastic labia majora, and/or large clitoris). Craniofacial-malformations may also be present and overlap with the Antley-Bixler

syndrome (OMIM 201750), a rare craniosynostosis syndrome presented in the perinatal period, that at the most severe end of the POR spectrum, can include craniosynostosis, choanal stenosis or atresia, stenotic external auditory canals, and hydrocephalus. Skeletal anomalies can include radiohumeral synostosis, neonatal fractures, congenital bowing of the long bones, camptodactyly, joint contractures, arachnodactyly, and clubfeet (Fluck et al., 2004). Genetically, the majority of those patients with mild to moderate skeletal malformations present compound heterozygosity for missense pathogenic variants, while all patients with severe clinical phenotypes carried a loss-of-function variant on one of the affected alleles (Krone et al., 2012). Krone and colleagues, in 2012, demonstrated that decreasing activity in *CYP19A1* gene may induce androgen accumulation in the placenta, leading to virilization of the female fetus and, less often, in the pregnant women. After birth, females with POR deficiency showed low androgens levels and virilization does not progress. Although literature does not report any hotspot on the gene, POR-inactivating pathogenic variants, including missense, frameshift, and splice site pathogenic variants, are scattered throughout the gene (Arlt et al., 2004). The most frequent pathogenic variant in Caucasians is p.Ala287Pro, while p.Arg457His is the most frequent pathogenic variant in Japanese population (Arlt et al., 2004). In one study of 30 patients with POR deficiency from 11 countries, 23 POR pathogenic variants including a deletion and a partial duplication were detected by MLPA, and only 22% of unrelated patients carried homozygous POR pathogenic variants (Krone et al., 2012). POR deficiency may present as a form of apparent 17,20 lyase deficiency due to pathogenic variants in the *POR* gene rather than the *CYP17A1* gene (Miura et al., 1996). In conclusion, POR deficiency presents as with manifestations that may resemble 21-OHD, 17-OHD, or a combination of the two enzyme deficiencies and in some an association with a skeletal disorder known as Antley-Bixler syndrome, may be found. The genes responsible for the several types of CAH are well characterized and pathogenic variants analysis are widely available. The severity of the disease is highly related to the enzyme defect and may influence the phenotype presentation. The high number of defective enzymes involved in the androgen's pathway and responsible for the several manifestations of CAH and based on the enzyme deficiency the clinical features are distinct and particularly, abnormalities of genitalia may be present (Table 2).

Table 2 - Main enzyme deficiencies in steroidogenic pathway.

Condition	Gene	Protein abnormality	Onset	Genitalia	Clinical features
Lipoid CAH	<i>StAR</i> 8p11.2	StAR Protein	Congenital	Female, with no sexual development	Salt wasting Low steroid products
	<i>CYP11A</i> 15q23-24	P450scc	Congenital	Female, with no sexual development	Salt wasting Low steroid products
3 β -HSD deficiency, classic type	<i>HSD3B2</i> 1p13.1	3 β -HSD	Congenital	Females virilized, males undervirilized	Salt wasting High DHEA, 17-pregnenolone, low androstenedione, testosterone, high K, low Na and CO ₂
3 β -HSD deficiency, non-classic type	-	3 β -HSD	Congenital	Normal genitalia with mild to moderate hyperandrogenism postnatally	None High DHEA, 17-pregnenolone, low androstenedione, testosterone
17 α -OH deficiency	<i>CYP17</i> 10q24.3	P450c17	Postnatal	Variable sexual development	Hypokalemic low-renin hypertension normal or decreased androgens and estrogen, elevated DOC, corticosterone
21-OH deficiency SV, classic type	<i>CYP21</i> 6p21.3	P450c21	Congenital	Females prenatally virilized, normal male genitalia	None Elevated 17-OHP, DHEA, and androstenedione, normal electrolytes
21-OH deficiency, non-classic type	<i>CYP21</i> 6p21.3	P450c21	Congenital	Males and females with normal genitalia at birth, hyperandrogenism postnatally	None Elevated 17-OHP, DHEA, and androstenedione on ACTH stimulation
11 β -deficiency, classic type	<i>CYP11B1</i> 8q24.3	P450c11B1	Postnatal	Females virilized with ambiguous genitalia, males unchanged	Low-renin hypertension Elevated DOC, 11-deoxycortisol; androgens, low K, elevated Na, CO ₂
11 β -deficiency, non-classic type	<i>CYP11B1</i> 8q24.3	P450c11B1	Congenital	Males and females with normal genitalia at birth, hyperandrogenism postnatally	None Elevated 11-deoxycortisol $\pm$ DOC, elevated androgens
P450 Oxido-reductase deficiency	<i>POR</i>	POR	Postnatal	Females virilized with ambiguous genitalia, males undervirilized	None Partial, combined, and variable defects of P450c21, P450c17 and P450 activity

I.2. Diagnosis of Congenital Adrenal Hyperplasia

The blocking of 21-OH enzyme may be affected differentially, and the percentage of normal enzyme function may be distinct. When the secretion of enzyme is severely affected an insufficient adrenal aldosterone for sodium reabsorption by the distal tubes occurs and results in severe forms, accompanied by cortisol deficiency and androgen excess. In most patients, it may be easier to achieve the clinical diagnosis of CAH form, although in some cases, particularly in those with the less severe form, the distinction between the CAH diagnosis due to 21-OHD or other endocrinological disease due to other defect on the cholesterol pathway, may cause some interrogations to the clinicians. Although the level of 17-OHP is a powerful biochemical diagnostic tool in classical CAH cases, this marker may be insufficient to diagnose the less severe cases.

2.1 Clinical forms of disease

CAH due to 21-OHD can be present in 3 subtypes according to clinical severity of 2 distinct forms, the classical and the non-classical form (Speiser et al., 2010). These two forms can be distinguished by the clinical presentation, hormonal levels and molecular genetic analysis. The classical form is divided into SW and SV clinical presentations (Table 3).

Table 3 - Main clinical characteristics in 21-OHD CAH forms.

Feature	21-OHD CAH	
	Classic	Non-Classic
Salt wasting	~75% of all individuals	Absent
Prenatal virilization	Females	Absent
Postnatal virilization	Females and males	Variable
Cortisol deficiency	~100%	Rare

The SW form affects more than 75% of the individuals presenting the classical form and is the most severe manifestation of the disease, resulting from the insufficient secretion of aldosterone for sodium reabsorption by distal renal tubules, accompanied by a cortisol deficiency and overproduction of androgens. By the other side, SV accounts for about 25% of the classic patients, presenting defective production of cortisol and an overproduction of adrenal androgen (Table 3).

In classic form, the prenatal exposure to androgens at crucial stages of sexual development, such as testosterone and Δ^4 -androstenedione, is determinative for the virilization of external genitalia in females' fetuses, often resulting in genital ambiguity at birth. On the opposite, individuals with the less severe of CAH due to 21-OHD - NC form - present only moderate enzyme deficiency and the clinical findings are not significantly serious, manifesting postnatally signs of hyperandrogenism (Witchel, 2012; Merke and Poppas, 2013).

2.1.1 Salt wasting form

SW form can affect newborns and occur, as early as, the first to fourth week of life. Newborns may experience failure to thrive, vomiting, dehydration, poor feeding, weight loss, hypotension, and hyperkalemia and hyponatremia, due to an electrolyte's decompensation. Individuals with SW forms with untreated or poorly controlled manifestations may present low levels serum concentration of chloride, sodium and total carbon dioxide, high potassium concentration levels and an inappropriately high potassium concentration in urine (Nimkarn et al., 2007). If treated, SW crisis can be reversible due to the administration of mineralocorticoids replacement. The adrenal crisis can be fatal in newborn males that were probably undiagnosed, in which normal genitalia that do not cause any suspicion to the clinicians. In those cases, they can experience a salt wasting crisis at home, after discharged from the hospital after birth and far from any kind of clinical emergency procedure. Contrarily, females with ambiguous genitalia, can be early detected and timely treated with corticoid replacement therapy. In cases with severe loss of 21-OH function, adrenal aldosterone secretion is insufficient for sodium reabsorption by the distal renal tubules, resulting in salt wasting as well as cortisol deficiency and androgen excess. Although an overt salt-

wasting crisis classifies the child as a salt-waster, some degree of aldosterone deficiency, determined by the adrenal capacity to produce aldosterone in response to renin stimulation, was found in all forms of 21-OHD CAH (Nimkarn et al., 2007). Infants with renal salt wasting have poor feeding, weight loss, failure to thrive, vomiting, dehydration, hypotension, hyponatremia, and hyperkalemic metabolic acidosis progressing to adrenal crisis (azotemia, vascular collapse, shock, and death).

2.1.2 Simple virilizing form

SV form of the disease is particularly worrisome, as manifestation of genital virilization may start in utero life, and the excess of adrenal androgens will lead to genital virilization in 46,XX individuals.

It is well accepted that virilization status affects other aspects of infant's development, such as external secondary characteristics (skin, hair, and genitalia), growth and maturation.

The degree of androgens excess may vary and the signs in affected females can be quite different, from enlargement of the clitoris, fusion of the labioscrotal folds to the formation of a urogenital sinus. The severity of virilization is often classified using the 5-points scale of Prader (Prader and Gurtner, 1955). As the anti-müllerian hormone (AMH) is not secreted in those patients, the müllerian ducts develop normally and a normal uterus and fallopian tubes are present in affected females. The achievement of the CAH form of the disease is not reachable based only on the degree of virilization of the affected females at birth. After birth, both females and males with SV CAH due to 21-OHD that were not compensated with glucocorticoids therapy, will develop pubic and axillary hair, acne, rapid linear growth and advanced bone age. However, the growth may be accelerated, potential height is reduced and premature epiphyseal fusion result in short adult stature. Even, if the replacement therapy with glucocorticoids started early in life, generally, the individuals do not acquire the expect adult height and the bone age is advanced compared to the chronologic age. Both, male and female individuals, properly treated with glucocorticoids therapy may present a normal onset for puberty, however, exceptions may occur even among individuals in whom the disease is well controlled (Trinh et al., 2007). Untreated males may also present

progressive penis enlargement and small testes. Untreated females' manifest hirsutism, baldness, clitoral enlargement, menstrual abnormalities and low fertility rates. There are several reasons indicated for this subfertility status, such as inadequate vaginal anatomy, elevated androgens levels leading to ovarian dysfunction and psychosexual behaviors that relates with gender identity and selection of sexual partner (Gastaud et al., 2007; Costa et al., 2016). Other manifestations, such as chronic anovulation, elevated progesterone levels or aberrant endometrial embryo implantation, have also been identified as related to subfertility in those patients (Witchel, 2012). Among the male patients, the main cause of subfertility is the existence of testicular adrenal rest tumors, which are thought to be originated from aberrant adrenal tissue. Additionally, hypogonadotropic hypogonadism may result from suppression of LH secretion by the pituitary gland and the excessive adrenal androgens and their aromatization products (Ogilvie et al., 2006). The deficiency of cortisol may also affect the development and function of the adrenal medulla, which leads to lower levels of epinephrine and metanephrine concentrations comparing to unaffected individuals (Merke et al., 2000). It should also be taken into account that glucocorticoid replacement therapy in previously untreated individuals bring a true precocious puberty due to the glucocorticoids release on the hypothalamic pituitary axis and the inhibition by estrogens derived from excess adrenal androgen secretion.

The prenatal exposure to androgens in female's fetus with classic forms of CAH is responsible for the virilizing effect on the external genitalia, alterations in childhood behaviour and a decrease in self-reported femininity, but not an increase in self-reported masculinity in adult females (Nimkarn et al., 2022). Differences in childhood play interests may also be related with gender dysphoria and heterosexual interest in adult life (Costa et al., 2016). Women with all forms of 21-OHD CAH refer high rates of bisexual and homosexual orientation, probably due to the degree of prenatal androgenization. Meyer-Bahlburg et colleagues, in 2008, correlated the bisexual and homosexual behaviour with global measures of masculinization of nonsexual behaviour and predicted independently based on the degree of both prenatal androgenization and gender dysphoria masculinization of child behaviour. Contrarily to the observed in adult females, males with 21-OHD CAH do not refer a general alteration in childhood play behaviour, nor gender dysphoria, or sexual orientation (Hines, 2004).

2.1.3 Non-classic form

Commonly, individuals with non-classic CAH (NC-CAH) form presents mild reduced synthesis of cortisol which has no clinical relevance. Non-classical CAH patients may present postnatally several symptoms of androgens excess, including, acne, hirsutism, premature development of pubic hair, faster growth with advanced bone age or short adult stature due to premature epiphyseal fusion (New, 2006). The earliest report of diagnosis of premature pubarche was detected in a 6 month-old infant. Usually, the clinical symptoms in NC-CAH patients start from age of 5 and often emerge in late childhood, adolescence and adulthood (Kohn et al., 1982; Whitchel, 1992).

i) Females with NC-CAH:

Females with NC form, are not usually virilized at birth, but it is difficult to predict which female patients will manifest signs of virilization. Postnatally, usually these patients may develop manifestations that include hirsutism, frontal baldness, delayed menarche, polycystic ovaries, menstrual irregularities, and infertility. The fertility rate among untreated women has been reported to be around 50% (Pang, 1997). Contrarily to newborns with SW CAH due to 21-OHD, that are at a high risk for life-threatening salt-wasting crises, neonates' patients with NC-CAH form only presented moderate enzymes deficiency and less often signs of hyperandrogenism (Tsuji et al., 2015).

The manifestations spectrum in NC-CAH due to 21-OHD patients is very broad and literature shows that around 60% of adult women manifest only hirsutism; approximately 10% exhibit hirsutism and menstrual irregularities; around 10% show only menstrual disorders and 2-10% manifest hyperandrogenism, but with higher prevalence in particular populations (New, 2006, Escobar-Morreale et al., 2008). Some manifestations on NC-CAH females may be difficult to distinguish between the clinical symptoms and findings of premature adrenarche from those of polycystic ovary syndrome (PCOS) in girls (Binay et al., 2014).

ii) Males with NC-CAH:

Few data related to NC-CAH form in male individuals is available, but typically, male patients present early beard growth, enlarged phallus with proportionately small testes but frequently with a normal gonadal function and normal sperm counts (New, 2006).

Bilateral adrenocortical incidentaloma was reported as an isolated finding in an adult male with NC-CAH (Nigawara et al., 2008).

Overall, the NC spectrum disease present suggestive findings that should be suspected in certain individuals, such as females who are virilized at birth, or become virilized postnatally, or manifest precocious puberty, adrenarche or pubarche; males with evident masculinization in childhood (i.e., premature adrenarche); newborns experiencing salt-losing crisis in the first days of life, or newborns with positive newborn screening. Karyotype analysis of patients with CAH due to 21-OHD CAH probably will show no alterations in those patients: 46,XX karyotype in females and 46,XY karyotype in males.

2.2. Biochemical screening markers

Normally, after the clinical evaluation of patients with suspicion of CAH due to 21-OHD, evaluation of 17-OHP in serum, constitutes an important biomarker for diagnosis and therapy monitorization.

Besides, the identification of high levels of 17-OHP, other findings may be crucial for a correct diagnosis in patients with CAH 21-OHD. In general, adrenal androgens may also be increased in males and females with CAH 21-OHD and particularly testosterone and androgen precursors (Δ^4 -androstenedione, DHEA) may be present elevated in affected females and prepubertal male infants. Also, renin activity in plasma is markedly increased in patients with classic SW CAH form. NimKarn and colleagues, in 2007, compared the plasma renin activity with aldosterone levels in patients with classic CAH. The concentration of aldosterone revealed to be significantly low compared to the plasma renin activity in SW patients, due to the impaired aldosterone synthesis. Hence, the reduced ratio of aldosterone to plasma renin activity observed can elucidate between SW and SV forms after the newborn period.

The 17-OHP is traditionally measured at single early morning (before 8 a.m.) and plasma concentration baselines levels in affected individuals are not always elevated, and 17-OHP levels can be normal in the NC form (Table 4).

Table 4 - 17-OHP levels related to CAH 21-OHD forms of the disease.

	Classic Form	Non-Classic Form	Unaffected
17-OHP level, baseline	>10.000 ng/dL or 300 nmol/L	200-10.000 ng/dL or 6-300 nmol/L ¹	<200 ng/dL or 6 nmol/L ¹
17-OHP level, after ACTH stimulation	>10.000 ng/dL or 300 nmol/L	1.000-10.000 ng/dL or 31-300 nmol/L	<1.000 ng/dL or 50 nmol/L

Adapted from Speiser et al., 2010.

Additionally, a 60-minutes ACTH stimulation test, in which the concentration of 17-OHP is measured baselined and at 60 minutes after the intravenous injection of a standard 250-µg of synthetic ACTH can be requested. The parameters for such analysis are defined by laboratories based on the method used and in adult females, however normal range is dependent of the menstrual cycle phase. Particularly, due to subjectivity associated with conventional biomarkers, a raised need to improve the biochemical assessment was pursued. The determination of serum levels of 11-oxyandrogens appeared as potential new androgen biomarkers for disease monitoring in patients with 21-OHD (Jha et al., 2020). Of all four oxyandrogens, 11-hydroxytestosterone (11-OHT), 11-ketotestosterone (11-KT), 11-hydroxyandrostenedione (11-OHA4), and 11-ketoandrostenedione (11-KA4), 11-OHT, and 11-KT were suggested as having an important role as biomarkers to monitor disease in patients with CAH due to 21-OHD, when conventional biomarkers are uninformative or discrepant (Jha et al., 2020).

2.2.1 Newborn Screening

Newborn screening for 21-OHD CAH is not widely implemented worldwide, despite its consensual application. During the past decade, neonatal screening programs have been implemented in an increasing number of countries over the world (Therrell et al., 1998; Speiser et al., 2018). The aim of the newborn screening for CAH addresses particularly two purposes: to identify children, especially males, with the classic form of 21-OHD

CAH who are at risk for life-threatening salt-losing crises and to facilitate a diagnosis of females with ambiguous genitalia, avoiding salt-losing crisis and neonatal death (Iñiguez et al., 2018). Most children are diagnosed and treated early to avoid these complications. So, it is consensual that newborn screening for CAH provides clear benefits in prevention of neonatal salt crisis and mortality, especially amongst boy's infants (Speiser et al., 2020).

In newborn screening programs for 21-OHD, the concentration of 17-OHP is measured on a filter paper blood spot obtained by the heel-stick technique broadly used for other newborn screenings. Most screening programs use a single test without a retest of samples, even in those samples with unsettled 17-OHP levels (Speiser et al., 2010). In order to improve the efficiency of screening programmes, some countries reevaluate samples with borderline first-tier results with a second-tier test (Chan et al., 2013). Immunoassay methods revealed a high false positive rate and other methods, such as, liquid chromatography-tandem mass spectrometer (LC-MS/MS), are being recommended for a second-tier test, in order to improve sensibility and specificity (Speiser et al., 2010; Heather and Nordenstrom, 2021). Other countries decided to analyse the concentration of different hormones, such as 17-OHP, 21-deoxycortisol (21-DOC), and cortisol, as a second-tier test on samples with a positive first-tier test result (Janzen et al., 2007). 21-DOC seems to be a key marker as it is not affected by timing of collection and revealed to have a better predictor status of 21-OH deficiency compared with 17-OHP (Held et al., 2022).

Although, even more countries have been implementing newborns screening programs, the existence of many false positive results block a wide world implementation. Votava et al., (2005) showed that newborns screening rarely detects individuals with the non-classic form of 21-OHD CAH (Zetterström et al., 2020). There are some aspects that should be taken into account on results from newborns screening programs. The results obtained from blood samples taken in the first 24 hours of life should be interpreted carefully as the 17-OHP levels are increased in all infants and may give false positive results. False positive results may also be detected in premature newborns or low birth-weight babies and an adjustment for gestational age and birth weight is mandatory (Wilson et al., 2014). Other factors, such as, stress caused by other illness, the fact that other forms of CAH caused by distinct enzyme deficiencies, including 11-OHD, 3 β -

hydroxysteroid dehydrogenase deficiency and P450 oxidoreductase, lead to elevated 17-OHP values on newborns screening (Miller, 2019). Beyond this, false negative results may also be detected in newborns treated with dexamethasone for the management of unrelated problems. Those facts suggest that replacement of 17-OHP by 21-DOC should reduce dramatically the number of false-positive results of newborns, avoiding the need of recall children for test, parental stress and extra expenses related with tests and appointments (Miller, 2019). This new second-tier approach may dramatically improve the screening specificity using a steroid profile (Tajima and Fukushi, 2016; Heather and Nordenstrom, 2021). The several improves in different countries have been made and show that the screening strategy can be more effective in the detection of infants that are at risk of developing adrenal salt crisis and thereby decreasing neonatal mortality. It is also possible that the screening, per se, also amend the outcome for patients, including a better knowledge of the disease and its treatment.

I.3. Epidemiology

CAH disease affects about 1:10.000 - 1:15.000 live births worldwide, data based on newborn screening records, that detect mainly classic CAH cases (Van der Kamp and Wit, 2004). Although, neonatal screening was not designed to detect the mild forms of disease, the prevalence of NC forms seems to be slightly common and affects approximately 1 in 1.000 individuals (Speiser et al., 1985).

This disease reveals that there is a predilection of specific genotypes in certain populations worldwide and within ethnic groups, fact that may be associated to genetic phenomena: unequal crossing over during meiosis, conversion of single nucleotide pathogenic variants from the pseudogene or result from an ancient founder effect (Hannah-Shmouni et al., 2017). In the United States and Europe, classic form of CAH accounts for 1:10.000 - 1:23.000 (Van der Kamp and Wit, 2004), 1:27.000 in New Zealand (Heather et al., 2015), 1:21.000 in Spain (Dulín and Ezquieta, 2018), 1:19.000 - 21.000 in Japan (Mikami et al., 1999; Pang, 2003), 1:6.000 in China and India (Zhong et al., 2016; Kaur et al, 2016) and approximately, 1:300 in Yupik Eskimos of Alaska (Pang et al., 1988). In contrast, NC-CAH due to 21-OHD may be detected in approximately 1:100/200 - 1.000 individuals, in the general population (Hannah-Shmouni et al., 2017). The first data, from Speiser et al., 1985, showed that the prevalence of NC-CAH, due to 21-OHD in the general population of New York City was estimated at 1:100, making NC form of CAH one of the most frequent autosomal recessive disorders in humans. Frequently, it is often mistaken for idiopathic precocious pubarche in children or polycystic ovary syndrome in young women. This form of the disease is particularly prevalent in individual populations, showing a high ethnic specificity. The highest ethnic-specific prevalence is found among Ashkenazi Jewish population, in which about 1:3 are carriers and 1:27 are affected (New et al., 2013), although other ethnic groups exhibit also high non-classic disease prevalence, such as Hispanics (1:40), Slavs (1:50), and Italians (1:300) (White et al., 1994).

In United States, the allele frequency of the most common pathogenic variants might vary considerably between different ethnicities. For example, the exon 7 pathogenic variant p.Val282Leu (c.1685G>T) is highly associated with NC-CAH in the Ashkenazi Jewish population of New York, but has not been observed in the Asian and Native

American populations, however, this variant may also occur in other populations. Among the Ashkenazi Jewish population of compound heterozygotes, the most common variants are the 3kb large deletion/p.Val282Leu and c.293-13A/C>G/p.Val282Leu (Wilson et al., 2007). The distribution of the disease and the prevalent variants is variable among populations and CAH seems to be quite rare in Asian, African American and East Indian populations of New York, while native American population exhibits a large gene deletion (New et al., 2013).

CAH due to other less common enzymes deficiencies may also have high prevalence in different populations. CAH owing to 11 β -hydroxylase deficiency (11 β -OHD) accounting for 5-8% of all cases and it occurs in 1 of every 100.000 live births in the general population and is more frequent in some populations of North Africa (Zachmann et al., 1983). For instance, data shows that among Moroccan Jews, the incidence was estimated to be 1 in 5.000 live births (Rösler et al., 1992) subsequently, it was shown to occur less frequently but remains more common than in other populations.

Other forms of CAH due to less common enzyme's defects are considered rare diseases and the incidence is unknown in the general population.

All those findings, pathogenic variants specificity through ethnics' diversity, are very important as they may point clinicians to a diagnosis of 21-OHD; nevertheless, there is a need for a complete gene testing study.

I.4. Genetics in Congenital Adrenal Hyperplasia

The genetic diagnosis of CAH is a complex process due to the extremely high variability of this genomic region. Genotyping is an important tool for diagnosis or carrier status confirmation, providing a prognosis of the disease severity that is essential for genetic counseling. Aspects, such as the presence of a pseudogene – *CYP21A1P* – with a high percentage of homology with *CYP21A2*, the coexistence of two or more variants on the same allele or even, the existence of more than one *CYP21/C4* unit repeated on the same chromosome, make the genetic analysis a challenging task in the genetic routine. *CYP21A2* (previously called *P450c21B*, *CYP21B* or *CYP21*) encodes 21-OH enzyme, a cytochrome P450 type II enzyme of 495 amino acids (Nebert et al.,1991; Higashi et al., 1986). The functional *CYP21A2* gene and its duplicated nonfunctional pseudogene (*CYP21A1P*), are located 30kb apart in the human leukocyte antigen (HLA) class III region in the major histocompatibility complex (MHC) locus on the short arm of chromosome 6 (band 6p21.3).

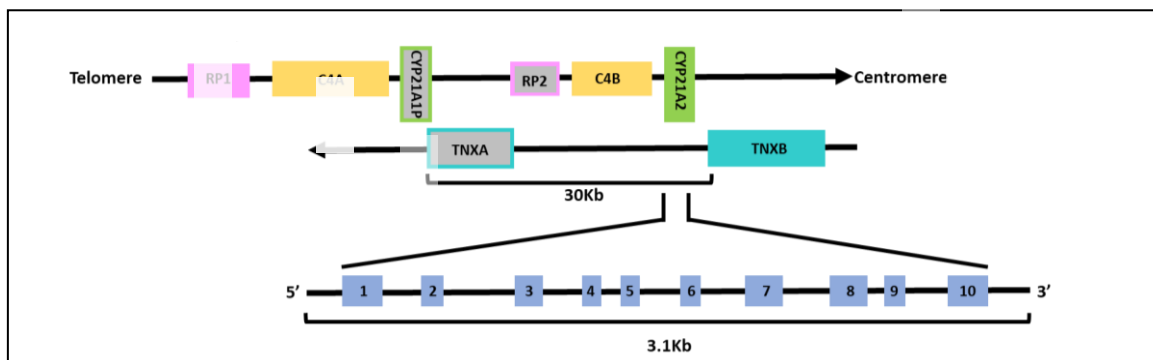


Figure 2 - The RCCX module: *CYP21A2* gene and its duplicated pseudogene (*CYP21A1P*) are located 30kb apart. Both genes are arranged in tandem repeat with the C4 (C4A and C4B) and RP (RP1 and RP2) genes. C4/*CYP21A* is flanked by telomeric RP (RP1 and RP2) and centromeric tenascin (TNXA and TNXB) genes, transcribed from the opposite strand. The RP, TNX, C4 and *CYP21* genes together compose the RCCX bimodule (RP1-C4-*CYP21A1P*-TNXA-RP2-C4-*CYP21A2*-TNXB). Enlarged area represents the 10 exons of *CYP21A2*. Active genes are solid colors and pseudogenes are in grey outlined with the color of the corresponding active gene.

The *CYP21A1P* gene is inactive due to the presence of several deleterious pathogenic variants in its coding region. Both gene and pseudogene share approximately 98% sequence homology and its adjacent and alternate location to each other pointed to an ancestral origin and to a selective pressure during evolution (Speiser et al., 1992) (Fig. 2).

Both genes are organized *in tandem* repeat with the *C4* (*C4A* and *C4B*) genes encoding the fourth component of complement and in turn, *C4/CYP21A2* is flanked by telomeric RP (*RP1* and *RP2*) and centromeric tenascin (*TNXA* and *TNXB*) genes. This rearrangement was probably due to an ancestral duplication of 30kb encompassing the *CYP21A2* and *C4* genes, and its high homology is responsible for the high frequency of genomic recombination. *RP1* encodes a serine/threonine nuclear protein kinase, *C4* encodes for the immune effector protein complement component, and *TNXB* encode for tenascin X, an extracellular matrix protein. The functional genes *RP1*, *CYP21A2* and *TNXB* genes, apart from *C4*, have a corresponding homologous pseudogene (*RP2*, *CYP21A1P* and *TNXA*, respectively). All these genes together constitute a structure, called RCCX module (*RP1-C4A-CYP21A1P-TNXA-RP2-C4B-CYP21A2-TNXB*). In the majority of the alleles, it exists in a bimodular way, with *CYP21A1P* gene taking the telomeric position and the *CYP21A2* gene in the centromeric position (Yang et al., 1999). Due to modular duplication or deletion events monomodular and trimodular haplotypes are also found in some populations (Blanchong et al., 2000; Saxena et al., 2009).

The high degree of sequence homology between gene and pseudogene is the key for recombination events and approximately 70-75% of *CYP21A2* disease causing pathogenic variants are transferred from pseudogene due to gene conversion or microconversion (Fig. 3).

Approximately 25–30% are chimera genes due to large deletions, also resulting from unequal crossing-over during meiosis (Concolino et al., 2009; Vrzalova et al., 2011). The misalignment can result in a 30kb gene deletion, which produces a nonfunctioning chimeric *CYP21A1P/CYP21A2* gene. Depending on the breakpoints, such genetic alterations result in different types of chimera genes between the homologous genes in the RCCX modules. Nine different *CYP21A1P/CYP21A2* chimeras have been reported, which have a CH-1 to CH-9 configurations, being the exon 3 of *CYP21A2* gene consider the most common breakpoint and a hotspot for recombination (Concolino et al., 2010;

Chen et al., 2012; Fanis et al., 2022). Additionally, and due to the complexity of RCCX structure, *TNXA/TNXB* chimeric genes, with the deletion of *CYP21A2* gene, leading to CAH-X syndrome have also be found in CAH patients (Fanis et al., 2022).

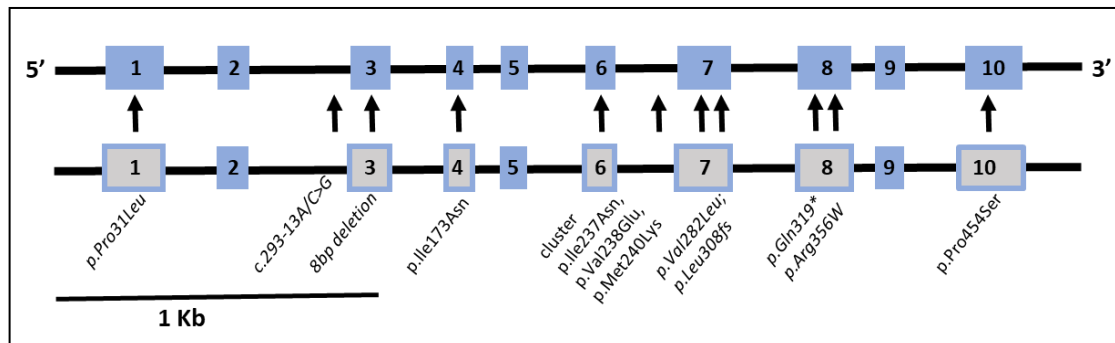


Figure 3 - *CYP21A2*, pseudogene *CYP21A1P* and recombination events: Unequal crossing-over through intergenic recombination results in transfer of deleterious pseudogene sequence to the active gene. The most common pseudogene-derived pathogenic variants found in the *CYP21A2* are shown by exons* (Adapted from Hannah-Shmouni & Chen, 2017).

*Nomenclature of *CYP21A2* variants is according to Ensembl transcript *CYP21A2-002*: ENST00000418967.6 (NM_000500 from National Center for Biotechnology Information).

De novo pathogenic variants may also occur but less frequently, in approximately 1–2% of cases, due to high variability of the *CYP21A2* locus and in those cases, are not transmitted by one of the parents (Speiser et al., 1992).

The molecular testing for the identification of biallelic pathogenic variants is of major importance for diagnosis confirmation and clarify familial segregation studies. Molecular approaches include mostly the single gene test strategy or less often, the multigene panels.

The single gene test involves the sequencing analysis of *CYP21A2* gene and the large rearrangement analysis, efficient to detect about 70-80% of the cases, and target deletion/duplication analysis is of particular importance in the identification of 20-30% of the cases. When single gene analysis, with selective assortment of functional gene, detect several pathogenic variants it is of major importance to determine if the pathogenic variants are in *trans* configuration (on separated chromosomes, one

inherited from each parent) or in *cis* configuration (on the same chromosome, representing one affected allele), caused by gene conversion events. In order to avoid diagnostic errors, parental segregation studies are recommended to determine if the pathogenic variants are in *cis* or *trans* configuration.

Due to the five major enzyme-mediated steps involved in the adrenal cortisol biosynthesis and the distinct impairments of the several enzymes, a differential diagnosis is usually taken in account. A multigene panel, including *CYP21A2* gene and other genes of interest, some of them not directly associated in the condition, may also be considered. This kind of approach should be carefully selected, as the multigene panel is mostly likely to identify the genetic cause of the condition limiting the identification of other variants of uncertain significance and other pathogenic variants non- underlying the phenotype (Chan et al., 2015).

The genetic analysis should be always taken in account, as for many patients with less severe forms of the disease, the identification of biallelic pathogenic variants on *CYP21A2*, will help on the confirmation of a diagnosis.

4.1 Molecular analysis

The molecular diagnosis constitutes a challenge for most of the genetic laboratories. For this reason, molecular analysis of *CYP21A2* gene should be performed by experienced laboratories, particularly for the analysis of CAH genotypes. The high homology between sequences typical of this HLA complex region can difficult the analysis and a major attention should be taken to avoid the pseudogene amplification, due to events like duplications, deletions and/or recombination of *CYP21A2*.

Previously, and during several years, most of the molecular genetic laboratories used the screening strategy for the detection of 10 most common pathogenic variants, as the instrument for molecular diagnosis. The strategy of target-site directly showed to fail in the diagnosis in about 10% of the patients and the newly strategy of sequencing the whole gene sequencing showed that the identification of new variants was more frequent than expected (Parajes et al., 2008).

Traditionally, the screening of the 10 most common pathogenic variants involves several molecular techniques, such as Polymerase Chain Reaction-Sequence Specific Primer (PCR-SSP), Polymerase Chain Reaction -Single Strand Conformation Polymorphism (PCR-SSCP), Restriction Fragment Length Polymorphism (RFLP) for large deletion/conversions, and Sanger sequencing (Fig. 4) (Marques et al., 2010).

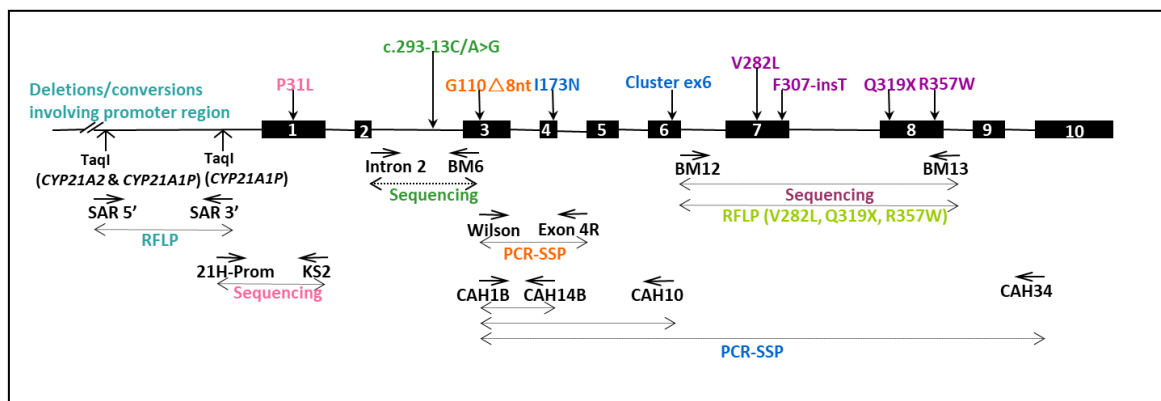


Figure 4 - Representation of the methodology used for screening of 10 most common variants causing 21-OHD: Numbered black boxes represent CYP21A2 exons. Horizontal arrows show the position of primers used for the six different PCR amplifications; Double arrows represent PCR amplified fragments. Restriction site positions for TaqI restriction enzyme in CYP21A2 and CYP21A1P are also represented (Adapted from Marques et al., 2010).

The PCR-SSCP technique was the elected for the identification of p.Gly111Valfs*21, p.Ile173Asn and exon 6 cluster (p.Ile236Asn, p.Val237Glu, p.Met239Lys), RFLP for large deletion/conversions extending to about 30Kb with breakpoints between exons 3 and 8 and the sequencing for the p.Pro31Leu, on exon 1, the intron 2 splice-site variants c.293-13A/C>G, and p.Val282Leu, on exon 7, p.Gln319* and p.Arg357Trp, on exon 8 (Fig. 4). RFLP allowed the confirmation of large deletion/conversions extending to about 30Kb with breakpoints between exons 3 and 8 of CYP21A2 gene. However, the complete characterization of the locus, regarding to the number of gene or pseudogene copies required a complex technique - Southern-blotting – which depends on radiolabeled probes for different genes and different combinations of restriction enzymes, at least two restriction enzymes (TaqI and BglII), to discriminate between deletion and

conversion events. This old-fashion techniques are labor intense, require the need of radioactivity and large amount of high-quality DNA, and have been gradually replaced by other methods, such as real-time PCR (Parajes et al., 2007) and multiplex ligation-dependent probe amplification (MLPA) technique, for the detection of large rearrangements (Concolino et al., 2009b). Screening for deletions and duplications of both *CYP21A2* and *CYP21A1P* using MLPA started to be more and more used as this technique has the advantage of being easy to set up, inexpensive and not subject to subjective analysis between laboratories worldwide (Concolino et al., 2009b). This technique allows the confirmation of point pathogenic variants detected by *Sanger* sequencing and avoids misinterpretation of a pathogenic genotype. In order of a non-misinterpreting results it is important to be aware of the high frequency of duplicated alleles in association with p.Gln319* variant and MLPA may help to clarify *CYP21A2*-gene copy number (Krone et al., 2002; Kleinle et al., 2009). Approximately, 20% of abnormal alleles present a deletion of 30-kb segment that encompasses the 3' end of the *CYP21A1P* pseudogene, all *C4B* complement gene, and the 5' end of *CYP21A2* gene.

The sequencing of the entire gene, contemplating the 10 exons and the intron-exon boundaries, showed to be of major importance in the obtention of an accurate diagnosis, particularly in cases where the phenotype does not correspond to the genotype or in those cases where just one pathogenic variant was detected, and the patient has clinical and biochemical evidence of CAH.

More recently, a well-established strategy for the study of complete functional *CYP21A2* gene has been replacing the original screening strategy. The presence of a high rate of homology between gene and pseudogene, and the existence of a significant number of SNPs in the functional gene turns hard the design of specific primers for the genetic diagnosis. A protocol that uses a long-range PCR that amplifies two partially overlapping fragments, respectively with 1517 and 2214 base-pairs (bp), avoiding the co-amplification of the pseudogene *CYP21A1P*, is now widely used (Loidi et al., 2006; Carvalho et al., 2020).

The PCR products may be purified and then, sequenced with internal forward and reverse primers covering all 10 exons and intron-exon boundaries of *CYP21A2* (Fig. 5). Although most of the pathogenic variants described could be properly detected by the screening approach, the complete gene sequencing can reveal both common variants

and rare and even de novo variants, enabling the clarification of clinical diagnosis of 21-OHD CAH patients.

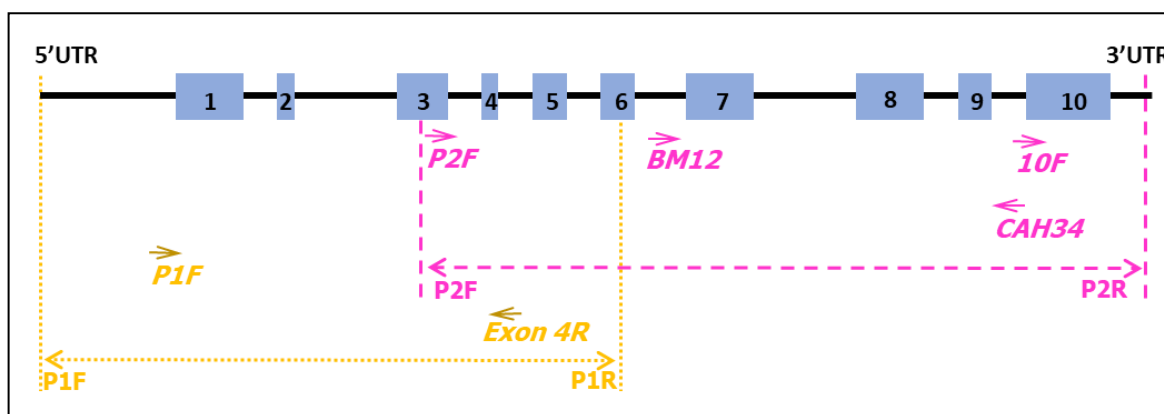


Figure 5 - Schematic representation of *CYP21A2* gene structure and strategy for complete gene sequencing: Numbered black boxes represent *CYP21A2* exons: Dot line (...) represent the first PCR - P1- amplification product (1517 bp) and dash line (- - -) represent the second PCR - P2 – amplification product (2214 bp). Arrows represent the primers used in 6 different sequencing reactions: P1F (5'-TCGGTGGGAGGGTACCTGAAG-3') and Exon4R (5'-TCGTCCTGCCAGAAAAGGAG-3') for P1 fragment and P2F (5'-TGTCCTGGGAGACTACTCC-3'), BM12F (5'-GAGGGATCACATCGTGGAGAT-3'), CAH34R (5'-CTCTGGAGTTCTGCCTGGCTCCAGG-3') and 10F (5'-AGAGGCCACATGAGTTCTGG-3') for P2 fragment) (Adapted from Carvalho et al., 2012).

Both PCR and MLPA methodologies demonstrate limitations, as it happens in many other molecular diagnosis. Particularly, allele drop-out events are a problematic situation as it may compromise the amplification and sequencing of one allele. Laboratory best practice guidelines recommended the study of familial segregation to investigate the possibility of allele drop-out when no heterozygous sequence is visualized (Baumgartner-Parzer et al., 2020).

Routine *Sanger* sequencing capillary electrophoresis can detect point sequence variants that may include small intragenic deletions or insertions and missense, nonsense and or splice-site variants. The long-range PCR is not able to detect the duplication of *CYP21A2* and MLPA is a reliable alternative for the detection of this gene rearrangements. Besides this, MLPA is unable to distinguish between CH-4 and CH-9, and classic CH-6 chimera

representation genes, due to the lack of a probe for intron 2 splice site variant, c.293-13A/C>G, crucial for the distinction of chimeras *CYP21A1P/CYP21A2* into the classical and attenuated phenotypes. The location of the *CYP21A2* gene on the RCCX module enhances the importance of the study of *TNXA/TNXB* chimeras in patients clinically diagnosed with CAH. Other methods, such as quantitative PCR or a microarray based strategy designed to detect single-exon deletions or duplications.

4.2 Genotype-phenotype correlation

CAH due to 21-OHD represents a spectrum of phenotypic heterogeneity, with a direct genotype-phenotype correlation achieved in approximately 90% of cases (Krone et al., 2000; Stikkelbroeck et al., 2003). Since most patients with CAH are compound heterozygotes carrying two different disease-causing pathogenic variants, the phenotype is defined by the pathogenic variant retaining the high rate of enzymatic activity (New et al., 2013). Generally, the disease severity in childhood can be accurately predicted by genotypes of SW and NC-CAH forms; while the most phenotypic variability is observed with the SV subtype (Jaaskelainen et al., 1997; Stikkelbroeck et al., 2003). Female patients had a rapid clinical diagnosis with classic CAH because of genital ambiguity; however, in countries that perform neonatal screening programs, classic CAH cases ascertained are similar between males and females (Held et al., 2020). Moreover, females are more likely to be diagnosed with the NC-CAH p.Val282Leu pathogenic variant, probably because males may be asymptomatic (Merke and Poppas, 2013). Most commonly, the large deletion that is associated with *CYP21A1P/CYP21A2* chimeric gene, often referred as a classic pathogenic variant, is responsible for the inactivation of *CYP21A2* gene and therefore are associated with a classic SW phenotype. This classic *CYP21A1P/CYP21A2* chimera contain the common intron 2 splice site variation (c.293-13A/C>G) and often multiple other pseudogene pathogenic variants. The 21-OH enzyme activity is less severely impaired when the deletion breakpoint occurs upstream the pathogenic variant c.293-13A/C>G within intron 2 of pseudogene, known by attenuated chimera. Attenuated chimeras have been described, CH-4 and CH-9, both harboring junction sites upstream of the common pseudogene pathogenic variant IVS2-13A/C>G,

carrying a weak but active *CYP21A1P* promoter and a NC pathogenic variant, p.Pro31Leu (c.92C>T), on exon 1, and result in a SV or NC phenotype (Chen et al., 2012). In a large cohort study of patients with CAH, 96% showed that large deletions detected were classic chimeras, where 4% were attenuated chimeras (Chen et al., 2012).

Although the high rates of concordance between genotype-phenotype, discordance may exist across all pathogenic variant groups. A high genotype-phenotype concordance is well established for p.Val282Leu, deletion 3Kb, 8 bp deletion, p.Gln319*, and p.Arg358Trp pathogenic variants. SV disease occurs in approximately 76% of CAH patients carrying the p.Ile173Asn pathogenic variant on one allele and a severe variant on the other, while 23% of these patients present with SW CAH, likely due to subtle variations in transcriptional regulation or downstream protein translation (New et al., 2013). Moreover, the intron 2 splicing pathogenic variant c.293-13A/C>G results primarily in SW CAH, however, approximately 20% of patients have SV phenotype (New et al., 2013).

Table 5 - Classification of most common *CYP21A2* pathogenic variants, based on *in vitro* data.

Group	Null	A	B	C
Phenotype	SW	SW	SV	NC
<i>In vitro</i> activity of <i>CYP21A2</i>	0%	<1%	1-5%	20-60%
Pathogenic variants	30kb deletion 8bp deletion exon 6 cluster p.Leu307fs p.Gln319* p.Arg358Trp	c.293-13A/C>G	p.Ile173Asn	p.Pro31Leu p.Val282Leu p.Pro454Ser

Rearrangements, like gene duplications may be responsible for incorrect report, as it happens with p.Gln319* pathogenic variant, that is sometimes associated with *CYP21A2* gene duplication. In this situation, the allele carrying the p.Gln319* coexists with a normal *CYP21A2*-like gene within the RCCX module, on a trimodular structure on the

same chromosome, not representing a pathogenic allele, as the normal gene is expressed (New et al., 2013).

Based on *in vitro* studies, *CYP21A2* pathogenic variants can be classified into 4 groups (Null, A, B, C) according to their severity and the proportion of normal enzyme activity (Table 5).

Deletions or nonsense pathogenic variants that affect critical enzyme functions, such as membrane anchoring, heme binding, or alter enzyme stability result in a complete loss of functionality (Null group) reflecting SW phenotypes. Missense pathogenic variants with 1–5% (B group) of normal enzyme activity are found in patients presenting SV phenotype and affect the transmembrane region or conserved hydrophobic regions (Amor et al., 1988; Higashi et al., 1988; Tusie-Luna et al., 1990). Group B correspond to an enzymatic activity lower than 1% because of the alternative splicing forms of mRNA and mainly associated with SW phenotype. Group C pathogenic variants, such as p.Val282Leu, p.Pro454Ser and p.Pro31Leu, cause an interference in oxidoreductase interactions, salt-bridge and hydrogen bonding networks, and are responsible for synthesis of 20–60% of normal enzyme activity as seen in NC-CAH (Helmberg et al., 1992; Tusie-Luna and White, 1995).

Genotype-phenotype variability in patients with CAH may result from several factors. Apart from the *CYP21A2* pathogenic variants, other genes that may have impact on the phenotype by modifying steroid action or salt balance, include the length of the CAG repeats of the androgen receptor, the highly polymorphic enzyme P450 oxidoreductase, splice pathogenic variants in RNA splicing factors and other genes encoding proteins other than cytochrome P450 type II enzyme that have 21-hydroxylase activity (Miller et al., 1997; L'Allemand, et al., 2000; Buchner et al., 2003; Kaupert et al., 2013; Moura-Massari et al., 2016). In order of that, a broad spectrum of phenotypic manifestations can occur, and genotype-phenotype discordances require further investigation. Although the knowledge that most of the cases of CAH are due to 21-OHD, there are several other enzymes that may be defective and responsible for other forms of CAH and to a good clinical diagnosis, analytical characterization should be performed.

4.3 Genetic counselling

Is consensual that genetic studies in CAH patients (classical and non-classical forms) are mandatory for an integrated and complete clinical and genetical evaluation. Family studies should include a detailed family pedigree, mainly siblings and parents, with a complete clinical, biochemical and genetic information of familial members. Those aspects are of major importance for achievement of segregation patterns.

On genetic counselling there are some major factors that should be taken into account concerning to the molecular strategy settled by the laboratory. There is a minimal risk for allele drop-out (ADO), particularly in low concentration DNA biological samples, therefore molecular analysis should be carefully interpreted by certified and experienced individuals at reliable laboratories. The identification of a at risk individual should happen in an appropriate time to ensure treatment options in preconceptionally context. Genetic study of relatives, partner or progenitors, should be ascertain prior to reproduction, when pathogenic variants were identified in the index case. In all circumstances, a hypothetical positive result should be carefully discussed with families before a genetic test, to address questions such as treatment, prognosis, morbidity and mortality. Even, neonatal manifestations should be discussed in the genetic counselling context. NC-CAH individuals are not often detected in newborns screening tests and therefore, their diagnosis is most common during childhood or early adult life, when the phenotype become more evident (Zetterström et al., 2020). Genetic testing for complete gene sequencing, or for target pathogenic variants previously identified in relatives, are recommended. Most frequently the pre-conceptional counselling is indicated, after clinical endocrinology diagnosis confirmation by blood hormone tests. If any risk is identified, prenatal diagnosis (PND) or preimplantation genetic testing (PGT) are available for couples at risk of having a child affected with CAH.

Since the most common type of CAH is due to 21-OHD, *CYP21A2* pathogenic variants are generally investigated, being some of them common among different populations. Particularly, in NC-CAH form patients, more than half of the patients have p.Val282Leu pathogenic variant (New et al., 2013). Less frequently, genes encoding for 11 β -hydroxylase - *CYP11B1*, 3 β -hydroxysteroid dehydrogenase - *HSD3B2* and StAR may also

present pathogenic variants and should further be investigated in case of absence of pathogenic variants in *CYP21A2* or suggestive biochemical findings (Polat et al., 2014). All forms of CAH are transmitted as an autosomal recessive pattern and the determination of the risk is dependent of the carrier status of the patient's parents and obviously, genetic counseling should focus on the future risk of having an affected child (Fig. 6).

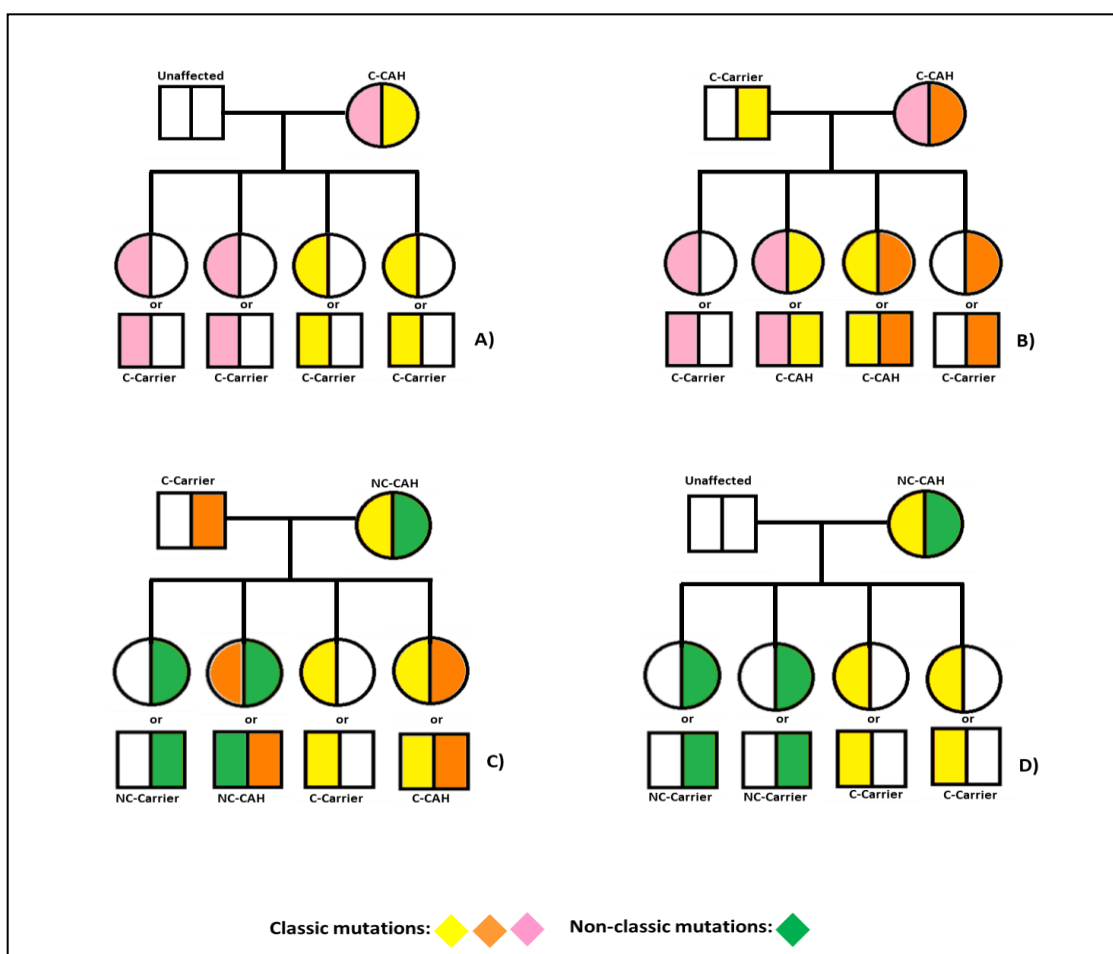


Figure 6 – Genetic risk in the offspring of a patient with CAH. A) If the partner of a patient with classic CAH is unaffected, then all children will be carriers of classic CAH; **B)** If the partner of a patient with classic CAH is a carrier of a classic pathogenic variant, then there is a 50% chance that a pregnancy will result in a child affected with classic CAH; **C)** If the patient has non-classic CAH, but carries a classic gene, and the partner is a carrier of a classic pathogenic variant, then there is a 25% chance of having a child with classic CAH; **D)** If the partner of a patient of non-classic CAH (carrying a classic pathogenic variant) is unaffected, then there is a 50% chance of having children carriers of the classic pathogenic variant.

Different scenarios can be analyzed based on familial information and, although the knowledge that phenotype is determined by the most functional allele, the evaluation of segregation pattern of the mutant alleles is of major importance to achieve the presence in *cis* or *trans* configuration and to evaluate *de novo* pathogenic variants.

In the case of a patient with classic CAH that has a partner that is unaffected, then all children will be carriers of classic CAH. If the partner of a patient with classic CAH is a carrier of a classic pathogenic variant, then there is a 50% chance that a pregnancy will result in a child with classic CAH and 50% of the offspring will be carriers of classic CAH. On the other side, if the patient has NC CAH, but carries a classic gene, and the partner is a carrier of a classic pathogenic variant, then there is a 25% chance of having a child with classic CAH, 25% of chance to having a child with NC-CAH, and 50% of the offspring will be carrier (half with the CAH pathogenic variant and the other half with the NC pathogenic variant). If the partner of a NC-CAH patient (carrying a classic pathogenic variant) is unaffected, then there is a 50% chance of having children's carriers of the classic pathogenic variant and 50% chance of having children's carriers of the NC pathogenic variant (Fig. 6). Even more, in the case where both parents are carriers, the risk for affected offspring is 25%, 25% for unaffected child and 50% of chances for carrier offspring, with different approaches on genetic counselling regarding to the severity of the pathogenic variant present in the carrier. As CAH due to 21-OHD transmits in a monogenic autosomal recessive way, concerns about the gender differences prevalence are not an issue, so the risk for both males and female's offspring is the same.

As the frequency of both forms of the disease is distinct, and the incidence of classic CAH is approximately 1:10.000-1:15.000, with a carrier rate of 1:50-1:71 (median 1:60), the chance of having a child with classic CAH is 1:120, when the partner status is unknown (Speiser et al., 1985; Van der Kamp and Wit 2004; Hannah-Shmouni et al., 2009; Hannah-Shmouni et al., 2017).

Regarding to NC-CAH, about two thirds of patients are compound heterozygotes, carrying one severe allele that causes classic CAH and other less severe that causes NC-CAH.

Reasoned on the basic genetic principles, the presence of one allele carrying a pathogenic variant result in a carrier status for the individual with no manifestation of the disease. Recent literature, also compare data from individual with two normal

CYP21A2 alleles and carriers, carriers may have slightly elevated 17-OHP levels when stimulated with ACTH and a high prevalence of late onset manifestations of hyperandrogenism and PCOS (Guarnotta et al., 2020).

Thus, molecular genetic testing by evaluation of pathogenic variants is of major importance in the clinical context and may improve a successful patient genetic counselling, underscoring the risks and benefits of genetic evaluation (Krone and Arlt, 2009).

In conclusion, genetic counselling should focus on the evaluation of risk of having an affected child and should be carried out by an experienced medical genetic counselor.

4.4 Prenatal diagnosis

The 21-OH deficiency is responsible for the impairment of mineralocorticoids and glucocorticoids, which translates to an elevation of ACTH, accumulation of precursors and shunt to the androgen pathway with consequently excessive androgen production. As adrenocortical function begins to develop around 7th week of gestation, a fetus with classical CAH is exposed to an excess of androgens at critical steps of sexual differentiation. Female fetuses affected with classical CAH show virilization of the external genital, due to excess of adrenal androgens, while female fetuses with NC-CAH are born with normal genitalia, as the prenatal levels of androgens are not high enough to cause genitalia virilization (New, 2006). By opposite, male fetuses are not diagnosed immediately after birth as they do not present genital ambiguity. The major source of androgens in males are the testes, not the adrenals and in order of that, the testes may be small due to suppression of follicle-stimulating hormone and luteinizing hormone from adrenal androgens converted to estrogen, resulting in reduced fertility or infertility (Ogilvie et al., 2006).

Newborn screening programs only identified and diagnosed postnatally infants affected with classical CAH forms of the disease, instead the PND that influence the management of disease during gestation or at birth.

Prenatal diagnosis is an invasive approach that uses fetal DNA from amniotic fluid, obtained by amniocentesis, or chorionic villus sampling, used to test for gender

determination and genetic status of the fetus. PND is recommended for couples who are at risk of having a child affected with CAH. First diagnosis through amniotic fluid evaluation started in 70's and molecular diagnosis was made based on HLA serotyping of cultured amniocytes (Pollack et al., 1979). This strategy presented disadvantages as it could not be performed before second trimester of gestation, when genital organogenesis had already occurred and a mismatch in time for therapeutic to avoid genital ambiguity had already gone.

Evaluation of 17-OHP levels on amniotic fluid was also a strategy previously adopted for CAH prenatal diagnosis. It had presented several limitations such as the diminished ability to detect SV and NC forms of the fetuses and the unreliability to diagnose when the mother was receiving treatment with glucocorticoids, due to the fetal adrenal androgen suppression (Pang et al., 1985).

In parallel, the analysis of chorionic villus sampling (CVS) during first trimester (8-12 weeks of gestation), allowed the evaluation of fetus at risk early in pregnancy, by direct identification of the most common pathogenic variants using several molecular biology approaches. These correlation between genotype and the expected phenotype could anticipate counselling to parents and reasoned decisions regarding to prenatal therapeutic options. Determination of the fetal sex could allow prenatal therapeutic decisions of 46,XX fetuses in risk for virilization, although administration of dexamethasone to the pregnant mother to reduce virilization of the genitalia is controversial and its administration should be minimized and carefully employed (Speiser et al., 2010). Therapy should start early in pregnancy before disease status is known, although a concern exists as there is a chance of exposing unaffected fetuses to unknown risk and the unknown long-term effects of *in utero* glucocorticoid exposure (Speiser et al., 2010; McCann-Crosby et al., 2018). Further, postnatal complication in CAH newborns can be avoid by administration of mineralocorticoids and glucocorticoids replacement at birth.

Current prenatal invasive options are not ideal due to the inability to provide a diagnosis before fetal adrenal organogenesis outset.

The discovery of cell free fetal (cff) nucleic acids in maternal circulation leads the determination of fetal sex from 7th week of gestation by the detection of Y fetal chromosome sequences in maternal plasma (New et al., 2014). Non-invasive techniques

for fetal sex determination prevent the unnecessary treatment of male fetuses, what represents 50% of all fetuses in an autosomal recessive disorder (Rijnders et al., 2001). The identification of fetal DNA on maternal circulation became a challenge because maternal plasma contains just a small fraction of fetal nucleic acids within maternal blood. As a result of this, were developed studies for identification of paternally inherited fetal DNA in maternal blood, that obviously was not present in maternal genome, to ensure the fetal origin (Lo et al., 1998). In 2002, Chiu and colleagues presented a strategy of exclusion of *CYP21A2* mutant alleles based on the inherited of affected or normal paternal allele from a couple carrier of different pathogenic variants and therefore not affected with CAH.

Posteriorly, the use of next generation sequencing technology allows the identification of fetal specific sequences in maternal plasma samples that were absent on maternal genome and deducing paternal contribution. In 2014, New published the first study in which a small cohort of at-risk pregnancies were analysed based on parental haplotypes establishment on *CYP21A2* locus. Those data showed to be promising as cff-DNA was obtained early enough in gestation allowing the starting therapeutic on only those who would benefit.

Thus, this more recent PND approach partially addresses some of the ethical concerns associated with prenatal therapy and, if used, would potentially avoid dexamethasone treatment of male fetuses and unaffected female fetuses.

For the purpose to overcome ethical questions and decision of a termination of pregnancy, the PGT emerged as an alternative that can be offered and represents a solution to decrease and likely disrupt the transmission of severe genetic diseases. Patients with multiple endocrine disease, in which the gene responsible for pathogenic variants is known, are candidates for PGT in order to reduce the chance for a genetic disease from their future offspring prior to pregnancy. Successful PGT in patients with CAH has already been reported however some difficulties are common regarding to single cell amplification and genes involved (Van de Velde et al., 1999; Simpson and Rechitsky, 2017). Those most recent approaches for molecular diagnosis allow single gene diagnosis and concomitantly, the analysis of aneuploidy, providing better pregnancy rates.

I.5. Future steps in CAH management

5.1 Gene therapy and stem-cell transplantation

Suitable treatment of CAH patients is challenging due to the requirement of a continuous and simultaneous control of several physiologic mechanisms. Conventional therapy for CAH requires a life-long glucocorticoid and mineralocorticoid in SW forms and a replacement and prevention of androgens excess production enough to maintain adrenal androgen suppression. Therapeutic strategies as bilateral adrenalectomy or treatment based on circadian hydrocortisone represent controversy because they may represent a complete adrenal insufficiency forcing an effective hormone substitution therapy or a demand of individualized physiological adaptations for a long period of time (Bornstein et al., 2020; Nordenström et al., 2022).

Novel and potential treatments are blooming up to improve the CAH patient's outcome. They include strategies focused on GnRH analogues, anti-androgens and aromatase inhibitors, for which there are ongoing clinical studies to support their use in CAH and aiming a restoration of the physiological feedback regulation of the HPA axis (Loechner et al., 2010; Bornstein et al., 2020).

However, as CAH is a monogenic disorder, and to the best of our knowledge well understood concerning to the pathogenesis and molecular level, gene therapy or stem-cell approaches for the treatment of CAH could be a clue for CAH cure. The gene therapy strategies involve animal models and specific and effective vectors to deliver the gene of interest. Few *in vivo* studies have been reported in adrenal field using reprogrammed mouse or human cells, and the first gene therapy approach for CAH was reported in H-2aw18 mice, a recombinant mouse cell line that harbor a deletion spanning the C4 gene and one of the two 21-OH gene, that shows typical characteristics of 21-OH deficiency. The introduction of a recombinant DNA fragment containing the murine genomic gene for 21-OH allow the rescue of lethal pathogenic variant, showing that this mouse cell line is an effectual model for gene therapy studies (Gotoh et al., 1994). Although, some limitations have been reported due to the lack of functionality to adrenal stimulus probably because of incomplete differentiation and limited steroidogenic potential of

cells before transplantation (Bornstein et al., 2020). Generation of steroid-producing cells directly from adult adrenocortical stem cells/progenitors could be also an option (Steenblock et al., 2018). Approaches involving donor or patient-derived stem cells in which a specific pathogenic variant was altered combined with biomaterials and encapsulation of adrenocortical stem cells in immune-isolation devices may presented as a reliable alternative therapy for patients suffering of CAH or other adrenal insufficiency (Bornstein et al., 2020).

Other studies have examined the selective adrenotropism for adenoviruses using a single intra-adrenal injection of adenoviral vector encoding *CYP21A2*, reenforcing the value of adenoviruses as good vector for gene therapy. These approaches restore the impaired adrenocortical function in 21-OH knockout mice and bovine adrenocortical cells due to functional and morphological changes in the adrenal cortex after transfection with recombinant adenoviruses (Alesci et al., 2002). Those new approaches using adenoviruses targeting 21-OH in the adrenal cortex are vibrant, but specificity, efficiency, and safety on targeting adrenal gland remain unclear. The development of new vectors, non-viral, viral and viral-recombinant (lentivirus, retrovirus, adeno-associated virus, *herpes simplex* virus) are needed for gene transfer in order to ensure a long-term DNA integration (Loechner et al., 2010). Further, the potential use of stem cells to afford functional adrenal cortical tissue may represent a cure for CAH. Stem cell therapy has evolved from early stages of using embryonic cells to advanced application that can repair and restore adrenal functionality. Different approaches have been settled to treat adrenal insufficiency with pluripotent stem cells (PSCs) and generation of human induced steroidogenic cells (hiSCs) from fibroblasts, blood-, and urine derived cells could be used for cell transplantation, after gene-editing. Such cells expressing steroidogenic enzymes are able to produce a wide different steroid hormone *in vitro* (Bornstein et al., 2020). Those strategies prone the creation of model of human disease by reprogramming steroidogenic cells for a curative cell replacement therapy for patients those family of disorders (Ruiz-Babot et al., 2018).

While gene therapy is likely to become an option for the treatment of CAH with these advances, the research specific to the gene therapy in CAH appears to be sparse. As conclusion, gene or stem-cell transplantation therapies could be a feasible option for treatment of CAH in a near future.

II. AIMS

Congenital Adrenal Hyperplasia was probably firstly mentioned in XIX century, by Luigi Crecchio, an Italian anatomist that provided a description of a case of probable CAH. This fact, the called “French disease” was as posteriorly published by Alfred Bongiovanni from De Crecchio ("*Sopra un caso di apparenzi virili in una donna*". Morgagni 7:154–188, 1865) in 1963 for the *The New England Journal of Medicine*. The clinical symptoms, as well as the world distribution of the disease have always been controversial. The molecular analysis was lately an important tool for the clarification of the trickiest clinical genotypes.

Therefore, we aimed to understand the Portuguese panorama on CAH due the most common enzyme deficiency – 21-OH. During several years, on the Genetics unit of Faculty of Medicine at Universidade do Porto were genotyped patients from Endocrinology department and Pediatric Endocrinology department of Centro Hospitalar Universitário de São João, Porto with phenotype or suspicion of CAH 21-OHD. Improvements on the molecular diagnosis were made along the years and coding sequence of the complete gene sequencing was investigated.

PAPER I - Mutational Characterization of Steroid 21-Hydroxylase Gene in Portuguese patients with Congenital Adrenal Hyperplasia. *Experimental Clinical Endocrinology & Diabetes* 2010; 118(8): 505-12.

In order to achieve the frequency of the ten most frequent pathogenic variants in Portuguese CAH patients, 84 individuals were studied: 10 salt-wasters, 6 simple-virilizers and 68 non-classical patients.

PAPER II - Comprehensive genetic analysis and structural characterization of *CYP21A2* mutations in CAH patients. *Experimental Clinical Endocrinology & Diabetes* 2012; 120: 535-539.

The aim of this work was a reanalysis by complete gene sequencing of a small cohort of NC and classic CAH patients previously screened for the 10 most common pathogenic variants, in order to detect additional pathogenic variants that could corroborate the phenotype.

PAPER III - A Multicenter Study on Genotype–Phenotype Correlation from a Portuguese Pediatric Cohort. *Hormone Research Paediatrics* 2019; 19:1-13.

This study reflects a conducting a nationwide genotype description of the CAH pediatric patients from several Portuguese pediatric endocrinology centers and aims to establish their genotype–phenotype correlation.

PAPER IV - The Complexities in Genotyping of Congenital Adrenal Hyperplasia: 21-Hydroxylase Deficiency. Corrigendum in: *Frontiers Endocrinology* 2020; 10: 432. Erratum for: *Frontiers Endocrinology* 2019; 10:432

This work aims to review the main aspects on the clinical and molecular analysis diagnosis of the deficiency of 21-hydroxylase due to *CYP21A2* pathogenic variants.

PAPER V - Congenital Adrenal Hyperplasia due to 21-Hydroxylase Deficiency: An Update on Genetic Analysis of *CYP21A2* gene. *Experimental Clinical Endocrinology & Diabetes* 2020; 128:1-5.

In this short review present an update on the genetic methods on *CYP21A2* gene variants analysis in CAH patients, performed in Genetics Unit of Pathology Department of Faculty of Medicine of University do Porto.

PAPER VI - Differences in hormonal levels between heterozygous *CYP21A2* pathogenic variant carriers, non-carriers, and females with non-classic congenital hyperplasia. *Archives of Endocrinology and Metabolism* 2022; 14:2359-3997000000437.

This study aims to characterize a population of Portuguese females suspected of having non-classic congenital adrenal hyperplasia due to clinical and biochemical criteria and who have undergone *CYP21A2* molecular analysis.

III. PUBLICATIONS

PAPER I

**Mutational Characterization of Steroid 21-Hydroxylase Gene in Portuguese patients
with Congenital Adrenal Hyperplasia**

Experimental Clinical Endocrinology & Diabetes 2010; 118(8): 505-12

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Mutational Characterization of Steroid 21-Hydroxylase Gene in Portuguese patients with Congenital Adrenal Hyperplasia

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Key words

- congenital adrenal hyperplasia
- 21-hydroxylase deficiency
- CYP21A2
- mutational frequency
- genetic diagnosis

Abstract

▼ Congenital adrenal hyperplasia (CAH) due to steroid 21-hydroxylase deficiency is a common inherited disorder of adrenal hormone biosynthesis due to mutations in the 21-hydroxylase gene, *CYP21A2*. Genotyping for ten of the most frequent mutations was performed in 84 Portuguese CAH patients: 10 salt-wasters, 6 simple-virilizers and 68 non-classical patients. The patients were diagnosed by a level of 17-hydroxyprogesterone above 10 ng/ml either in basal conditions or after an ACTH 0.25 mg IV Test. A variety of genotyping techniques were used to detect these ten mutations. *CYP21A2* mutations were

detected in 91.7% (77/84) of the patients. The frequency of alleles carrying two or more *CYP21A2* mutations (9.5% – 16/168) is higher than in other populations. The most frequent mutations identified in our population were V281L (41.7%) and deletions/conversions involving the promoter region of the *CYP21A2* gene (28.3%). A decreased frequency of IVS2-12C/A>G mutation (5.6%) was the most characteristic feature of our population. This study allow the characterization of the mutational spectrum of CAH patients, mainly non-classical CAH, with 21-hydroxylase deficiency from Portugal showing specific genetic features of this population which reveals differences with worldwide countries.

Introduction

▼ Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency is one of the most common autosomal recessive metabolic disorders with an estimated carrier frequency of 1 in 60 (Pang et al., 1988). Defects of the steroid 21-hydroxylase protein prevent the conversion of progesterone to deoxycorticosterone and 17-hydroxyprogesterone to 11-deoxycortisol, resulting in impaired aldosterone and cortisol synthesis, respectively. The decrease in cortisol biosynthesis leads to loss of negative feedback inhibition, increased ACTH secretion, and subsequent androgen excess. Deficiency of both cortisol and aldosterone synthesis results in salt wasting CAH (SW-CAH), the most severe form of the disease, with early manifestation after birth and ambiguous external genitalia in girls. In simple virilizing CAH (SV-CAH), only cortisol synthesis is deficient and is manifested by subclinical salt loss, different degree of virilization of external genitalia in girls and early precocious pubarche. Additionally, if stressed, simple virilizers may have mild salt loss. Nonclassical CAH (NC-CAH), also called late-

onset CAH, is characterized primarily by signs of androgen excess such as premature pubarche, hirsutism, menstrual disturbances, and infertility (reviewed in Merke and Bornstein, 2005). This disorder is caused by mutations in the *CYP21A2* gene, that encodes an adrenal microsomal cytochrome P-450 enzyme specific for steroid 21-hydroxylation (P-450c21 or P-450XXI) (Carroll et al., 1985). *CYP21A2* is located in the highly polymorphic HLA histocompatibility complex on chromosome 6p21.3, along with a pseudogene, *CYP21A1P* (White et al., 1986). Both *CYP21A2* and *CYP21A1P* contain 10 exons spaced over 3.1 kb and are located adjacent to and alternating with the *C4B* and *C4A* genes encoding the fourth component of serum complement (White et al., 1985). Their sequences are 98% identical in exons and approximately 96% identical in introns (Rodrigues et al., 1987). Over 95% of the mutations represent intergenic recombinations between *CYP21A2* and *CYP21A1P*. Approximately 75% represent deleterious mutations found in the pseudogene that are transferred to *CYP21A2* during mitosis by gene conversion. About 20% are meiotic recombinations due to unequal cross-

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ing over during meiosis which can produce a wide variety of rearrangements depending on the breakpoints, such as gene duplications and gross gene deletions encompassing the C4 and CYP21A2 (White et al., 1988; Speiser et al., 1992; Tusie-Luna et al., 1995; Higashi et al., 1998). One commonly reported deletion is the deletion of a 30-kb segment that includes the 3' end of CYP21A1P, all C4B, and the 5' end of CYP21A2. This allele carries a single non-functional chimeric gene with its 5' and 3' ends corresponding to CYP21A1P and CYP21A2, respectively (White et al., 1988; Lee et al., 2000). More than 100 additional mutations account for the remaining 5% (reviewed in Speiser and White, 2003). One to two percent of affected alleles are *de novo* mutations (Speiser et al., 1992).

The clinical severity of the disease is determined by the extent of the impaired enzymatic activity. Typically, the phenotype corresponds to the less severely affected alleles. Mutations are generally classified as severe, moderate or mild. Hence, the salt wasting patients typically have severe/severe genotypes, simple virilizing patients will have severe/moderate or moderate/moderate genotypes, and nonclassic patients can have severe/mild, moderate/mild or mild/mild genotypes. However, in about 10–20% of cases, the correlation between phenotype and genotype is poor (Speiser et al., 1992; Wedell et al., 1994; Krone et al., 2000).

According to The Human Gene Mutation Database (HGMD)-2009, more than 118 different mutations have been reported within the CYP21A2 gene. Large gene conversions, gene deletions, an 8bp deletion in exon 3, F306-L307insT, Q318X and R356W are severe mutations that result in complete inactivation of 21-hydroxylase activity. The splice mutation in the second intron (IVS2-12C/A>G) and the I172N represent moderate mutations. The first one results in very low but measurable enzyme levels and the I172N mutation results in 1% of normal enzymatic activity. V281L and P30L represent mild mutations that have been associated with 30–40% of residual enzymatic activity (reviewed in White and Speiser, 2000).

The aim of this study was to determine the CYP21A2 mutation profile in a cohort of Portuguese Congenital Adrenal Hyperplasia patients, by assaying for the 10 most frequent CYP21A2 gene mutations.

Material and methods

Patients

CYP21A2 gene analysis was performed in 84 Portuguese patients with CAH due to 21-hydroxylase deficiency, diagnosed at S. João Hospital in Porto. Fifty-four relatives, corresponding to the families of 29 patients, were also studied. Patients were diagnosed according to standard clinical criteria, including determination of serum 17-hydroxyprogesterone (17OHP) before and after ACTH stimulation. Patients with SW-CAH were characterized by the presence of adrenal crisis (hyperkalemia, hyponatremia and dehydration), virilised external genitalia in females and high levels of serum 17OHP concentration. Patients with SV-CAH were characterized by the presence of ambiguous genitalia or clitoromegalia in girls, no salt wasting crisis, pseudoprecocious puberty, growth acceleration, advanced bone age, and elevated levels of serum 17OHP. Patients with NC-CAH were identified by mildly basal elevated levels of serum 17OHP (≥ 10 ng/ml) and by signs of androgen excess as hirsutism, menstrual disturbance and infertility (White and Speiser, 2000). Ten patients were clas-

sified as salt wasters, 6 as simple virilizers and 68 as non-classical patients. Seventy-one of these patients were females and thirteen were males. The discrepancies in the number of patients with different forms of the disease result from the fact that most of the patients referred to our Department for mutational screening are adult females, diagnosed in the Department of Endocrinology of S. João Hospital, presenting signs and symptoms of androgen excess.

To analyse the frequency of the missense variant A265V in the general population, we screened 105 individuals (210 chromosomes), consisting of 40 males and 65 females, of a control population.

Sample preparation and mutational analysis

DNA was extracted from peripheral blood leukocytes using a proteinase K/SDS digestion protocol followed by salting out/chloroform extraction as described before (Mullenbach et al., 1989).

Genotyping for 10 known disease-causing mutations was performed by Polymerase Chain Reaction – Sequence Specific Primer (PCR-SSP), Restriction Fragment Length Polymorphism (RFLP) or direct DNA sequencing. The 10 mutations ascertained included large deletions or conversions events involving the promoter region of the CYP21A2 gene (del/conv of promoter region), P30L in exon 1, an intron 2 splicing mutation (IVS2-12C/A>G), an 8bp deletion in exon 3 (G110Δ8nt), I172N in exon 4, cluster of three point mutations (L236N, V237E, M239K) in exon 6 (E6 cluster), V281L and a T-insertion (F306-L307insT) in exon 7, and Q318X and R356W in exon 8 (◊ Fig. 1).

Detection of large deletions/conversions involving the CYP21A2 promoter region

Large deletions/conversions involving the promoter region of the CYP21A2 gene were detected by a PCR-based assay with primers SAR5' and SAR3' (Table 1), followed by digestion with *Taq I* restriction enzyme (Siegel et al., 1995). Although the PCR product is a mixture of both CYP21A2 and CYP21A1P sequences, the CYP21A1P amplicon contains two *Taq I* restriction sites at positions -740 and -210, and CYP21A2 amplicon contains only the -210 *Taq I* site (◊ Fig. 1). After *Taq I* digestion, the ratio of CYP21A2/CYP21A1P could be estimated by the relative intensity of the fragments after gel electrophoresis and ethidium bromide staining, using the Image Master TotalLab software (Pharmacia Biotech). PCR reaction was performed in a total volume of 25 µl. Reaction mixture contains 1X REDTaq PCR Reaction Buffer, 100 µM of each dNTP, 0.3 µM of each primer, 1 µl of DNA at a concentration of 100 ng/µl, and 0.5 units of REDTaq DNA Polymerase (Sigma). Samples were subjected to denaturation at 94 °C for 3 min followed by 30 cycles of 94 °C for 30s, 60 °C for 30s and 72 °C for 1 min, ending by extension at 72 °C for 7 min. PCR generated a CYP21A2 product of 656bp and a CYP21A1P product of 659bp. These products were digested with the restriction enzyme *Taq I* (Bioron) and analysed by electrophoresis on a 1.5% agarose gel. The CYP21A2 products generated a 582bp and a 74bp fragments and the CYP21A1P products generated a 532bp, a 74bp and a 53bp fragments. In normal subjects, equal-intensity 582bp and 532bp bands could be detected. If one of the CYP21A2 alleles was deleted or resulted from a conversion involving the promoter region, the intensity of the 582bp band decreased relative to that of the 532bp (◊ Fig. 2B). If both the CYP21A2 alleles were deleted/converted, only the 532bp band could be detected (Siegel et al., 1995). This method does not dis-

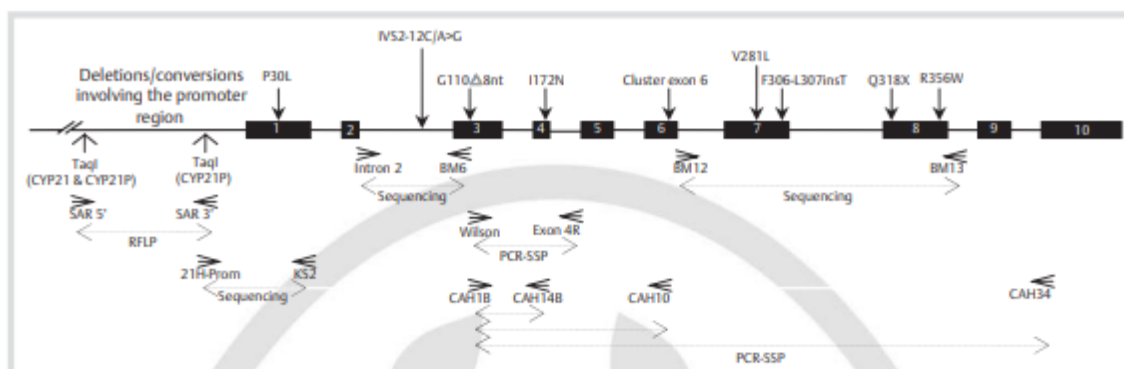


Fig. 1 Schematic diagram of the structure of the *CYP21A2* gene and the methodology used for the detection of 10 mutations causing 21-hydroxylase deficiency. Numbered black boxes represent *CYP21A2* exons. Horizontal arrows show the position of primers used for the six different PCR amplifications. Double arrows represent PCR amplified fragments. Restriction site positions for *Taq I* restriction enzyme in *CYP21A2* and *CYP21A1P* are also represented.

Table 1 PCR primers used for the analysis of the *CYP21A2* gene.

Primer	Sequence	Sense	Specificity	Position	Mutation
SAR 5'	5'-CTC AAA CCA GCT CAA GGT GGG G-3'	+	<i>CYP21A2</i>	Promotor	A Del/Conv
SAR 3'	5'-AGT CTC ATT GGC CTT GGG ACG TG-3'	-	<i>CYP21A2</i> & <i>CYP21A1P</i>	Promotor	A
21H-Prom	5'-CGG ACA CTA TTG CCT GCA CA-3'	+	<i>CYP21A2</i>	Promotor	B P30L
KS-2	5'-ACC CTC TCC GTC ACC TCG-3'	-	<i>CYP21A2</i> & <i>CYP21A1P</i>	Intron 1	B
Intron 2	5'-TGG GCA GAC TTT GCT GGC AGA G-3'	+	<i>CYP21A2</i> & <i>CYP21A1P</i>	Exon 2	C IVS2-12C/A>G
BM6	5'-CCA GAG CAG GGA GTA GTC TG-3'	-	<i>CYP21A2</i>	Exon 3	C
Wilson ¹	5'-ACT ACC CGG ACC TGT CCT TGG TC-3'	+	<i>CYP21A1P</i> *	Exon 3	D G110Δ8nt
Exon 4R	5'-TCG TCC TGC CAG AAA AGG AG-3'	-	<i>CYP21A2</i>	Intron 4 & Exon 5	D
ApoB1F	5'-CTC TGG GAG CAC AGT ACG AAA AAC CAC TT-3'	+	APOB	-	D
ApoB1R	5'-AAT GAA TTT ATC AGC CAA AAC TTT TAC AGG-3'	-	APOB	-	D
CAH14B ²	5'-CTT GTC TCC GAA GGT GAG GTA ACT GT-3'	-	<i>CYP21A1P</i> *	Exon 4	E I172N
CAH10 ³	5'-TGC TGC CTC AGC TGC TTC TCC TCG T-3'	-	<i>CYP21A1P</i> *	Exon 6	E Cluster Exon 6
CAH1B	5'-CCG GAC YTG TCC TTG GGA GAC TAC TG-3'	+	<i>CYP21A2</i>	Exon 3	E
CAH34	5'-CTC TGG AGT TCT TGC CTG GCT CCA GG-3'	-	<i>CYP21A2</i> & <i>CYP21A1P</i>	Exon 10	E
BM12	5'-GAG GGA TCA CAT CGT GGA GAT-3'	+	<i>CYP21A2</i>	Exon 6	F V281L, F306-L307insT, Q318X, R356W
BM13	5'-GCT TTC CTC ACT CAT CCC CAA-3'	-	<i>CYP21A2</i> & <i>CYP21A1P</i>	Intron 8	F

^{1,2,3} Sequence-specific primers. Nucleotides in bold indicates modification sites to confer mutation specificity

(*) denote mutations generated by intergenic recombinations between *CYP21A2* and *CYP21A1P*. Therefore, these primers will have specificity for the *CYP21A1P* and for the G110Δ8nt, I172N and E6 cluster mutated *CYP21A2*, respectively. A-F correspond to different PCR reactions. Y (CAH1B) correspond to a mixture of C and T

tinguish between a total gene conversion and a *C4+CYP21A2* deletion since the promoter region of both arrangements encompasses the *Taq I* site at position -210. Moreover, a partial gene conversion involving the promoter region can also encompass the *Taq I* site.

Sequencing

The P30L, IVS2-12C/A>G, V281L, F306-L307insT, Q318X and R356W mutations were detected by direct DNA sequencing. For P30L detection, a 562bp fragment was generated with primers 21H-Prom and KS2 (Table 1), encompassing whole exon 1 and the promoter-intron boundary (Siegel-Witchel et al., 1996). The PCR reaction was done in a total volume of 50μl using 1μl of genomic DNA (100ng/μl), in 1X Buffer, 25 mM of MgCl₂, 100μM of each dNTP, 0.5μM of each primer and 1.25 units of Taq Polymerase (Fermentas). Samples were subjected to denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 30s, 64 °C for 30s and 72 °C for 2 min, ending by extension at 72 °C for 7 min.

For detection of IVS2-12C/A>G mutation, a 377 bp fragment was generated with primers *intron 2* and *BM6* (Table 1), covering whole intron 2 and exon-exon boundaries. PCR reaction was carried out in 50μl reaction mixture containing 1X PCR Buffer, 100μM of each dNTP, 0.5μM of each primer, 1μl of genomic DNA (100ng/μl) and 1.25 units of AmpliTaq Polymerase (Applied Biosystems). Samples were subjected to denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 30s, 64 °C for 30s and 72 °C for 2 min, ending by extension at 72 °C for 7 min.

For V281L, F306-L307insT, Q318X and R356W detection, an 824bp fragment was generated with primers *BM12* and *BM13* (Table 1), covering part of exon 6 and whole exons 7 and 8. PCR reaction was carried out in 50μl reaction mixture containing 1X PCR Buffer, 100μM of each dNTP, 0.5μM of each primer, 1μl of genomic DNA (100ng/μl) and 1.25 units of AmpliTaq Polymerase (Applied Biosystems) (Siegel et al; 1994). Samples were subjected to denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 30s, 64 °C for 30s and 72 °C for 2 min, ending by extension at 72 °C for 7 min.

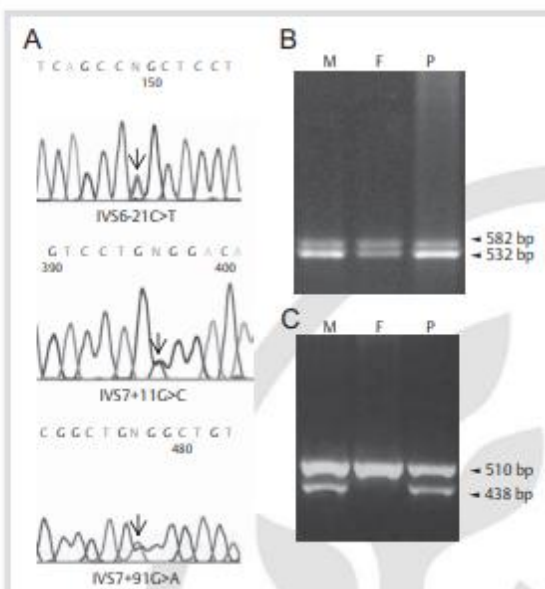


Fig. 2 Genotyping of a simple-virilizing patient and their parents for three intronic variants (A), deletions/conversions involving the promoter region of the *CYP21A2* gene (B) and G110Δ8nt mutation (C). (A) DNA sequences showing the variant IVS6-21C>T in intron 6 and the variants IVS7+11G>C and IVS7+91G>A in intron 7 (arrows). All of them were detected in the proband and in his father. (B) Analysis for the detection of deletions/conversions involving the promoter region of the *CYP21A2* gene by PCR with primers SAR 5' and SAR 3' followed by digestion with *TaqI* restriction enzyme. The digested fragments were analysed in a 1.5% agarose gel. The proband (P) and his mother (M) show higher intensity of the 532bp band (*CYP21A2P*) relative to the 582bp band (*CYP21A2*). They are carriers of a del/conv of promoter region in one of the alleles. (C) Detection of the G110Δ8nt mutation by PCR-SSP. The proband (P) and his mother (M) are carriers of G110Δ8nt. The PCR products were analysed in a 1.5% agarose gel. Lane M, mother; lane F, father; lane P, proband.

The PCR products were purified with a commercially available *GFX™* PCR DNA and Gel Band Purification Kit (GE Healthcare) and sequenced using the *BigDye® Terminator v1.1 Cycle Sequencing Kit* (Applied Biosystems). The reaction products were analysed on an automated *ABI PRISM™ 310 Genetic Analyser* (Applied Biosystems).

PCR-SSP

G110Δ8nt, I172N and E6 cluster mutations were detected by PCR-SSP (○ Fig. 2C; ○ Fig. 3). This method is designed only to screen the presence of the mutations and it cannot distinguish their heterozygous or hemi/homozygous state. For the detection of G110Δ8nt mutation, primers Exon 4R, *Wilson*, *ApoB1F* and *ApoB1R* were used (Table 1). Exon 4R is an upstream primer specific for the functional *CYP21A2* gene (Witchel et al., 1997). *Wilson* is a primer specific for G110Δ8nt mutation since it only binds when the mutation is present. In presence of the G110Δ8nt mutation, *Wilson* and Exon 4R primers generate a 438bp product (○ Fig. 2C). *ApoB1F* and *ApoB1R* are used as an internal control (product of 510bp) for apolipoprotein B gene to assure PCR amplification. The PCR reaction was done in a total volume of 25μl using 1μl of genomic DNA (100ng/μl), 1X REDTaq PCR Reaction Buffer, 100μM of each dNTP, 0.5μM of each primer, and 0.5 units of REDTaq DNA

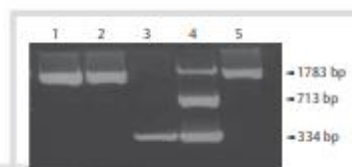


Fig. 3 Analysis of the I172N in affected patients by PCR-SSP with primers *CAH1B*, *CAH14B*, *CAH10* and *CAH34*. Primers *CAH1B* and *CAH34* generate a control band of 1783bp. In presence of I172N, primers *CAH1B* and *CAH14B* generate a 334bp band and in presence of exon 6 cluster mutations, both primers *CAH1B* and *CAH10* generate a 713bp. This method only screens the presence/absence of the mutations and it cannot distinguish between heterozygosity and hemi/homozygosity. Lane 1, lane 2 and lane 5, negatives samples for I172N and exon 6 cluster; lane 3, positive for I172N mutation; lane 4, positive control for I172N and exon 6 cluster mutations.

Polymerase (Sigma). The PCR products were separated in a 2% agarose gel stained with ethidium bromide.

For the detection of both I172N and E6 cluster mutations, a single PCR reaction was performed with primers *CAH14B*, *CAH10*, *CAH1B* and *CAH34* (Rothberg et al., 1998) (Table 1). *CAH1B* is the upstream primer that provides specificity for the functional gene because the pseudogene has the G110Δ8nt mutation in this region. *CAH14B* and *CAH10* are primers specific for I172N and the E6 cluster, respectively. They generate a 334bp and/or a 713bp products in presence of I172N and/or E6 cluster, respectively (○ Fig. 3). *CAH34* is a downstream non-discriminating control primer to show amplification in the absence of both mutations; used with *CAH1B* generate a control fragment of 1783bp. PCR reaction was carried out in 20μl reaction mixture containing 1X AccuPrime PCR Buffer II, 0.9μM of *CAH14B* primer, 0.45μM of *CAH10* primer, 0.9μM of *CAH34* primer, 2.1μM of *CAH1B* primer, 1μl of genomic DNA at a concentration of 100ng/μl and 0.3 units of AccuPrime Taq DNA Polymerase HIFI (Invitrogen). The PCR products were separated in a 1.5% agarose gel.

RFLP

For the analysis of the missense variant A265V, the PCR product obtained with the primers *BM12* and *BM13* (Table 1) was digested with *HinPII* (New England Biolabs; normal fragments: 267bp, 466bp, 90bp; mutant fragments: 733bp, 90bp), and the digestion products were separated and visualized in 2% agarose gel stained with ethidium bromide.

Results

In the present study, 84 patients from Portugal with CAH due to 21-hydroxylase deficiency were genotyped. Additionally, 54 relatives, mainly parents ($n=44$) but in few cases siblings ($n=8$) and offspring ($n=2$) were genotyped to evaluate allele segregation. Mutations in the *CYP21A2* gene were detected in 91.7% of the patients (77/84). From all patients analysed, 57.1% (48/84) presented 2 mutations, 15.5% (13/84) presented 3 mutations, 1.2% (1/84) presented 4 mutations and 1.2% (1/84) presented 5 mutations. Contrary, 8.3% (7/84) did not present any mutation and 16.7% (14/84) presented a single mutation (Table 2). The most frequent mutation was V281L (41.7%), followed by del/conv of promoter region (28.3%), Q318X (10.1%), IVS2-12C/A>G

Table 2 Genotype and phenotype in 84 Portuguese patients with CAH due to 21-hydroxylase deficiency.

Genotype	N° of patients	Expected phenotype	Observed phenotype			Relatives
			SW	SV	NC	N° of individuals
del/conv promoter region; G110Δ8nt/del/conv promoter region	1	SW	1	0	0	1
del/conv promoter region; G110Δ8nt/G110Δ8nt	1	SW	1	0	0	2
del/conv promoter region; G110Δ8nt/Q318X	1	SW	1	0	0	2
G110Δ8nt/F306-L307insT	1	SW	1	0	0	1
Q318X/R356W/Q318X	1	SW	1	0	0	2
Q318X/Q318X	1	SW	1	0	0	2
IVS2-12C/A>G/Q318X	1	SW/SV	1	0	0	2
IVS2-12C/A>G/IVS2-12C/A>G	2	SW/SV	1	1	0	2
del/conv promoter region/I172N	2	SW/SV	0	2	0	1
IVS2-12C/A>G/I172N	2	SW/SV	1	1	0	4
del/conv promoter region/V281L	21	NC	0	0	21	2
del/conv promoter region; Q318X/V281L	1	NC	0	0	1	1
del/conv promoter region; F306-L307insT; Q318X; R356W/V281L	1	NC	0	0	1	0
del/conv promoter region; Q318X; R356W/V281L	1	NC	0	0	1	0
del/conv promoter region; I172N/V281L	1	NC	0	0	1	0
del/conv promoter region; V281L/V281L	5	NC	0	0	5	5
G110Δ8nt/V281L	1	NC	0	0	1	0
G110Δ8nt; Q318X/V281L	1	NC	0	0	1	0
IVS2-12C/A>G/V281L	2	NC	0	0	2	2
F306-L307insT/V281L	1	NC	0	0	1	0
Q318X/V281L	5	NC	0	0	5	2
Q318X; R356W/V281L	1	NC	0	0	1	0
P30L/-	1	NC	0	0	1	0
V281L/V281L	8	NC	0	0	8	1
del/conv promoter region/-	2	normal	0	0	2	3
del/conv promoter region; G110Δ8nt/-	1	normal	0	1	0	2
I172N/-	2	normal	0	1	1	7
V281L/-	8	normal	0	0	8	3
Q318X/-	1	normal	0	0	1	2
-/-	7	normal	1	0	6	5
total	84		10	6	68	54

(-) No mutation detected

(5.6%), G110Δ8nt (4.8%), I172N (4.4%), R356W (2.4%), F306-L307insT (1.9%) and P30L (0.9%) (Table 3). The E6 cluster of mutations was not detected in this study (Table 3). From the 54 relatives, 41 (76%) showed CYP21A2 gene mutations (always the same as in the index case).

Homozygosity was found in 11/84 patients (13.1%) and compound heterozygosity was found in 51/84 patients (60.7%). Complete genotypes and clinical phenotypes from all patients are shown in Table 2.

Twenty-nine of the 84 patient's relatives were available to verify segregation of alleles and confirm the determination of the patient genotype. For the remaining patients, the genotype was determined on the basis of the mutations detected and the well-defined clinical phenotype of the patient. Most of these patients had NC-CAH and carried several severe mutations and the mild V281L mutation. For this reason, it was assumed that V281L was present as a single mutation in one allele and the other chromosome carried the multiple mutations.

The genotype [del/conv of the promoter region/V281L] was the most frequent, occurring in 25% (21/84) of the patients. All of them had NC-CAH, as would be expected due to the presence of the mild V281L mutation in one allele. The second most frequent genotype was [V281L/V281L], present in 9.5% (8/84) of the patients (Table 2).

The overall genotype-phenotype correlation was 75% (63/84) and 21 patients did not show the expected phenotype (Table 2).

Sixteen alleles, corresponding to sixteen patients, carried more than one mutation, corresponding to 9.5% (16/168) of the alleles with multiple mutations in this population. The most frequent complex haplotype was [del/conv of promoter region; V281L] which was observed in 5 of the 168 alleles analysed, followed by [del/conv of promoter region; G110Δ8nt] in 4 alleles and [Q318X; R356W] in 2 alleles. All the remaining complex haplotypes analysed, namely [del/conv of promoter region; Q318X], [G110Δ8nt; Q318X], [del/conv of promoter region; I172N], [del/conv of promoter region; Q318X; R356W] and [del/conv of promoter region; F306-L307insT; Q318X; R356W] were present in only one allele (Table 2).

Direct sequencing of CYP21A2 gene revealed some already described polymorphisms and their observed allelic frequencies are shown in Table 4. The 1675C>T (-4AUG), 3bp INS (Leu10INS) and 3323 G>C (S268T) have been reported as polymorphisms. S268T and the C to T transition at position -4 retained full enzymatic activity after transfection [21] (Wu and Chung, 1991; Wedell et al., 1992; Nikoshkov et al., 1997). LEU10INS was reported in one normal allele and it seems to be a polymorphism of 21-hydroxylase generating a protein with either 494 or 495 aminoacids (Rodrigues et al., 1987).

The missense variation A265V in exon 7 of CYP21A2 gene was found only in three unrelated female NC patients (Table 4). One female carried A265V in association with a del/conv and Q318X in the same allele, and the mild V281L mutation in the other

Table 3 Allelic frequencies of ten *CYP21A2* gene mutations in 84 Portuguese patients with CAH due to 21-hydroxylase deficiency. The mutations are grouped according to the predicted enzymatic activity.

Type	Enzyme activity	Mutation	Alleles studied	Alleles with mutation	N° of alleles in SW-CAH patients	N° of alleles in SV-CAH patients	N° of alleles in NC-CAH patients	Allelic frequency (%)
severe	null	del/conv of promoter region	138	39	4	3	32	28.3
		G110Δ8nt	168	8	5	1	2	4.8
		E6 cluster	160	0	0	0	0	0
		F306-L307insT	162	3	1	0	2	1.9
		Q318X	168	17	6	0	11	10.1
		R356W	168	4	1	0	3	2.4
moderate	potentially not null	IVS2-12C/A>G	160	9	4	3	2	5.6
		I172N	160	7	1	4	2	4.4
mild	residual	P30L	108	1	0	0	1	0.9
		V281L	168	70	0	0	70	41.7

Table 4 Allelic frequency and description of sequence variations/polymorphisms found by sequencing analysis in 84 Portuguese patients with CAH due to 21-hydroxylase deficiency.

	Position of nucleotide substitution ¹	Position of aminoacid substitution	Gene position	Alleles studied	Alleles with variation	Allelic frequency (%)	HGMD accession number	NCBI SNP reference
upstream	1675 C>T		Promoter region	158	10	6.3	CR921046	–
non-synonymous-coding	3-bp INS	Leu10INS	E1	158	119	75.3	–	–
	3323 G>C	S268T	E7	168	19	11.3	CM994664	rs6472
	3314 C>T	A265V	E7	168	3	1.8	–	–
synonymous-coding	1793 T>C	L39L	E1	158	11	7.0	–	rs6468
	1813 A>C	P45P	E1	154	30	19.5	–	rs6464
	3264 C>G	L248L	E7	168	85	50.6	–	rs6477
intronic variants	IVS6 + 12A>G		I6	152	36	23.7	–	rs6458
	IVS6 + 13C>T		I6	152	36	23.7	–	rs6459
	IVS6 + 21C>T		I6	168	5	3	–	rs6465
	IVS7 + 11G>C		I7	168	4	2.4	–	rs6442
	IVS7 + 91G>A		I7	168	1	0.6	–	rs6444

¹ GenBank Ref. ID M12792

allele. In the other one, A265V was detected with V281L in the same allele, presenting the severe Q318X mutation in the other chromosome. The third one presented a [del/conv;V281L/V281L] genotype but allele segregation was not possible since parents were not available for analysis. The presence of the alteration A265V was screened by restriction enzyme analysis in 105 control individuals and was found in heterozygosity in two of them (one male and one female), resulting in an allelic frequency of approximately 1%.

The IVS6 + 12A>G and IVS6 + 13C>T variants represent consecutive base changes and are always associated. They are normally present in the *CYP21A1P* and have been detected with a high frequency during this study (23.7%).

The remaining three substitutions, IVS6 + 21C>T, IVS7 + 11G>C and IVS7 + 91G>A (○ Fig. 2A) were detected with lower frequency (≤3%) and are not present in the *CYP21A1P*. The silent mutation 3264 C>G is present in the *CYP21A1P* and usually appears in association with the IVS6 + 12A>G and IVS6 + 13C>T intronic variants. It is also present with a very high frequency (50.6%) in this study.

Discussion

In this study we describe the spectrum and frequency of mutations as well as the genotype-phenotype correlation in a cohort of 84 patients with CAH due to 21-hydroxylase deficiency from Northern Portugal. Most of the patients (81% – 68/84) were diagnosed with the non-classic form of the disease, being referred to the Endocrinology due to endocrine disturbances caused by androgen excess.

Severe mutations of the *CYP21A2* gene were detected in 43.5% (73/168) of the chromosomes and are present on at least one allele in 61.8% (42/68) of NC patients here studied.

V281L is the most frequent mutation, present in 41.7% of all alleles and in 51.5% (70/136) of alleles from NC-CAH patients. In fact, 83.8% (57/68) of the NC-CAH patients presented this mutation. This result is in accordance with the mean allelic frequency of 40–50% reported for V281L mutation in NC-CAH patients (Ezquieta et al., 1995; Krone et al., 2000; Speiser et al., 2000; Deneux et al., 2001; Friães et al., 2006; Wilson et al., 2007). Furthermore, this result is in concordance with the previous reported higher prevalence of the V281L mutation and the NC

form of the disease in Southern Europe and North Africa when compared with Northern Europe (Kostaska et al., 2003). The two point mutations, I172N and Q318X, seem to vary in frequency along with the North-South axis in Europe: the low frequency for I172N found in this study is similar to that reported in Spain and Italy, respectively (Ezquieta et al., 2002; Carrera et al., 1996), and much lower than in Sweden, Finland and Germany, respectively (Wedell et al., 1994; Levo and Partanen, 1997; Krone et al., 2000). However, the decreased frequency of this mutation can be explained by the underrepresentation of patients presenting the classic form of the disease in this study, especially the simple-virilizing form, since this is a typical mutation of the SV form. The Q318X mutation is more frequent in Northern Portugal population than in North European countries, but similar to Italy, Spain and Portugal, respectively (Carrera et al., 1996; Ezquieta et al., 2002; Friães et al., 2006). The high frequency of G110Δ8nt mutation found in this study differs from most studies worldwide except for the higher frequency (7.4%) reported in the Czech Republic population (Kostaska et al., 2003). The high frequency of these two mutations, Q318X and G110Δ8nt, is particularly interesting since these are severe mutations typical of classic forms of the disease. Since the number of classic patients in our study is small, this observation might represent a clear increase in Q318X and G110Δ8nt mutations, even in patients presenting milder forms of the disease. This variability in mutation frequencies may also reflect the different origins of the populations due to the migration processes throughout the centuries. Surprisingly, our study presents a very low frequency for the IVS2-12C/A>G mutation (5.6%), comparing to other populations (Wilson et al., 2007). In most populations, IVS2-12C/A>G is one of the most common mutations identified (Wedell et al., 1994; Lako et al., 1999; Stikkelbroeck et al., 2003; Dolzan et al., 2005) and it is even the most frequent mutation in the United States, Mexican, German and Japanese populations, respectively (Wilson et al., 1995; Ordoñez-Sánchez et al., 1998; Asanuma et al., 1999; Krone et al., 2000). This difference could be explained by the under representation of the classic form of the disease in our cohort of patients. Nevertheless, there may be a lower frequency of the IVS2-12C/A>G mutation in South Europe countries, as have been noted in previous studies of Spanish and Portuguese populations, with an IVS2-12C/A>G frequency of 17.5% and 9.8%, respectively (Ezquieta et al., 2002; Friães et al., 2006).

This study shows a high frequency (9.5%) of alleles with multiple mutations. This result differs from many studies (Wedell et al., 1994; Wilson et al., 1995; Asanuma et al., 1999; Deneux et al., 2001; Stikkelbroeck et al., 2003; Dolzan et al., 2005; Friães et al., 2006) where the percentage of alleles with two or more mutations range from 2 to 5.5%. However, it has also been described a high frequency (16.2%) of alleles carrying multiple mutations in the Portuguese-related Brazilian population (Witchel et al., 2000). It is likely that complex alleles with two or more mutations resulted from large gene conversions or multiple mutation events.

In the present study, concordance between genotype and phenotype was observed in 75% (63/84) of the patients. Additionally, in patients carrying mutations on both alleles, we obtained a total concordance between genotype and phenotype. Twenty-one patients presented only one or no allele mutated. These patients are potential candidates to carry undetected mutations present in a non-studied region of the CYP21A2 gene that might

adversely affect activity and/or to carry mutations within the regulatory region of the gene. It has been described that the regulatory region of the pseudogene has at least five times less transcriptional activity as compared with the promoter region of the functional CYP21A2 gene (Chang and Chung, 1995). It is also possible that the NC phenotype in patients with only one mutation depends on individual variations such as modifying genes.

Additionally, some sequence variations were detected in these CAH patients. The screening of the alteration A265V in 105 controls resulted in an allelic frequency of 1%. This alanine is not conserved in 5 species analysed, human, pig, bovin, mouse and rat, evidencing that this alteration is probably a polymorphism rather than a mutation. A recent study, based on functional *in vitro* activity of mutant A265V, confirmed that A265V is an allelic variant rather than a disease-causing amino acid change. Additionally, the location of A265 on the protein molecule, analysed on a computerized CYP21A2 structural model reveals that the amino-acid change to valine does not have a significant impact on protein structure which supported the *in vitro* results (Bleicken et al., 2008).

In conclusion, the mutation screening of CYP21A2 gene used in this study allowed the identification of mutations in 91.7% of the patients, with a genotype/phenotype correlation of 75%. The results obtained in Northern Portuguese population showed a high frequency of V281L and deletion/conversion mutations and a very low frequency for the IVS2-12C/A>G mutation, with severe mutations being observed in about 67% of non-classic patients. Overall, this study highlights the importance of genetic diagnosis of 21-hydroxylase deficiency as a complement to clinical analysis and as a pre-requisite for appropriate genetic counselling.

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Conflict of Interest: None.

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PAPER II

Comprehensive genetic analysis and structural characterization of *CYP21A2* mutations in CAH patients

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Comprehensive Genetic Analysis and Structural Characterization of CYP21A2 Mutations in CAH Patients

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Key words

- CYP21A2
- congenital adrenal hyperplasia
- 21-hydroxylase
- novel mutation

Abstract

▼ Congenital Adrenal Hyperplasia (CAH) due to 21-hydroxylase deficiency is a common autosomal recessive disorder caused by mutations in the steroid 21-hydroxylase gene (*CYP21A2*). Complete DNA sequencing of *CYP21A2* was performed in 5 patients, 3 non-classic and 2 classic forms of the disease, that were previously screened for the 10 most common mutations, in order to detect additional mutations that could justify the phenotype of the patients. 5 mutations were identified with the whole gene extended analysis. The mutations, p.Pro432Leu

and p.Ala434Glu, the first previously reported by our group and the second a novel one were structurally analyzed with ICM-Pro software regarding biochemical properties such as protein stability, accessibility to surface and hydrophobicity, in order to elucidate their effects on the *CYP21A2* protein. The 2 affected residues, Pro432 and Ala434, were also studied for conservation purposes in order to predict the severity of both mutations with PolyPhen-2 software and were considered as "probably damaging". Prediction of clinical severity, based on molecular modeling and sequence conservation, was in accordance with the patient's clinical diagnosis.

Introduction

▼ Congenital Adrenal Hyperplasia (CAH) is an autosomal recessive disorder caused by a partial or complete deficiency of one of the several enzymes required in the steroidogenic pathways. 90-95% of the cases of CAH are due to deficiency of 21-hydroxylase, one of the enzyme members of the cytochrome P450 family, that catalyzes the conversion of 17-hydroxyprogesterone (17-OHP) to 11-deoxycortisol, a precursor of cortisol, and the conversion of progesterone to deoxycorticosterone, a precursor of aldosterone. Cortisol deficiency results in the ACTH-induced accumulation of substrate precursors, such as 17-OHP and progesterone, and an increased secretion of adrenal androgens (Bachelot et al., 2008).

CAH due to 21-hydroxylase deficiency is classified into 2 distinct clinical phenotypes, classical CAH (CAHc) and non-classical CAH (CAHnc). The former is detectable at birth or during childhood, and the latter is less severe, with clinical onset at a later age. CAHc is further divided into 2 major forms: the salt-wasting form, in which 21-hydroxylase activity is almost completely to completely lost, and the simple virilizing form in which ade-

quate levels of aldosterone are produced allowing the maintenance of normal sodium homeostasis (Speiser et al., 1992).

Patients with CAHnc synthesize normal amounts of cortisol and aldosterone but have slightly elevated levels of sex hormone precursors. Many non-classical cases remain undiagnosed because of the absence of phenotypic manifestations and the low base-line levels of 17-OHP. Females are usually born with non-ambiguous external genitalia, but may show mild clitoromegaly. Symptoms of androgen excess in children may include acne, premature development of pubic hair and accelerated growth associated with advanced bone age. Among older females, delayed menarche, hirsutism, menstrual disorders, impaired fertility, polycystic ovaries and temporal baldness may occur. Males with CAHnc may have early beard growth and an enlarged phallus with relatively small testis (White and Speiser, 2000; Gonçalves et al., 2007).

Deficiency of 21-hydroxylase is caused by mutations in the *CYP21A2* gene. *CYP21A2* contains 10 exons spaced along 3.1 kilobases (kb) in the human leukocyte antigen (HLA) class III region on the short arm of chromosome 6 (6p21.3),

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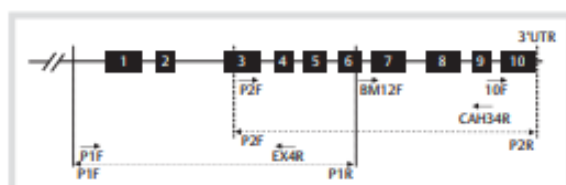
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Table 1 Clinical data of the patients.

Patient	Age	Sex	17-OHP (ng/dL)	Clinical Diagnosis
1	5	F	235	Nonclassical
2	17	F	1410 ¹	Nonclassical
3	9	M	1250 ¹	Nonclassical
4	10	F	6749	Classical
5	39	M	6600	Classical

¹Values registered after ACTH stimulation**Fig. 1** Schematic representation of *CYP21A2* gene structure and strategy for whole gene sequencing. Numbered black boxes represent *CYP21A2* exons. Dot line (—) represent the first PCR - P1 - amplification product (1517 bp) and dash line (---) represent the second PCR - P2 - amplification product (2214 bp). Arrows represent the primers used in 6 different sequencing reactions (P1F and Exon4R for P1 fragment and P2F, BM12F, CAH34R and 10F for P2 fragment).

30 kb apart from the pseudogene *CYP21A1P*. The *CYP21A2* and *CYP21A1P* loci are adjacent to and alternating with the genes encoding the fourth component of serum complement, factor *C4B* and factor *C4A* respectively (Speiser and White, 2003). The sequence homology between *CYP21A2* and *CYP21A1P* is 98% for the exons and approximately 96% for the introns (Rodrigues et al., 1987). *CYP21A1P* became inactive as a result of having accumulated several deleterious mutations along time. Intergenic recombinations between *CYP21A2* and *CYP21A1P* are responsible for over 95% of mutations causing 21-hydroxylase deficiency. Around 75% are deleterious mutations transferred from the pseudogene by gene conversion, during mitosis. Gene conversions can be quite large, involving the transfer of more than one pseudogene mutation, or be "microconversions", limited to a single point mutation (Huynh et al., 2009). About 20% are meiotic recombinations owing to unequal crossing over during meiosis. These can lead to gene duplications and gross gene deletions encompassing the *C4* and *CYP21A2* (White and Speiser, 2000). The frequent deletion of a 30-kb segment includes the 3' end of *CYP21A1P*, all *C4B* and the 5' end of *CYP21A2*. This allele carries a single non-functional chimeric gene with its 5' and 3' ends corresponding to *CYP21A1P* and *CYP21A2*, respectively (White et al., 1988; Lee et al., 2000). The remaining 5% corresponds to more than 130 additional mutations, the majority being missense mutations. *De novo* mutations account for 1–2% of pathogenic alleles (Speiser et al., 1992). The majority of patients with 21-hydroxylase deficiency are compound heterozygotes for 2 or more *CYP21A2* mutations and the clinical severity of the disease in such cases is determined by the mutation associated with the highest residual enzyme activity. Like in other populations, targeted mutational screening for a panel of common *CYP21A2* mutations and gene deletions detects more than 90% of disease-causing alleles in affected Portuguese individuals (Marques et al., 2010). Herein, we report the results of extended mutational analyses of the *CYP21A2* gene in 5 Portuguese patients with 21-hydroxylase deficiency, including two with the classical phenotype, in whom

targeted mutation screening had not been informative, and the *in silico* characterization of 2 novel *CYP21A2* mutations (p.Pro432Leu and p.Ala434Glu) identified in these patients.

Material and Methods

Patients and population controls

5 Portuguese patients with CAH apparently due to 21-hydroxylase deficiency, according to standard clinical and biochemical diagnostic criteria (Nieman LK, UpToDate 2011) were selected for extended mutational analysis of the *CYP21A2* gene after the screening for 10 common pathogenic mutations revealed just 1 or no mutations. Table 1 summarizes the most relevant demographic and clinical data of the patient cohort. Genomic DNA samples of 100 healthy Portuguese adults were used as population controls for the novel *CYP21A2* allelic variants identified in this study.

CYP21A2 gene sequencing and multiplex ligation-dependent probe amplification analysis

Patients had been initially screened for the 10 most common disease-causing mutations by Polymerase Chain Reaction-Sequence Specific Primer (PCR-SSP) for p.Gly110fs, p.Ile172Asn and exon 6 cluster (p.Ile236Asn, p.Val237Glu, p.Met239Lys), Restriction Fragment Length Polymorphism (RFLP) for large deletion/conversions involving the *CYP21A2* promoter region and direct DNA sequencing for p.Pro30Leu, c.290-13 A/C>G, p.Val281Leu, p.Leu307PhefsX5, p.Gln318X and p.Arg356Trp (Marques et al., 2010). Subsequently, the whole genomic sequence of the functional *CYP21A2* gene was selectively amplified by Polymerase Chain Reaction (PCR) into 2 partially overlapping fragments, P1 and P2 (Fig. 1), respectively with 1517 and 2214 base-pairs (bp), avoiding the co-amplification of the pseudogene *CYP21A1P* (Loidi et al., 2006). The PCR reaction mixture contained 10X Expand Long Template Buffer, 2.5 pmol/μL of each dNTP, 2.5 pmol/μL of each primer, 1 μL of DNA at a concentration of 100 ng/μL, and 1.5 units of Expand Long Template Enzyme mix (Roche®). PCR conditions consisted of initial denaturation at 94 °C for 2 min; 35 cycles of denaturation at 94 °C for 15 s, annealing at 59 °C or 62 °C respectively for the P1 and P2 fragments for 30 s, and extension at 68 °C for 2 min; and final extension at 68 °C for 7 min. The PCR products were purified with GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare) and sequenced with internal primers that covered the entire *CYP21A2* gene (P1F: 5'-TCG GTG GGA GGG TAC CTG AAG-3'; Exon 4R: 5'-TCG TCC TGC CAG AAA AGG AG-3'; P2F: 5'-TGT CCT TGG GAG ACT ACT CC-3'; BM12F: 5'-GAG GGA TCA CAT CGT GGA GAT-3'; CAH34R: 5'-CTC TGG AGT TCT TCC CTG GCT CCA GG-3'; 10F: 5'-AGA GGC CAC ATG AGT TCT GG - 3') using BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) (Fig. 1). The reaction products were analyzed on an automated ABI PRISM™ 310 Genetic Analyser (Applied Biosystems).

Screening for deletions and duplications of the *CYP21A2* gene and *CYP21A1P* pseudogene was performed by Multiplex Ligation-dependent Probe Amplification (MLPA), using the P050-CAH Kit (MRC-Holland).

Nomenclature and molecular models

CYP21A2 mutations are described according to standard molecular genetics nomenclature (Ogino et al., 2007) using Ensembl transcript CYP21A2-002: ENST00000448314 as the reference sequence.



3-dimensional visualization and analyses of the functional and structural consequences of the 2 novel CYP21A2 mutations were based on the crystallographic model of human 21-hydroxylase (Robins et al., 2006).

Bioinformatic structural and functional analyses of the novel CYP21A2 missense variants

The ICM-Pro software (version 3.7, Molsoft LLC, La Jolla, CA) was used to predict and characterize the effects upon the structure of 21-hydroxylase of mutations p.Pro432Leu and p.Ala434Glu, using as reference the latest human CYP21A2 molecular model PDB:2CEG (Protein Data Bank-RCSB, accessed on the 2010/12/10, at <http://www.rcsb.org/pdb>) (Robins et al., 2006).

Additional *in silico* analyses of the 2 novel missense CYP21A2 mutations were performed with ClustalW2 (European Bioinformatics Institute, UK, accessed on the 2010/01/14, at <http://www.ebi.ac.uk/>) (Chenna et al., 2003), that compare conservation of the affected amino acid positions across mammalian orthologues, and with PolyPhen-2 (Adzhubei et al., 2010), which predicts the impact of an amino acid substitution upon the structure and function of a human protein using straightforward physical and comparative considerations.

Results

Direct sequencing of the entire CYP21A2 gene in 5 Portuguese CAH patients (Table 1) allowed the detection of 4 mutations that had not been identified by a panel of common pathogenic mutations. 2 of them were missense mutations already reported to the Human Gene Mutation Database (HGMD® Professional 2011.3 accessed on the 2011/11/30, at <http://www.biobase-international.com/>) (Stenson et al., 2009) – p.Pro453Ser and c.289+5G>A. The remaining 2, both in exon 10, were p.Pro-



Fig. 2 Evolutionary conservation study of the p.Pro432Leu and p.Ala434Glu mutations among CYP21A2 orthologues [human CYP21A2 (Uniprot accession number, A28HY6), pig (P15540), dog (Q9N2C70), cow (P00191), sheep (Q7M366), mouse (P03940), rat (Q64562), puffer fish (Q752G0) and eel (Q722R9)], in addition to other mammalian cytochrome P450s [human CYP2C8 (P10632), human CYP2C9 (P11712), human CYP3A4 (P08684), rabbit CYP2B4 (P00178) and rabbit CYP2C5 (P00179)] using ClustalW2 multiple sequence alignment. The location of the affected residues is marked.

432Leu, recently reported by our group (Carvalho et al., 2010), and p.Ala434Glu, an entirely novel CYP21A2 mutation. Mutation p.Pro453Ser was present in 3 patients and p.Val281Leu in 2 patients. The intronic splice site mutation c.289+5G>A and mutations p.Pro432Leu and p.Ala434Glu were each detected in a single case (Table 2, Fig. 3). Table 3 summarizes the data of bioinformatic predictions of the pathogenicity of the latter 2 CYP21A2 mutations, based on molecular modeling, thermodynamic considerations and sequence conservation. Results of multiple sequence alignment with ClustalW are shown in Fig. 2. Mutations p.Pro432Leu and p.Ala434Glu are located in helix L, one of the 2 helices that “sandwich” the heme (Barbato et al., 2006). Although leucine is larger than proline, mutation p.Pro432Leu is conservative for charge and polarity (Fig. 4). The accessibility to surface of mutation p.Pro432Leu is estimated at 0.28, a value that exceeds those of residues in the inner core of the molecule. In mutation p.Ala434Glu, the hydrophobic alanine residue at position 434 is replaced by the hydrophilic and larger glutamic acid, causing a change in polarity (Fig. 4). The accessibility to surface of this mutation is estimated at 0.00. Mutations p.Pro432Leu and p.Ala434Glu were predicted by PolyPhen-2 to be “probably damaging” and none was identified in the screening of 200 CYP21A2 alleles from healthy controls.

Discussion

Screening for the 10 most common CYP21A2 pathogenic mutations has been widely used as standard diagnostic approach to CAH due to 21-hydroxylase deficiency (White and Speiser, 2000). As this methodology is labor intensive and cannot explain the clinical diagnosis in up to 25% of the cases, the importance of complete gene sequencing for the detection of new mutations cannot be overemphasized (Marques et al., 2010). In this study, the extended mutational analysis was performed in order to explain the diagnosis of CAH in cases that had not been resolved by selective screening.

Mutation p.Pro453Ser, in exon 10, was the only CYP21A2 mutation identified in patient #1, a 5-year-old girl who presented with precocious pubarche and whose plasma 17-OHP levels were in the range usually found in children with CAHnc (Nieman LK, UpToDate 2011). The genetic basis for CAHnc in this patient remained unclear and it is possible that she carries another mutation that was missed in this study.

Extended gene analysis by automated sequencing allowed genetic confirmation of the clinical diagnosis in all other patients. Patient #2, a female teenager with a CAHnc phenotype, is a compound heterozygote for 2 exon 10 mutations, p.Pro453Ser and p.Pro432Leu. Patient #3, 9-year-old boy with CAHnc, is a compound heterozygote for mutations p.Val281Leu

Patients	Initial Screening	Whole gene sequencing	Genotype
1	–/–	p.Pro453Ser (+/–)	p.Pro453Ser/–
2	–/–	p.Pro432Leu (+/–) p.Pro453Ser (+/–)	p.Pro432Leu/p.Pro453Ser
3	p.Val281Leu/–	p.Val281Leu (+/–) p.Pro453Ser (+/–)	p.Val281Leu/p.Pro453Ser
4	Del/conv/p.Val281Leu	p.Val281Leu (+) c.289+5 G>A (+)	Del/conv/p.Val281Leu, c.289+5 G>A
5	–/–	p.Ala434Glu (+/+)	p.Ala434Glu/p.Ala434Glu

(–/–) no mutation detected; (+/–) heterozygosity; (+/+) homozygosity; (+) hemizyosity; novel mutation is underlined

Table 2 Results of the complete CYP21A2 gene sequencing.



and p.Pro453Ser. Mutation p.Val281Leu is the most common in the Portuguese population (Friães et al., 2006; Marques et al., 2010).

In patient #4, who presented at birth with a simple virilizing form of CAH, the initial screening detected a deletion/conversion in one of the *CYP21A2* alleles, together with mutation p.Val281Leu. With extended gene sequencing, an intronic splice site mutation in intron 2 (c.289+5G>A) was additionally identified. Mutation c.289+5G>A decreases the consensus value of intron 2 splice donor site from 70.9% to 59.3% (Mount, 1982) and it has been reported to suppress this donor splice site in epidemiological studies of CAH (Friães et al., 2006). MLPA revealed a large deletion/conversion, spanning from the *C4B* gene to the exon 3 of *CYP21A2*, in one of the *CYP21A2* alleles. A normal MLPA profile was obtained for exons 4, 6 and 8. The genetic study of the parents and the only sister of patient #4 revealed that her father is heterozygous for the large deletion/conversion, her mother is homozygous for the p.Val281Leu and heterozygous for the c.289+5G>A mutation, and that her sister inherited the large deletion/conversion from the father and mutation p.Val281Leu from the mother.

The p.Ala434Glu was the only *CYP21A2* mutation identified in patient #5, an adult male with CAH. Although the parents of this patient were not available for genetic screening, no additional abnormalities could be identified by restriction enzyme analysis for deletions/conversions in the promoter region using *TaqI* (Marques et al., 2010), or by MLPA. These findings favor the

interpretation that the patient is indeed homozygous for mutation p.Ala434Glu.

In silico analysis of novel mutations is a valuable tool for predicting their pathogenicity and clinical severity. Residue p.Pro432 is located near the heme, but outside the inner structural core, and its mutation to leucine does not change the molecular polarity. Residue p.Pro432 is fairly conserved among *CYP21A2* orthologues but not in other mammalian cytochrome P450 enzymes. The partial conservation of residue p.Pro432, its surface exposure and the preservation of polarity are all suggestive that mutation p.Pro432Leu does not severely affect 21-hydroxylase activity, which is concordant with the observed clinical phenotype. In contrast, residue p.Ala434 lies buried in the inner core of the protein and is strictly conserved among orthologues and other cytochrome P450 enzymes, which denote its structural importance. Furthermore, its replacement by a glutamic acid in mutation p.Ala434Glu is non-conservative for polarity. The changes in residue size, hydrophobicity and polarity may interfere with neighboring residues that are in contact with the heme and unsettle the helix structure, severely affecting 21-hydroxylase activity (○ Fig. 4b), which is concordant with the observed clinical phenotype. The preceding data are in agreement with calculations of the free energy of both mutations, and with PolyPhen-2 prediction that mutations p.Pro432Leu and p.Ala434Glu are "probably damaging", with HumDiv scores of 0.858 and 0.999, respectively.

In conclusion, the complete sequencing of the *CYP21A2* gene is a reliable method for detection of common as well as rare alleles,

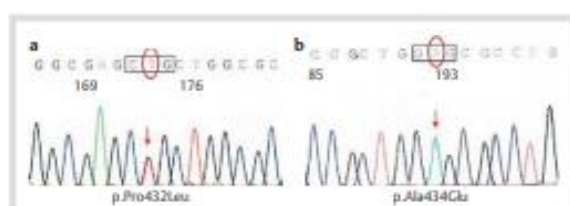


Fig. 3 Electropherograms obtained by direct sequencing. **a** Mutation p.Pro432Leu in heterozygous state. The base change from cytosine (C) to thymine (T) in one of the alleles of *CYP21A2* gene, leads to an amino acid substitution from proline (Pro) to leucine (Leu) in codon 432 of exon 10; **b** Mutation p.Ala434Glu in homozygous form. The nucleotide change from cytosine (C) to adenine (A) leads to a substitution of amino acid alanine (Ala) to glutamic acid (Glu) in codon 434 of exon 10.

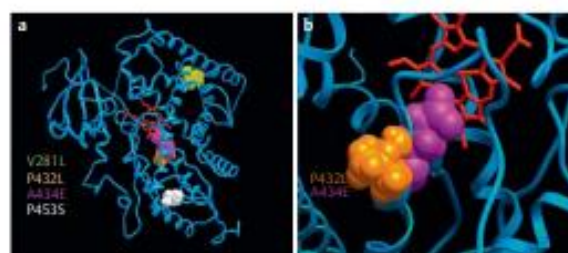


Fig. 4 *CYP21A2* 3-dimensional molecular model. **a** Overall view of the *CYP21A2* protein structure with the heme represented, as well as all missense mutations identified through direct DNA sequencing: Red, heme; Yellow, mutation p.Val281Leu; Orange, p.Pro432Leu; Pink, p.Ala434Glu; White, p.Pro453Ser. **b** Close-up view of the p.Pro432Leu (orange) and p.Ala434Glu (pink) mutations.

Table 3 Prediction of severity of *CYP21A2* gene mutations based on molecular modeling and sequence conservation.

Mutation	Zigosity	Exon	Secondary Structure	Accessability	Energy*	Hydrophobicity Difference	Molecular Mechanism	Residue Conservation	Polyphen-2 result	Predicted Severity
P432L	Heterozygote	10	Helix L	0.28	-2.098	5.4	Polarity conserved	50% conservation in orthologues (4 out of 8)	0.858 (probably damaging)	Nonclassical
A434E	Homozygote	10	Helix L	0.00	-1.933	-5.3	Buried residue changes from nonpolar to polar	Strictly conserved	0.999 (probably damaging)	Classical

Each of these mutations was evaluated according to the following parameters: protein stability, by calculating free energy by comparison of normal and mutant structures, changes in surface accessibility, alterations of hydrophobicity by the Kyte and Doolittle parameters (Kyte and Doolittle, 1982) and structural information from visual inspection of the model. Accessibility of less than 0.20 is considered to represent the inner core of the protein. Regions with values above 0 are hydrophobic in character. Conversely, negative values correspond to hydrophilicity. *Calculated according to the Electrostatically Driven Monte Carlo (EDMC) method with a low-cost energy function (ECEPP/3). Energy in kcal/mol

and enabled the identification of novel mutations. A more extended analysis of the promoter region (150–400bp before ATG) may be indicated in selected cases.

Conflict of interest: None.

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PAPER III

**A Multicenter Study on Genotype–Phenotype Correlation from a Portuguese
Pediatric Cohort**

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CYP21A2 Gene Pathogenic Variants: A Multicenter Study on Genotype–Phenotype Correlation from a Portuguese Pediatric Cohort

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Keywords

Congenital adrenal hyperplasia · 21 Hydroxylase deficiency · CYP21A2 · Genotype–phenotype correlation

Abstract

Background: Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency (21OHD) is an autosomal recessive disorder characterized by 3 overlapping phenotypes:

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salt-wasting (SW), simple virilizing (SV), and non-classic (NC). We aimed at conducting a nationwide genotype description of the CAH pediatric patients and to establish their genotype–phenotype correlation. **Methods:** CAH patients were recruited from Portuguese pediatric endocrinology centers and classified as SW, SV, or NC. Genetic analysis was performed by polymerase chain reaction (sequence specific primer, restriction fragment length polymorphism) or direct Sanger sequencing. Genotypes were categorized into 4 groups (0, A, B, and C), according to their predicted enzymatic activity. In each group, the expected phenotype was compared to the observed phenotype to assess the genotype–phenotype correlation. **Results:** Our cohort comprises 212 unrelated pediatric CAH patients (29% SW, 11% SV, 60% NC). The most common pathogenic variant was p.(Val282Leu; 41.3% of the 424 alleles analyzed). The p.(Val282Leu) variant, together with c.293-13A/C>G, p.(Ile173Asn), p.(Leu308Thr), p.(Gln319*), and large deletions/conversions were responsible for 86.4% of the mutated alleles. Patients' stratification by disease subtype revealed that the most frequent pathogenic variants were c.293-13A/C>G in SW (31.1%), p.(Ile173Asn) in SV (46.9%), and p.(Val282Leu) in NC (69.5%). The most common genotype was homozygosity for p.(Val282Leu; 33.0%). Moreover, we found 2 novel variants: p.(Ile161Thr) and p.(Trp202Arg), in exons 4 and 5, respectively. The global genotype-phenotype correlation was 92.4%. Group B (associated with the SV form) showed the lowest genotype–phenotype correlation (80%). **Conclusion:** Our cohort has one of the largest NC CAH pediatric populations described. We emphasize the high frequency of the p.(Val282Leu) variant and the very high genotype–phenotype correlation observed.

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Introduction

Congenital adrenal hyperplasia (CAH) consists of a group of autosomal recessive inborn errors of adrenal steroidogenesis. Deficiency of 21-hydroxylase (21OHD) accounts for 95% of the CAH cases. 21OHD results in defective conversion of 17-hydroxyprogesterone (17OHP) to 11-deoxycortisol and leads to hypocortisolism and hyperandrogenism due to accumulation of precursor steroid hormones. Although 3 major clinical forms are described (classic salt-wasting [SW], classic simple virilizing [SV], and non-classic [NC]), this disorder represents a continuous phenotypic spectrum [1].

In countries where there is no neonatal screening for 21OHD CAH, such as Portugal, patients with the classic

SW form are diagnosed in the neonatal period with different degrees of virilization in females and potentially life-threatening adrenal crisis in males (typically, failure to thrive, dehydration, hyponatremia, and hyperkalemia at the second week of life). For the classic SV form, female infants show clitoromegaly or atypical genitalia without clinical signs of SW, and male infants and toddlers manifest early virilization (pubarche, growth spurt, body odor). The NC patients usually present premature pubarche in school age or hirsutism, severe acne, oligomenorrhea, infertility in adolescence and adulthood, or they can also be asymptomatic, especially in males.

Classic CAH has a variable prevalence among Europeans and North Americans (1 in 10,000 to 1 in 16,000 live births) [2], while NC CAH affects around 1 in 100 to 1 in 1000 individuals [3, 4], with a higher prevalence in some populations, such as Hispanics [5], and a very low prevalence in others, for instance, the Chinese [6]. The frequency of heterozygote carriers is approximately 1 in 55 in the European population [7]. NC CAH heterozygote carrier-ship is found in approximately 15% of the American Ashkenazi Jews and 9.5% of the American Caucasians [4].

The gene encoding human steroid 21-hydroxylase, *CYP21A2*, and its highly homologous neighboring pseudogene, *CYP21A1P*, are located on chromosome 6p21.3. Although they are more than 95% identical, the pseudogene cannot be translated into a functional enzyme. Around 95% of pathogenic variants causing 21OHD occur due to recombination between *CYP21A2* and *CYP21A1P*. Approximately 75% of these variants are deleterious and transferred by conversion from the pseudogene during meiosis. The conversion can involve more than 1 pseudogene variant, or “microconversions,” limited to a single-point variant. The remaining 20–25% of CAH may be due to recombination owing to unequal crossing over during meiosis that can lead to gene duplications and gross gene deletions involving *CYP21A2* and other contiguous genes [3, 8].

Pathogenic variants in the *CYP21A2* gene cause different degrees of dysfunction on enzymatic activity. Therefore, pathogenic variants leading to a complete inactivation of 21OH are usually associated with the SW phenotype; pathogenic variants that reduce enzyme activity to 2–7% cause the SV phenotype; whereas those that coexist with a residual enzymatic activity in the range of 20–60% result in the NC phenotype. As CAH is an autosomal recessive disorder, an affected individual must present pathogenic variants on both alleles, and the parents are heterozygous for 1 variant, with the phenotype being conferred by the less deleterious pathogenic variant.

Several studies have shown a high correlation between genotype and phenotype in 21OHD [8–11], predominantly for the most severe and milder genotypes [12–14]. However, the largest and most heterogeneous cohort of CAH patients, who comprises data from 1507 families with at least 1 member diagnosed with CAH, found around 39% of nonconcordance between genotype and phenotype [13].

It is believed that the prevalence of CAH in Portugal, mainly for the NC form, is very high (similar to the European reported data), even though the exact prevalence is unknown. Two previous smaller studies analyzed the *CYP21A2* mutational spectrum in Portuguese CAH patients [15, 16]. However, these studies, like many others, included a small number of patients and represented single centers.

Thus, we aimed to perform a multicenter study, which intended: (1) to recognize the most frequent *CYP21A2* pathogenic variants in the Portuguese pediatric population; (2) to establish a genotype–phenotype correlation; and (3) to identify differences between our population and others previously described.

Materials and Methods

Patients

A total of 212 unrelated Caucasian patients with CAH were retrospectively recruited from 13 Pediatric Endocrinology centers from tertiary and secondary hospitals. This population is representative of Portugal's territory, as there were patients from all Portuguese districts.

In Portugal, there is no national database of CAH patients, so they were enrolled from clinical records from each center involved, after personal contact. Furthermore, there is currently no universal newborn screening for CAH.

Inclusion criteria were a 21OHD diagnosed under the age of 18 and genetic study with an identified pathogenic variant in homozygosity or compound heterozygosity. Patients without molecular confirmation or with only 1 variant identified (heterozygotes) were excluded.

Phenotype Classification

The phenotype was assigned by the referring endocrinologist and was based on clinical and laboratorial criteria.

The classic SW form was defined as adrenal crisis (hypovolemia, hyponatremia, hyperkalemia, hyperreninemia), atypical genitalia in females, and elevated basal 17OHP.

The classic SV form was defined as ambiguous external genitalia in females or early virilization in males (premature pubarche, growth acceleration, and advanced bone age) and elevated 17OHP, but with no evidence of SW.

The NC form was defined as normal genitalia in newborns and infants and clinical signs of hyperandrogenism that appear later in childhood (pubarche, advanced bone age, apocrine body odor) or

in adolescence (hirsutism, acne, oligomenorrhea), associated with elevated 17OHP (basal >200 ng/dL and post-ACTH >1,200 ng/dL).

The difference between the SV and the NC forms was established regarding atypical genitalia versus normal genitalia in females and the age of onset of clinical symptoms in males (those presenting before 3 years-old were classified as SV).

Sample Preparation and *CYP21A2* Gene Analysis

Screening for the 10 Most Common Pathogenic Variants

Initially, only *CYP21A2* gene evaluation by genotyping the 10 most known pathogenic variants was performed, by polymerase chain reaction (PCR), sequence specific primer, restriction fragment length polymorphism, or direct DNA Sanger sequencing. The initial screening for 10 pathogenic variants in the majority of the centers included large deletions or conversions events involving the promoter region of the *CYP21A2* gene (del/conv of promoter region), p.(Pro31Leu) in exon 1, an intron 2 splicing pathogenic variant c.293–13A/C>G, an 8 bp deletion in exon 3 (p.[Gly111Valfs21]), p.(Ile173Asn) in exon 4, cluster of 3 point pathogenic variants in exon 6 – exon 6 cluster (p.[Ile236Asn], p.[Val237Glu], p.[Met239Lys]), p.(Val282Leu) and a T-insertion p.(Leu308PhefsX5) in exon 7, and p.(Gln319*) and p.(Arg357Trp) in exon 8 [14]. Screening strategy contemplated only the previous pathogenic variants, and no other variants were detected. The study of the large deletions/conversions involving the promoter region of the *CYP21A2* gene were detected by a PCR-based assay followed by digestion with Taq I restriction enzyme [17].

Complete *CYP21A2* Gene Sequencing

The complete analysis of functional *CYP21A2* gene was performed by selectively amplifying by PCR into 2 partially overlapping fragments avoiding the coamplification of the pseudogene *CYP21A1P* [18, 19]. All coding sequences and intron–exon boundaries were sequenced with internal primers that covered the entire *CYP21A2* gene. In patients were no mutations, or just 1 mutation, was detected by screening of most common mutations, complete gene sequencing was a complementary technique.

Multiplex Ligation-dependent Probe Amplification

Screening for deletions and duplications of the *CYP21A2* gene and *CYP21A1P* pseudogene was performed by multiplex ligation-dependent probe amplification, using the P050 – CAH Kit (MRC-Holland).

Nomenclature and Molecular Models *CYP21A2*

Nomenclature of *CYP21A2* variants is according to Ensembl transcript *CYP21A2*-002: ENST00000418967.6 (NM_000500 from National Center for Biotechnology Information).

Bioinformatic structural and functional analysis of the 2 novel missense *CYP21A2* variants were performed using the PolyPhen-2 [20], the Mutation Taster [21] and the UniProt [22] bioinformatics tools.

Categorization of Pathogenic Variants According to Predicted Enzyme Activity

Disease-causing pathogenic variants were divided into 5 groups according to the expected 21-hydroxylase activity, a strategy de-

Table 1. Genotype groups according to the predicted severity of the enzyme deficiency

Group	Enzyme activity	Pathogenic variant	Expected phenotype
Group 0	Null	Del/Conv; c.69G>A (p.Trp23*); c.292+5G>A [15]; c.332_339delGAGACTAC (p.Gly111Valfs21); E6 cluster (c.710T>A (p.Ile237Asn), c.713T>A (p.Val238Glu), c.719T>A (p.Met240Lys); c.874G>A (p.Gly292Ser); c.920_921insT (p.Leu308Phefs); c.955C>T (p.Gln319*); c.1069C>T (p.Arg357Trp); c.1225C>T (p.Arg409Cys); c.1273G>A (p.Gly425Ser); c.1280G>A (p.Arg427His); c.1333C>T (p.Arg445*) [18]	Salt-wasting
Group A	<2%	c.293-13A/C>G (homozygous or compound heterozygous with a group 0 pathogenic variant)	Salt-wasting
Group B	~2%	c.518T>A (p.Ile173Asn) (homozygous or compound heterozygous with groups 0, or A pathogenic variants)	Simple virilizing
Group C	~20–60%	c.844G>A (p.Val282Leu); c.92C>T (p.Pro31Leu); c.1360C>T (p.Pro454Ser); c.13*G>A [28] (homozygous or compound heterozygous with groups 0, and A or B pathogenic variants)	Nonclassic
Group D	Unknown	New pathogenic variants (c.482T>C [p.Ile161Thr]; c.604T>C [p.Trp202Arg])	

scribed previously [1, 3, 9, 11, 13, 14, 23–28], as summarized in Table 1. Group D included the new variants detected in 2 patients, with unknown *in vitro* influence on enzymatic activity.

Hormone Assays

17OHP measurements were performed at each collaborating center and expressed in ng/dL. The stimulation test was performed with administration of 250 µg synthetic ACTH (synacthen®), and 17OHP was measured at 0 and at 60 min post-ACTH.

Statistical Analysis

Statistical analysis was performed using SPSS® version 20. Nonparametric tests were applied to compare independent samples, and chi-square tests were used to determine the association between categorical variables. *p* values below 0.05 were regarded as statistically significant.

Results

Characterization of the Population

From our 212 CAH patients, 61 presented a SW form, 24 a SV form, and 127 a NC form, and basic laboratorial and clinical findings are described in Table 2.

The majority of the NC CAH patients were females (80.3%) and only 16.5% (21/127) were diagnosed after the age of 10 years.

All but 1 male newborn with the SW form presented with adrenal crisis. In females, the majority of the salt-wasters were born with atypical genitalia and, in 1 case, complete Prader V virilization was found. For the SV forms, all the females presented atypical genitalia, and males presented very precocious pubarche (median age 2.3 years). In the NC form, the main clinical sign at presentation was precocious pubarche (median age 6.95 years) in both sexes.

Salt-wasters presented higher levels of basal 17OHP than SV (median 16,700 vs. 9,470 ng/dL; *p* < 0.05). The ACTH stimulation test was performed in 43.3% of the NC patients.

Allele Frequencies

In our population, 46.7% of the patients were homozygous, and 53.3% were compound heterozygous. Of the 424 unrelated alleles, 38 (8.2%) carried 2 or more pathogenic variants in *cis*, which were appropriately assigned to the paternal or maternal chromosomes after progenitor analysis. There was no history of consanguinity in any of our patients. Molecular analysis allowed the identification of 20 different point pathogenic variants. Large rearrangements, such as *CYP21A2* deletions and large gene conversions, were found in 6.7% of the CAH patients (Table 3). The most common pathogenic variant found was p.(Val282Leu). In 424 unrelated chromosomes, p.(Val282Leu) (41.3%), c.293-13A/C>G (14.1%), p.(Gln319*) (10.2%), p.(Ile173Asn; 8.7%), p.(Leu308Thr; 5.4%), plus large deletions and conversions (6.7%), were the most prevalent pathogenic variants accounting for 86.4% of the affected alleles.

Patients' stratification by disease subtype revealed that the most frequent pathogenic variants were c.293-13A/C>G in SW patients (31.1%), p.(Ile173Asn) in SV patients (46.9%) and p.(Val282Leu) in NC patients (69.5%). The most common genotype was the homozygosity for the p.(Val282Leu) pathogenic variant in 33% of the patients (70/212), all of whom had NC CAH, except for a boy with an adrenal crisis in the second week of life (Table 4, case 4).

Table 2. Key characteristics of the population (*n* = 212)

	Classic SW	Classic SV	NC	<i>p</i> value
Patients, <i>n</i> (%)	61 (28.8)	24 (11.3)	127 (59.9)	
Male, %	55.7	25	19.7	<0.05 [†]
Female, %	44.3	75	80.3	
Age at diagnosis (median; Pc25; Pc75), years	0.08 (0.04–0.11)	1.4 (0.03–2.98)	6.95 (5.45–8.87)	<0.05*
<i>Clinical presentation</i>				
Males	Adrenal crisis 97.1% Family screening 2.9%	Precocious pubarche 83.3% Family screening 16.7%	Precocious pubarche 92.0% Family screening 8.0%	
Females	Adrenal crisis 22.2% Atypical genitalia 74.1% Family screening 3.7%	Atypical genitalia 100%	Precocious pubarche 67.7% Hirsutism 20.6% Clitoromegaly 4.9% Family screening 2.9% Acne 2.9% Amenorrhea 1.0%	
Basal 17OHP basal (median; Pc25; Pc75), ng/dL	16,700 (5,535–59,820)	9,470 (4,473–21,238)	1,250 (705–2,475)	<0.05*
Post ACTH 17OHP (median; Pc25; Pc75), ng/dL			5,300 (3,246–8,178)	
Homozygotes, %	34.4	12.5	59.1	<0.05 [†]
Family history of CAH, %	18	16.7	16.5	0.40 [†]

* Nonparametric test.
[†] χ^2 -test.
SW, salt-wasting; SV, simple virilizing; NC, nonclassic;

Molecular analysis allowed the identification of 2 novel sequence variants, p.(Ile161Thr) and p.(Trp202Arg), in exons 4 and 5, respectively.

Among the NC patients, 60.6% (77/127) had 2 presumed NC alleles (group C), while 39.4% (50/127) were compound heterozygotes for a classic and a NC allele (one allele from group C and the other from group 0, A, or B). We found no differences between these patients regarding the age at presentation (median 6.94 vs. 6.95 years; *p* = 1.0) or basal 17OHP levels (1,204 vs. 1,394 ng/dL; *p* = 0.64). Compound heterozygotes for a classic and a NC allele appeared to have higher stimulated 17OHP levels than homozygotes/compound heterozygotes for NC alleles (7,280 vs. 4,250 ng/dL), but this difference was not significant (*p* = 0.11).

Characterization of the Novel Variants

Bioinformatics analysis was performed to predict the possible impact of the novel missense variants, p.(Ile161Thr) and p.(Trp202Arg), on the structure and function of the CYP21A2 protein. Using the PolyPhen and Mutation Taster bioinformatics software, the 2 novel

variants were predicted to be “damaging” and “disease causing,” respectively, with a high pathogenicity score for the altered amino acid. Multiple alignments results showed that the Isoleucine (Ile) at codon 161 and the Tryptophan (Trp) at codon 202 were fully conserved through the species (Fig. 1). The p.(Trp202Arg) variant was found in a female with atypical genitalia that presented a SW form and had this novel pathogenic variant in one allele and a large conversion in the other. Based on these data, we believe that this pathogenic variant is associated with a very low enzymatic activity. The p.(Ile161Thr) variant was identified in a female with normal genitalia in infancy that presented a precocious pubarche at the age of 5 and was classified as a NC. She had this novel pathogenic variant in one allele and the p.(Ile173Asn) in the other. We believe that this variant is associated with a mild reduction of the 21OH activity.

Prenatal Diagnosis

We had 4 cases of prenatal diagnosis, all of them due to family history (affected sibling or parent), with 2 with genotypes predictive of SW (c.293-13A/C>G/c.293-13A/

Table 3. Distribution of disease-causing pathogenic variants according to the phenotypes

Pathogenic variant	Number of alleles Observed phenotype, n (%)			
	classic SW (% of total variants identified in the SW phenotype patients)	classic SV (% of total variants identified in the SV phenotype patients)	nonclassic (% of total variants identified in the NC phenotype patients)	total (% of total variants identified in all CAH patients)
p.(Val282Leu)	9 (6.8)		182 (69.5)	191 (41.3)
c.293-13A/C>G	41 (31.1)	9 (18.4)	15 (5.7)	65 (14.1)
p.(Gln319*)	14 (10.6)	3 (6.1)	11 (4.2)	47 (10.2)
p.(Ile173Asn)	3 (2.3)	23 (46.9)	14 (5.3)	40 (8.7)
p.(Leu308Phefs)	15 (11.4)	7 (14.3)	3 (1.1)	25 (5.4)
Del/conv*	23 (17.4)	2 (4.1)	6 (2.3)	31 (6.7)
p.(Gly111Valfs21)	6 (4.5)	2 (4.1)	6 (2.3)	14 (3.0)
p.(Pro31Leu)	2 (1.5)		11 (4.2)	13 (2.8)
p.(Arg357Trp)	9 (6.8)	1 (2.0)	2 (0.8)	12 (2.6)
p.(Pro454Ser)			8 (3.1)	8 (1.7)
p.(Gly425Ser)	2 (1.5)	1 (2.0)		3 (0.6)
p.(Arg445*)	1 (0.8)		1 (0.4)	2 (0.4)
c.292+5G>A	2 (1.5)			2 (0.4)
E6 cluster**	2 (1.5)			2 (0.4)
p.(Gly292Ser)	1 (0.8)			1 (0.2)
p.(Arg409Cys)			1 (0.4)	1 (0.2)
p.(Arg427His)			1 (0.4)	1 (0.2)
c.13*G>A		1 (2.0)		1 (0.2)
p.(Trp23*)	1 (0.8)			1 (0.2)
p.(Ile161Thr)†			1 (0.4)	1 (0.2)
p.(Trp202Arg)†	1 (0.8)			1 (0.2)

424 alleles evaluated. 462 pathogenic variants identified, 38 (8.2%) in *cis*. * Del/conv – large deletion and large gene conversion – Del/conv *n* = 17; Del_CYP21A2 *n* = 10; Conv_CYP21A2 *n* = 4. ** E6 cluster – p.(Ile236Asn), p.(Val237Glu), p.(Met239Lys). † Novel pathogenic variant.

C>G and c.293--13A/C>G/p.[Arg357Trp]) and 2 with genotypes predictive of NC CAH (c.293-13A/C>G/p.[Val282Leu] and p.[Val282Leu]/p.[Val282Leu]). The 2 patients with genotypes predictive of NC CAH were a male born in 2015 and a female born in 2016. They presented no clinical manifestations of CAH and were referred to the pediatric endocrinology outpatients' clinics. Their follow-up includes the evaluation of growth, signs of virilization or precocious puberty twice a year, and annual bone age. Both of them are asymptomatic and were not started on any medication.

Genotype-Phenotype Correlation

The global genotype-phenotype correlation was 92.4%. The correlations for the pathogenic variant groups were Group 0 (*n* = 34): 85.3%, Group A (*n* = 28): 89.3%, Group B (*n* = 25): 80%, and Group C (*n* = 123): 95.9%. The difference between groups was not statistically significant (*p* = 0.08). A genotype-phenotype discordance was seen

in 17 patients (Table 4). Patients 1–5 presented a SW form, but the genotype would suggest a NC or SV form. Complete gene sequencing was performed for patient 1, but in patients 2–5 only the most frequent 10 pathogenic variants were screened. For Pt5, the pathogenic variants p.(Val282Leu) and p.(Leu308Phefs) occurred *de novo*, probably on the maternal allele. If we exclude patients without entire gene sequencing (Pt2–5), the genotype-phenotype correlation rises to 93.8%.

Patients 6–9 were diagnosed with a SV form. For Patient 6, we would expect a SW form, as the girl has the c.293-13A/C>G pathogenic variant in one allele and 2 null pathogenic variants in the other allele (p.[Gly111Valfs21] and p.[Gln319*]). This girl was diagnosed in the first day of life, as she presented severe virilization (Prader IV). She started therapy within some days. There is no record of dehydration, electrolyte imbalance, or hyperreninemia, so the phenotype was classified as SV. Likewise, Patient 7, who was also diagnosed in the first

Table 4. Details of patients without genotype–phenotype concordance

Cases	Gender	Age at diagnosis, years	Clinical presentation	Genotype	Observed phenotype	Expected phenotype	Molecular analysis
1	Male	0.07	Adrenal crisis	p.(Pro311Leu)/p.(Arg445*)	SW	NC	Full CYP21A2 sequencing
2	Male	0.04	Adrenal crisis	c.293-13A/C>G/p.(Ile173Asn)	SW	SV	Screening for 10 most common variants
3	Female	0.05	Adrenal crisis	p.(Ile173Asn)/p.(Ile173Asn)	SW	SV	Screening for 10 most common variants
4	Male	0.08	Adrenal crisis	p.(Val282Leu)/p.(Val282Leu)	SW	NC	Screening for 10 most common variants
5	Female	0.05	Atypical genitalia	p.(pro311Leu)/p.(Val282Leu), p.(Leu308Phefs)	SW	NC	Screening for 10 most common variants
6	Female	0.00	Atypical genitalia	c.293-13A/C>G/p.Gly111ValfsTer21, p.(Gln319*)	SV	SW	Full CYP21A2 sequencing
7	Female	0.00	Atypical genitalia	Del/conv in homozygosity	SV	SW	Screening for 10 most common variants
8	Female	0.00	Atypical genitalia	p.(Val282Leu)/p.(Gln319*)	SV	NC	Full CYP21A2 sequencing
9	Female	0.05	Atypical genitalia	c.13*G>A/p.(Leu308Phefs)	SV	NC	Full CYP21A2 sequencing
10	Male	7.00	Precocious pubarche	p.(Gly111Valfs21)/Del/conv	NC	SW	Screening for 10 most common variants
11	Female	6.97	Precocious pubarche	c.293-13A/C>G/p.(Gln319*)	NC	SW	Screening for 10 most common variants
12	Male	4.59	Precocious pubarche	c.293-13A/C>G/p.Arg427His	NC	SW	Full CYP21A2 sequencing
13	Female	10.7	Clitoromegaly	p.(Pro311Leu), c.293-13A/C>G, p.(Gly111Valfs21), p.(Val282Leu), p.(Leu308Phefs)/p.(Gln319*)	NC	SW	Screening for 10 most common variants
14	Male	5.68	Precocious pubarche	p.(Ile173Asn)/c.293-13A/C>G	NC	SV	Full CYP21A2 sequencing
15	Female	9.71	Precocious pubarche	p.(Ile173Asn)/p.(Ile173Asn)	NC	SV	Screening for 10 most common variants
16	Female	6.35	Precocious pubarche	p.(Ile173Asn)/p.(Ile173Asn)	NC	SV	Screening for 10 most common variants
17	Female	4.74	Clitoromegaly	p.Gln319*/p.Gln319*, CYP21A2 duplication	NC	No disease	Full CYP21A2 sequencing

day of life due to atypical genitalia, started early treatment and never had an adrenal crisis, despite the genotype showed a large deletion on both CYP21A2 alleles.

Patients 8 and 9 both showed atypical genitalia at birth and were classified as SV, even though the genotype favored a NC phenotype. In both of them, complete sequencing of the gene was performed, and no other pathogenic variant was identified.

Patients 10–16, classified as NC, presented between 4 and 10 years of age with signs of virilization (precocious pubarche or clitoromegaly). Their genotype would suggest a SW form in Patients 10–13 or a SV form in Patients 14–16.

Discussion

In our cohort of 212 CAH patients diagnosed under the age of 18, 85 (40%) were classic and 127 (60%) were NC. Within the classic form, SW accounted for 61 (72%) and SV for 24 (28%), which is close to the generally accepted incidences (approximately 75% of classic CAH are salt-wasters). To the best of our knowledge, this is one of the largest NC 21OHD pediatric populations reported. Comparing our results to other studies of distinct populations clearly demonstrates that there are ethnic specificities within the CYP21A2 variants (Table 5). We empha-

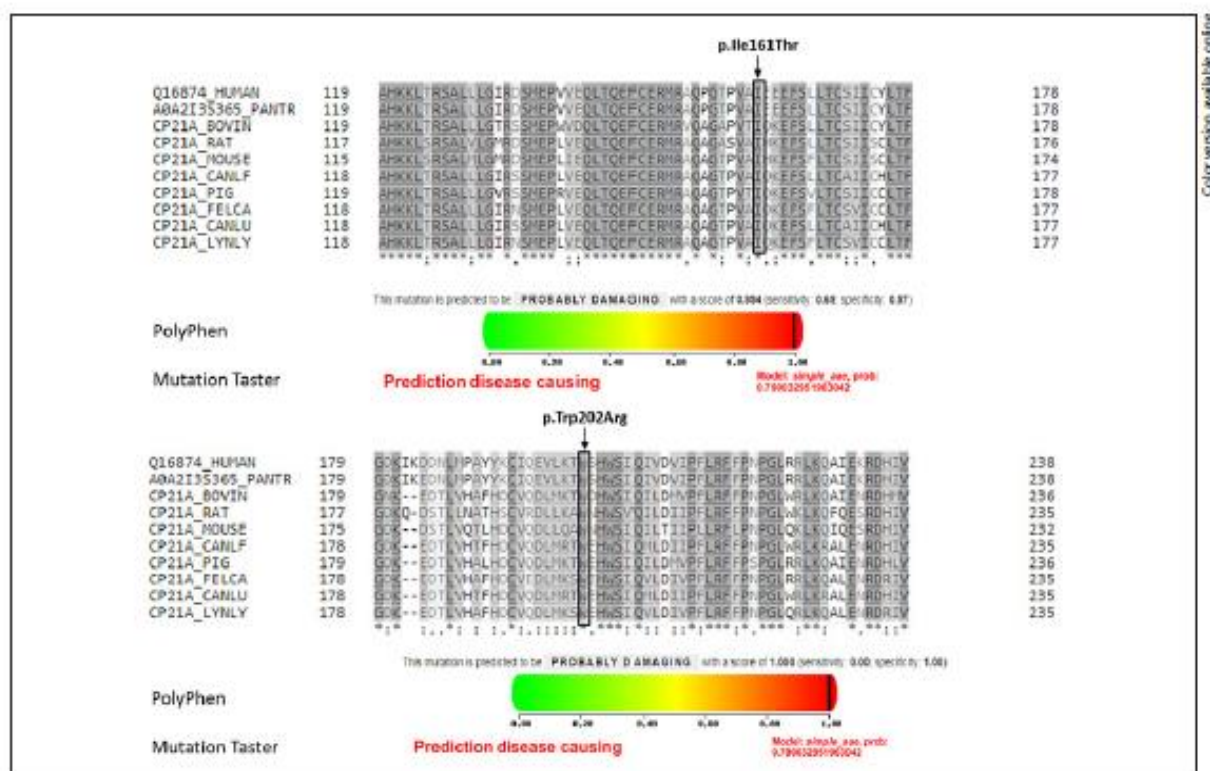


Fig. 1. Evaluation of two novel *CYP21A2* missense variants, p.(Ile161Thr) and p.(Trp202Arg). Multiple species alignments were performed using UniProt program for the amino acid conservation analysis. The predicted effect of the variants on the protein was estimated with the online prediction programs PolyPhen-2

and Mutation Taster, which are shown at the bottom of each sequence alignments. For each variant, a high pathogenicity score for the altered amino acid was obtained. Studies on functional enzymatic activity in vitro are needed for full characterization of those novel variants.

size the fact that more than 80% of the NC CAH patients were females, which may raise attention to the underdiagnosing of NC CAH in males.

In our cohort, only 16.5% of the NC CAH individuals were diagnosed after the age of 10. This might be explained by 2 factors. First, a large number of CAH adolescents remain underdiagnosed or even asymptomatic. Second, we have only included in this work CAH patients with genetic confirmation, ruling out those that are diagnosed and treated as CAH, but do not have molecular corroboration. The Endocrine Society suggests genotyping only when results of ACTH-stimulated 17OHP are equivocal or cannot be performed or for purposes of genetic counseling [29]. In our opinion, however, genotyping is an important diagnostic tool that should be offered to all CAH children and adolescents.

In this study, we have only included patients with at least one pathogenic variant in both *CYP21A2* alleles,

excluding those with no pathogenic variant or heterozygotes carriers. The reason was that sequencing was not routinely done in all the patients. In the older patients, only the 10 most frequent pathogenic variants were screened, lacking the analysis of some other exons.

Our series is mainly composed of NC patients, and this may explain the highest frequency for p.(Val282Leu; 41.3% of the alleles). In other populations, the p.(Val282Leu) pathogenic variant accounts for approximately 35–55% of NC CAH [11, 25, 30–35]. This high frequency is also found in Galicia (63.2%) [18], in the northwest of the Iberian Peninsula. Portugal and Galicia share a very similar ethnic background.

The most prevalent genotype in our population was the homozygosity for p.(Val282Leu), detected in 33% of the individuals, which has been previously reported in high frequency in the Ashkenazi Jewish population [13].

Table 5. Comparison between our 21OHD CAH population and others previously described in what regards the proportion of the CAH forms and the frequency of the different pathogenic variants

Country	Alleles, Frequency of phenotypes, n	Frequency of pathogenic variant(of alleles),													
		SW	SV	NC	p.(Val282Leu)	c.259-13A/C>G	p.(G1031P)	p.(Ile179Asn)	Del/comp*	p.(Gln308Phe)	p.(Gly111Val)21	p.(Phe311Leu)	p.(Arg357Trp)	p.(Pro154Ser)	ESCIinter**
Present study	Portugal	424	288	113	599	413	14.1	10.2	8.7	5.4	3.0	2.8	2.6	1.7	0.4
New et al. [13], 2013	Heterogeneous	3,005	ND	ND	ND	23.9	22.9	ND	8.2	3.5	20.0	ND	3.6	2.6	2.1
Loddi et al. [18], 2006	Spain	266	6.5	5.1	88.4	63.2	9.3	3.4	2.3	5.6	1.9	1.1	1.1	3.6	1.1
Buhat et al. [52], 1995	France	298	32.6	17.1	41.9	16.7	20.5	3.9	8.9	22.9	1.2	2.7	0	0	5.0
Radano et al. [33], 2000	Italy	114	32.8	14.8	52.5	24.5	21.0	4.3	7.95	18.4	0	1.8	2.6	2.6	1.8
Dracopoulou-Vakoni et al. [43], 2001	Greece	222	44.1	30.6	25.2	10.8	29.3	8.6	13.5	13.5	0	2.5	11.3	0.45	3.6
Lado et al. [53], 1999	UK	284	100	100	0	ND	30.3	ND	ND	ND	45.0	ND	ND	ND	ND
Krone et al. [11], 2000	Germany	310	53.5	30.2	6.4	2.9	30.3	4.8	19.7	27.4	0.3	1.6	2.6	4.5	0.3
Dolan et al. [44], 2005	Austria, Czech Republic, Hungary, Slovakia, Slovenia	696	63.5	26.4	10.1	3.4	31.2	2.6	14.5	30.6	1.6	1.0	3.7	2.4	0.7
Grigoriou Sidi et al. [40], 2005	Romania	66	47.4	52.6	0	ND	40.9	ND	12.1	16.7	ND	12.1	7.6	1.5	ND
Mlack et al. [38], 2015	Serbia	100	31.1	24.6	44.3	4.6	18.5	11.1	2.8	14.1	2.8	7.4	13	11.1	1.9
Dunic et al. [12], 2017	Croatia	186	70.9	29.1	0	0	34.9	4.8	11.3	18.8	ND	2.2	5.9	16.7	ND
Siddhoo et al. [39], 2003	The Netherlands	370	66.7	14.9	18.4	2.2	28.1	3.5	12.4	31.9	0.3	4.3	0.3	8.4	3.0
Levo et al. [54], 1997	Ireland	102	61.0	33.9	5.1	3.9	11.8	4.0	29.4	40.1	0	0	0	2	2.0
Chlason et al. [55], 1999	Denmark	136	ND	ND	4.4	ND	33.8	ND	10.3	ND	8.8	36.0	ND	2.2	2.2
Wiedell et al. [56], 1998	Sweden	400	ND	ND	5.7	ND	26.6	ND	19.8	ND	2.4	32.2	ND	3.0	1.6
Bechaga et al. [41], 2004	Brazil	430	43.4	34.6	21.9	1.8	34	7.3	14	35	3	1.7	1	11	1
Martino et al. [9], 2011	Argentina	866	35.7	36.5	47.8	26.2	20.6	6.7	8.2	11.2	ND	0.8	0.7	4.2	1.4
Ordóñez-Sánchez et al. [57], 1998	Mexico	94	44.7	48.9	6.4	8.5	47.9	4.2	11.7	1.0	1.0	2.1	8.5	7.4	2.1

Table 5. (continued)

Country	Alleles, Frequency of phenotypes, n	Frequency of pathogenic variant(of alleles),													
		SW	SV	NC	p(Val281Leu)	c293-130/C>G	p(C141S)	p(Ile175Asn)	Del/conv*	p(Leu308Phe)	p(C111VAla21)	p(Ang367Tyr)	p(Pro54Ser)	ExCluster**	
Finckelstein et al. [24], 2011	364	51.6	26.3	22.1	12.6	23.4	3.3	12.6	30.5	0.3	0.5	0.8	3.6	0.5	1.1
Charfeddine et al. [30], 2012	100	44.0	28.0	28.0	12.0	6.0	26.0	8.0	22.0	ND	ND	1.0	1.0	ND	1.0
Wang et al. [6], 2016	460	43.9	26.1	30.0	0.2	36	4.6	14.3	26.4	1.7	4.3	0.2	5.9	ND	1.3
Bathani et al. [45], 2012	88	81.8	18.2	0	1.1	14.7	15.9	5.7	31.9	ND	0	0	7.95	ND	2.3
Choi et al. [46], 2012	144	73.6	26.4	0	ND	28.5	2.8	15.3	31.3	ND	ND	ND	9.0	ND	1.4
Koyama et al. [58], 2002	98	67.3	23.1	9.6	0	39.8	3.1	14.3	51	0	4.1	4.1	15.3	ND	51

* Del/conv - large gene rearrangement (large deletion and large gene conversion).

** Ex-cluster - p(Ile256A to), p(Val257Glu), p(Met259Lys).

ND, not determined.

* Del/conv - large gene rearrangement (large deletion and large gene conversion).

** ES cluster - p(D256A), p(Ile257Glu), p(Met239Lys).

ND, not determined.

Portugal has a widely known population of Spanish-exile Sephardic Jews. The origins of Ashkenazi Jews remain highly controversial, but some models point to a Mediterranean/West European ancestry [36] that could explain these similarities.

In the majority of the published series (Table 5), NC patients have always been less frequent than classic ones, and the most common pathogenic variant is c.293-13A/C>G, usually related to the classic CAH. In Portuguese patients with classic CAH, the most frequent variant is also the c.293-13A/C>G, in 14.1% of the total alleles and 31.1% of the SW ones. The second most frequent variant in the SW form is the p.(Gln319*), similarly to that described in a Spanish population [37].

An interesting finding is that we have detected a very low number of deletions or large conversions (6.7%) compared to the previously published in general population, 25% [3, 8], but once again similar to Spain [18].

The percentage of homozygous genotypes observed in the present study (46.7%) is higher than in the majority of the countries (Serbia 13% [38], China 16.1% [6], USA 21% [24], Tunisia 24% [30], the Netherlands 28.3% [39], Croatia 33% [12], Romania 33.3% [40], Brazil 39% [41]), even though not one case of consanguinity was reported. Alleles with multiple pathogenic variants accounted for 8.2% of unrelated alleles. The percentage of alleles carrying 2 or more pathogenic variants in *cis* is very variable in previous publications (6.5% in Serbia [38] to 21.6% in Spain [42]). This is related to the methodology used for the diagnosis and the segregation studies, which are extremely important for genetic counseling.

Our global genotype-phenotype correlation was 92.4%, slightly higher than the average worldwide genotype-phenotype correlation, estimated in 80–90% [10, 11, 43]. Usually, the correlation is higher than 80–90% for the pathogenic variants associated with low enzymatic activity and a SW phenotype (groups 0 and A) and for the pathogenic variants of the group C (NC phenotype), but lower for the group B pathogenic variant (usually associated with a SV phenotype) [6, 9, 11–13, 24, 39, 44–46]. In our population, the correlation was also lower for group B associated pathogenic variant (80% compared to exceeding 90% concordance on groups 0, A, and C), but with no statistical significance observed.

The lack of a genotype-phenotype correlation in some of our patients may be because sequencing was not always done, so Patients 2–5, who had more severe phenotypes than that predicted by the genotype, may have another

variation *in cis* or in the promotor. Patients 6 and 7 were females diagnosed on the first day of life due to atypical genitalia, for whom therapy with hydrocortisone and fludrocortisone was immediately started. These girls did not present an adrenal crisis, and therefore were classified as SV, but this early therapeutic intervention may have masked SW. Patients 10 and 13 had a NC phenotype despite having a genotype compatible with a SW form. One explanation could be the presence of a *CYP21A2* duplication, in a trimodular haplotype in the allele harboring the more deleterious variant. For both cases, only the 10 most common variants were analyzed, and the evaluation of duplicated genes by multiplex ligation-dependent probe amplification or Southern-blotting was not conducted. The existence of a trimodular haplotype has been reported in many populations [24, 38, 47, 48]. For instance, a random sample of the Spanish population showed that 7% of the individuals carried at least one chromosome with duplicated *CYP21A2* [18]. The majority of unaffected individuals carried a *CYP21A2* duplicated haplotype with the p.(Gln319*) pathogenic variant, in a trimodular genotype [13, 24]. At least one of our patients (Patient 17) had a *CYP21A2* duplication – a girl that carries the p.(Gln319*) pathogenic variant in one allele and the *CYP21A2* duplication *in cis* with the p.Gln319* in the other allele, who expresses a NC form of 21OHD, instead of the expected absence of 21OHD symptoms.

Patients 14–16 have a group B genotype, usually associated with a SV CAH, but present a NC phenotype. Despite previous reports showed a lower genotype-phenotype correlation for p.(Ile173Asn) [13, 44], it is possible that these patients had unrecognized mild clitoromegaly at earlier ages. Patients 11–14, carried the c.293-13A/C>G splice variant, which can be “leaky” such that a small amount of the normal transcript is made resulting in phenotypic heterogeneity [49]. Thus, despite the classification of CAH into 3 different clinical forms, the disease represents a continuous phenotypic spectrum [1].

Basal 17OHP levels in our patients with NC 21OHD were higher than the recommended cutoff levels (basal median 1,250 ng/dL/post-ACTH median 5,300 ng/dL vs. the recommended basal >200 ng/dL and post-ACTH >1,200 ng/dL). Moreover, as previously reported, NC 21OHD patients that are compound heterozygotes for a severe pathogenic variant present higher levels of ACTH-stimulated 17OHP than homozygotes/compound heterozygotes for milder alleles [34].

Finally, as there is no newborn screening for CAH in Portugal, the majority of the males in our sample (97.1%)

and a substantial number of females (22.2%) were diagnosed after a neonatal adrenal crisis (median age at diagnosis: 31 days of life). The only SW boy who did not experience adrenal crisis had a prenatal diagnosis of 21OHD because his mother is a CAH patient and his father was genotyped and is a carrier.

A pilot study for universal newborn screening was conducted in Portugal in the 80s but was abandoned because of a delay in the communication of the results, due to the methodology used at that time, and therefore could not be able to anticipate the adrenal crisis [50]. However, the current technology for neonatal screening has evolved, and it is recommended by the Endocrine Society [51] since it allows the identification of the majority of classic CAH during the first days of life. Without universal screening, as we can easily see in our cohort, newborn males, who have no genital abnormalities to raise suspicion, are particularly at risk for an adrenal crisis.

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Statement of Ethics

All procedures performed in this study were in accordance with the National Committee for Data Protection and with the 1964 Helsinki Declaration or Comparable Ethical Standards. This study was approved by the local Ethics Committee.

Disclosure Statement

The authors have no conflicts of interest to declare.

Author Contributions

R.S.-S.: conception and design of the work, acquisition, analysis and interpretation of data, drafting the work, and writing the paper. R.C.: conception and design of the work, acquisition of data, drafting the work, and paper revision. L.L., F.E., L.S., C.B., A.A., G.B., R.C., Teresa Bernardo, P.V., A.L.L., L.R., and M.J.O.: acquisition, analysis and interpretation of data, drafting the work, and paper revision. M.F. and A.M.: analysis and inter-

pretation of data and paper revision. R.M.: acquisition, analysis and interpretation of data, drafting the work, and technical editing of the manuscript. B.C.: conception and design of the work, analysis and interpretation of data, writing and reviewing the paper critical, and scientific advisor. A.G.: characterization of the novel mutations, writing and reviewing the paper critical,

and scientific advisor. R.O.: writing and reviewing the paper critical and scientific advisor. V.R., J.R.S., B.M., A.M.G., A.M., J.C., and A.S.: acquisition of data and review of the final version. T.B.: conception and design of the work, acquisition, analysis and interpretation of data, and critical review of the paper.

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PAPER IV

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

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

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

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

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

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

MOLECULAR GENETIC TESTING

CYP21 Gene Structure

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

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

gene and one copy of the *CYP21A1P* pseudogene is also possible and has been described in carriers of the p.(Gln319*) pathogenic variant and of chimeric *CYP21A1P/CYP21A2* genes (31–34).

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

CYP21A2 Pathogenic Alterations

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

This missense pathogenic variant results from a transition of a CCC to a TCC and was initially described as not present in the pseudogene (63, 64). Although the functional mechanism is not clearly explained it corresponds to a decrease of 50–68% of 17-OHP and 20–46% of progesterone utilization (65). It occurs in a number of different populations and suggests that *CYP21A1P* may carry P453S as an occasional polymorphism and that this pathogenic variant is transferred to *CYP21A2* in the same way as the other pathogenic variants frequently causing 21-hydroxylase deficiency (2).

Other Pathogenic Variants

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

of these pathogenic variants are missense, and require functional studies to be classified.

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

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

Polymorphisms

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

GENOTYPING AND PREGNANCY

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

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

GENOTYPE-PHENOTYPE CORRELATIONS

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

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

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

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

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

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

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

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

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

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

There are several examples that, in spite of the general assumption that there is a relatively high concordance between genotype and phenotype there is some variability, particularly in the moderately affected patients (75–77). The pathogenic variants designated as IVS2-13 (c.293-13A/C>G) and I172N (p.(Ile173Asn)) for instance result in variable degrees of

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

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

21-hydroxylase activity (possibly through alternative splicing) hence explaining that patients that would generally be expected to be Simple Virilizing cases may sometimes be Salt-Wasting and others also be closer to NCAH (51, 60). Another example is the P30L (p.(Pro31Leu)) pathogenic variant which can be associated with cases of NCAH as well as cases of SV-CAH (64).

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

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

PREVALENCE IN DIFFERENT ETHNIC POPULATIONS

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

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

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

Single-Stranded Conformation Polymorphisms (SSCP)

If double stranded DNA is denatured and then quickly returned to native conditions, it will remain in a single-stranded state with a characteristic conformation that can be detected by a change in the mobility of the segment during polyacrylamide gel

electrophoresis under non-denaturing conditions. This method can detect novel pathogenic variant that would be missed by allele-specific approaches, but has some complexity in execution and interpretation (Figure 7) (74, 92).

Allele-Specific PCR (ARMS)

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

Ligation Detection Reaction (LDR)

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

D-HPLC

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

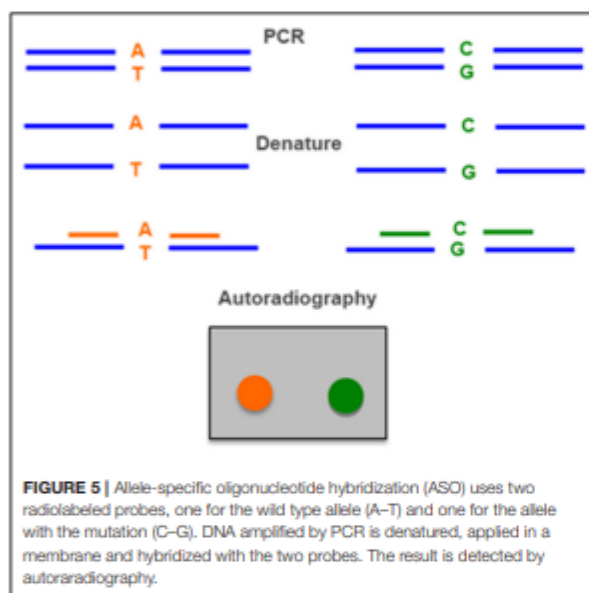


FIGURE 5 | Allele-specific oligonucleotide hybridization (ASO) uses two radiolabeled probes, one for the wild type allele (A-T) and one for the allele with the mutation (C-G). DNA amplified by PCR is denatured, applied in a membrane and hybridized with the two probes. The result is detected by autoradiography.

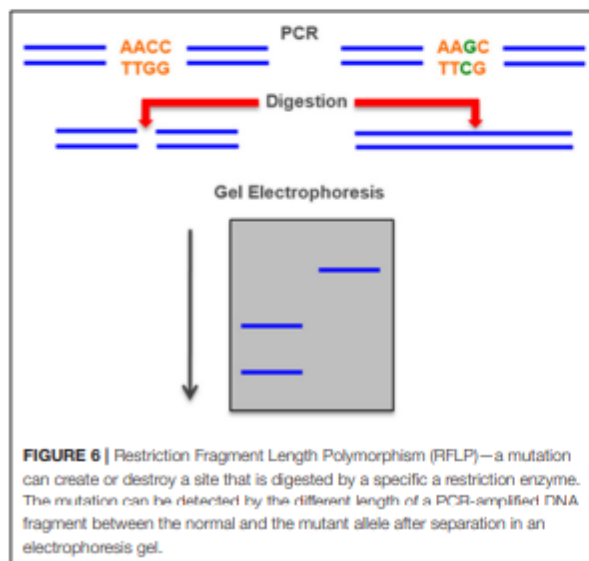


FIGURE 6 | Restriction Fragment Length Polymorphism (RFLP)—a mutation can create or destroy a site that is digested by a specific restriction enzyme. The mutation can be detected by the different length of a PCR-amplified DNA fragment between the normal and the mutant allele after separation in an electrophoresis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

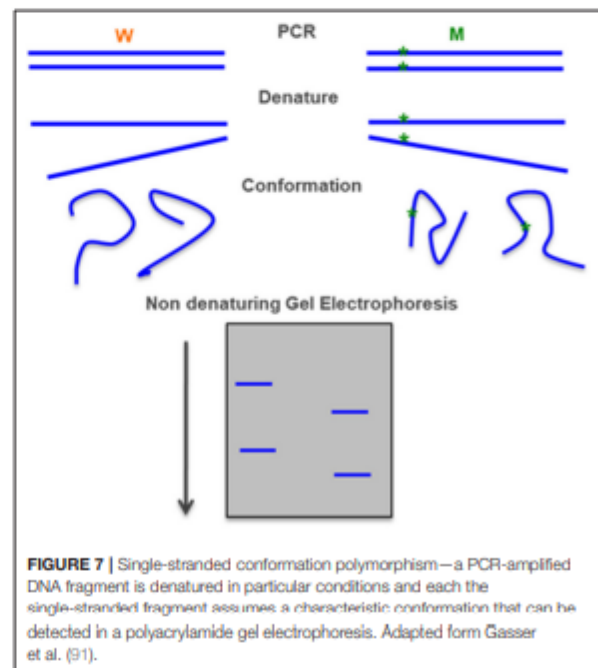


FIGURE 7 | Single-stranded conformation polymorphism—a PCR-amplified DNA fragment is denatured in particular conditions and each the single-stranded fragment assumes a characteristic conformation that can be detected in a polyacrylamide gel electrophoresis. Adapted from Gasser et al. (91).

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

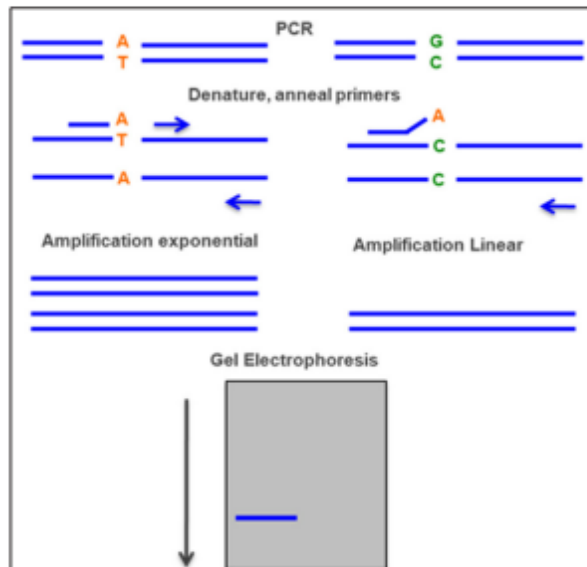


FIGURE 8 | Allele-specific PCR—two PCR reactions are done simultaneously, with the same primer in one end and two different primers on the other end, one with the normal sequence and the other with the mutant. The rate of amplification is much higher with the specific primer and can be detected by gel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

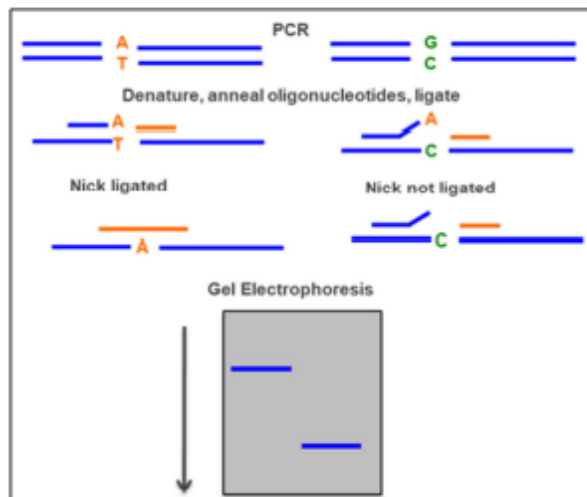


FIGURE 9 | Ligase detection reaction—DNA ligase preferentially seal adjacent oligonucleotides hybridized to a DNA sequence when there is a perfect complementarity at the nick junction. A single base mismatch generates a different fragment detected by gel electrophoresis.

Minisequencing and Multiplex Minisequencing

In minisequencing, a primer is hybridized to DNA next to a variant nucleotide site and extended with DNA polymerase by a single appropriate dideoxynucleotide triphosphate (ddNTP) that matches the nucleotide at the target site. This method can be used in a multiplex reaction with primers elongated at the 5' end with a poly(T) track of different sizes to facilitate electrophoretic separation of the diagnostic products (Figure 11) (97).

DUPLICATIONS AND DELETIONS

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

Southern-Blot

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

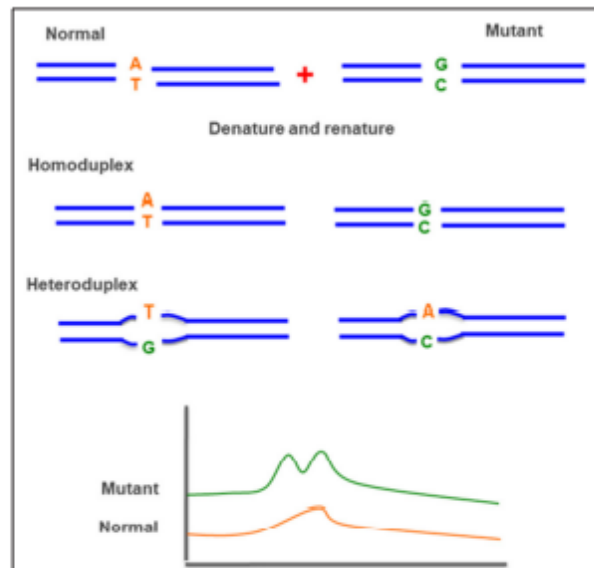
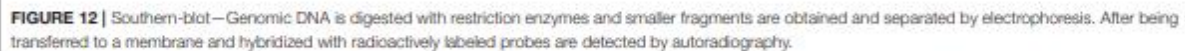
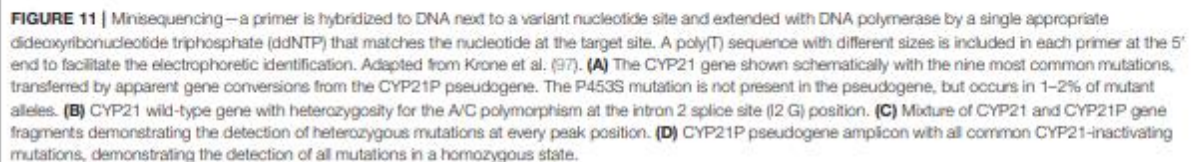
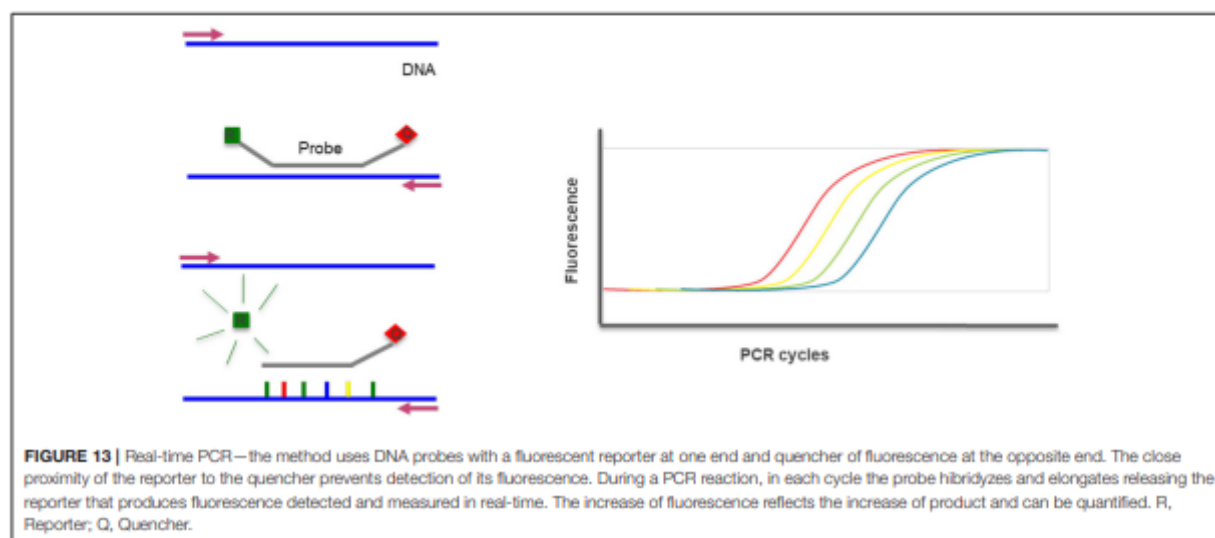


FIGURE 10 | Denaturing high pressure liquid chromatography (D-HPLC)—uses heteroduplex formation between normal and mutated DNA strands. The different conformations are detected by ion-pair reverse-phase liquid chromatography.



Real time PCR is a technique where the progressions of a PCR reaction can be monitored in real time and simultaneously

quantify the amount of product amplified. The method is based on the detection of the fluorescence produced by a reporter molecule which increases, as the reaction proceeds.



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

MLPA

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

DNA SANGER SEQUENCING

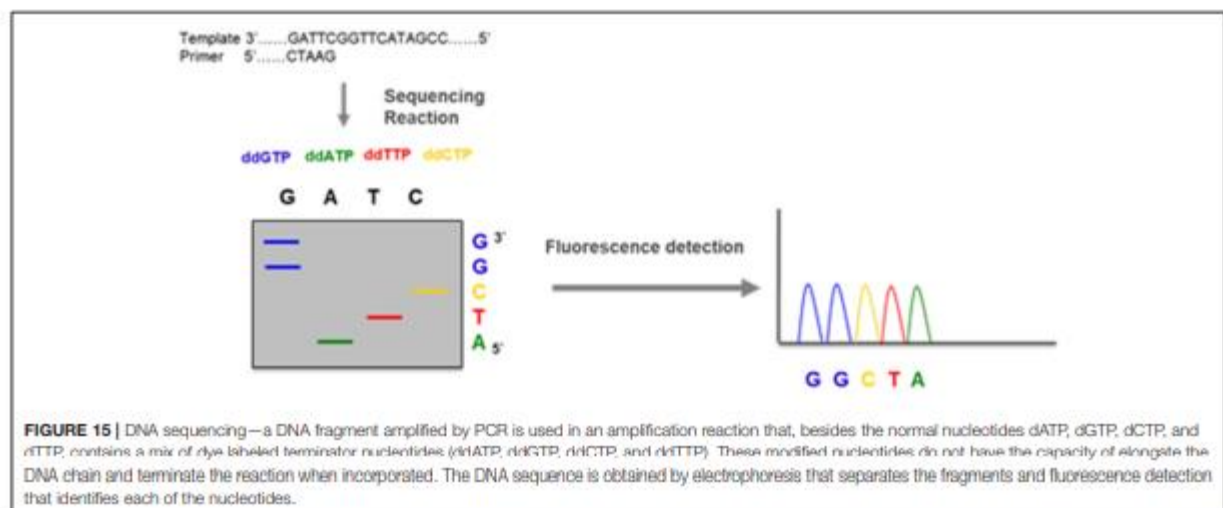
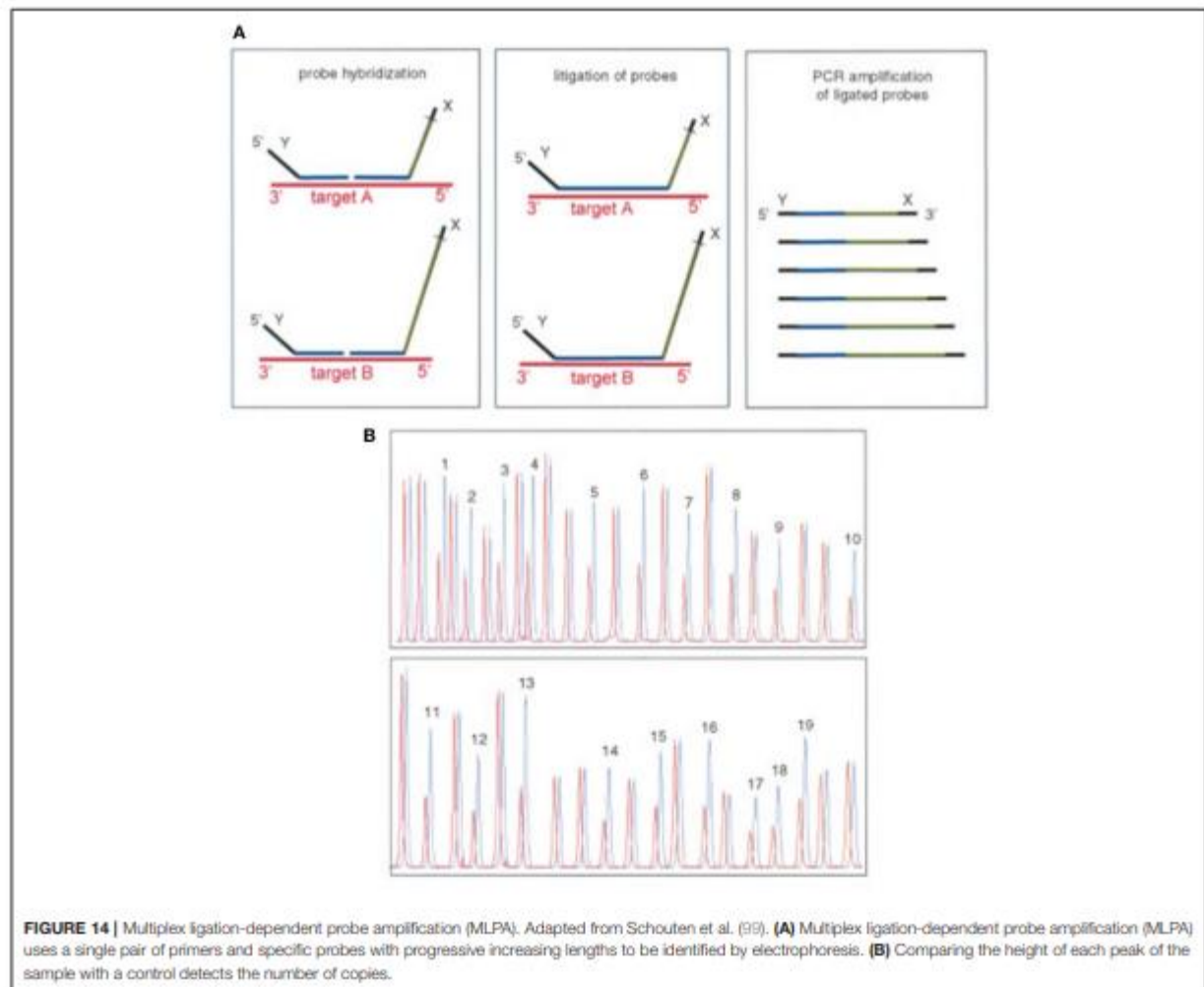
Nowadays, in many hospitals, a whole gene sequencing together with MLPA has become the standard procedures to genotype the *CYP21A2* gene in cases of 21OH Deficiency. This is the method

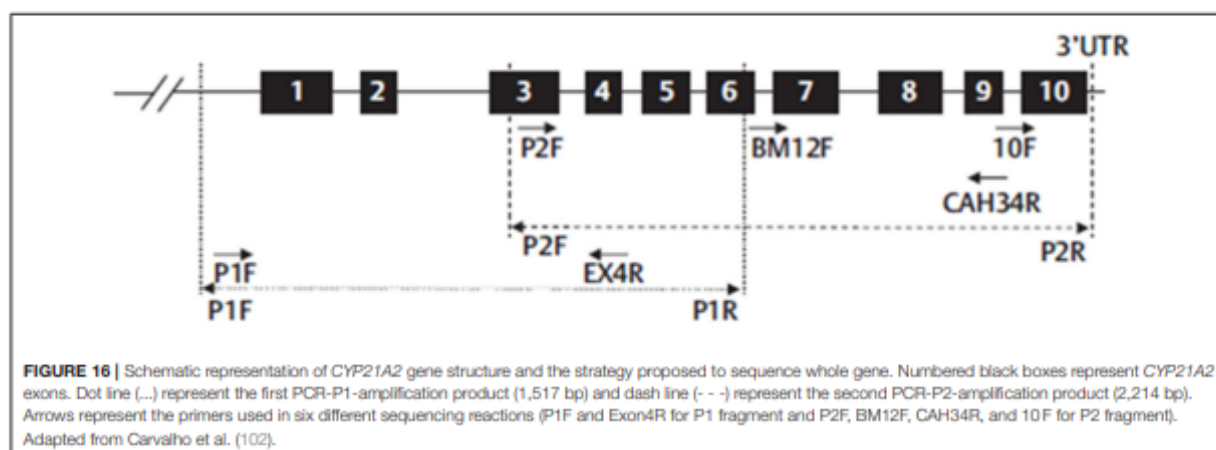
elected to detect pathogenic variants not screened by the targeted analysis and is also able to detect novel sequence variants. It usually covers the coding regions and the flanking intron-exon regions of the gene. *CYP21A2* whole genomic sequence may be performed selecting the functional *CYP21A2* gene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

NEW ASPECTS IN GENOTYPING

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

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





CONCLUSION

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

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

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

The diagnosis is first confirmed through 17OH-progesterone determinations which can be very high, moderately high or even normal at basal conditions needing confirmation through an ACTH stimulation test. The defining cut-off is generally considered to be between 10 and 15 ng/ml, either basally or

post-ACTH. In the case of women these dosages should be done in the follicular phase of their menstrual cycles and in every patient the blood samples should be collected early in the morning. Newborn screening programs are very important as they permit the identification of severe cases at the ideal time for treatment thus being life-saving in many situations. In consequence of this screening programs, survival is no longer the major issue and has been replaced by the need to improve the patients' quality of life.

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

AUTHOR CONTRIBUTIONS

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

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PAPER V

**Congenital Adrenal Hyperplasia due to 21-Hydroxylase Deficiency: An Update on
Genetic Analysis of *CYP21A2* gene**

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Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency: An Update on Genetic Analysis of CYP21A2 Gene

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ABSTRACT

Congenital Adrenal Hyperplasia is a group of genetic autosomal recessive disorders that affects adrenal steroidogenesis in the adrenal cortex. One of the most common defects associated with Congenital Adrenal Hyperplasia is the deficiency of 21-hydroxylase enzyme, responsible for the conversion of 17-hydroxyprogesterone to 11-deoxycortisol and progesterone to deoxycorticosterone. The impairment of cortisol and aldosterone production is directly related to the clinical form of the disease that ranges from classic or severe to non-classic or mild late onset. The deficiency of 21-hydroxylase enzyme results from pathogenic variants on CYP21A2 gene that, in the majority of the cases, compromise enzymatic activity and are strongly correlated with the clinical severity of the disease. Due to the exceptionally high homology and proximity between the gene and the pseudogene, more than 90 % of pathogenic variants result from intergenic recombination. Around 75 % are deleterious variants transferred from the pseudogene by gene conversion, during mitosis. About 20 % are due to unequal crossing over during meiosis and lead to duplications or deletions on CYP21A2 gene. Molecular genetic analysis of CYP21A2 variants is of major importance for confirmation of clinical diagnosis, predicting prognosis and for an appropriate genetic counseling. In this review we will present an update on the genetic analysis of CYP21A2 gene variants in CAH patients performed in our department.

Introduction

Congenital Adrenal Hyperplasia (CAH), OMIM #201910, comprises of a group of genetic disorders inherited in an autosomal recessive pattern caused by a complete or a partial defect in one of the enzymes involved in the steroidogenesis pathway. One of the most common defective enzyme is the 21-hydroxylase (21OH), responsible for impairment of cortisol biosynthesis, that accounts for about 90–95 % of CAH patients. Steroid 21-hydroxylase enzyme,

which is expressed in the cytoplasm of the adrenal cortex, is encoded by CYP21A2 gene. The existence of pathogenic variants of CYP21A2 gene may be directly correlated to the clinical phenotype of CAH due to the impairment of enzymatic activity. As a result of different degrees of enzyme dysfunction, CAH presents in three major clinical forms: Classical salt wasting (SW), Classical simple virilising (SV) and Non-classical form (NC).

In this review, we will present an update on the genetic diagnosis of CAH due to 21-hydroxylase deficiency (21OHD), which is the most common cause of CAH.

Epidemiology and Etiology of 21OHD

CAH results from a defect in one of the several enzymes of the adrenal steroidogenesis pathway and clinical manifestations depend on the enzyme involved, type of pathogenic variant and the hormone level that is overproduced or deficient. Following the most common 21OHD, 11-beta hydroxylase (11OHase) deficiency and 3-beta hydroxysteroid dehydrogenase (3βHSD) deficiency are the other main enzymes involved in CAH. Less commonly, p450 oxidoreductase deficiency, 17 hydroxylase deficiency and lipoid CAH (StAR deficiency) may also be associated with other forms of CAH [1, 2]. The 21OH enzyme is involved in the glucocorticoid and mineralocorticoid pathways and is responsible for the conversion of 17-hydroxyprogesterone (17OHP) to 11-deoxycortisol and progesterone to deoxycorticosterone. Cortisol deficiency stimulates ACTH secretion, which in turn drives the production of androgens (DHEA and androstenedione) due to an increase in the precursors proximal to the block in the glucocorticoid pathway. DHEA and androstenedione are converted into testosterone which is responsible for virilization; one of the major clinical manifestations of CAH due to 21OHD.

CAH due to 21OHD has a worldwide incidence of approximately 1:14 000 to 1:18 000 births, based on neonatal screening and national cases registries [1]. Late onset NC-CAH may affect around 1:100 to 1:1000 individuals [3, 4], showing a higher prevalence within some specific sub-groups, specifically Eastern European Jews, Yugoslavs, Mediterranean and Hispanics [5]. Since the prevalence of C-CAH and NC-CAH has been reported to vary among populations, the expected heterozygote frequency for 21-OHD also varies considerably and a C-CAH carrier frequency of 1:55 was been estimated based on results obtained from newborn screening programs [6, 7]. However, one study conducted on middle European population, showed a CAH frequency of 1:10, higher than previously published [7]. Moreover, for certain ethnic groups such as the Ashkenazi Jews, the carrier frequency was reported to be 1:3, thus making it to be the highest ever reported for the disease [8].

Clinical evaluation of CAH patients

The severity of clinical manifestations of 21OHD may distinguish CAH into the classic, (which is more severe), and the NC form. The classic form is further divided into the SV form (~25 % of individuals) and SW form (≥75 % of individuals) in which aldosterone production is impaired. In classic 21OHD CAH, virilization of external genitalia due to prenatal exposure to potent androgens, such as testosterone and Δ4-androstenedione at critical stages of sexual development, often results in atypical genitalia at birth. In affected females, the excess of androgen production results in different degrees of virilization, including clitoromegaly, fusion of the labioscrotal folds, and formation of a urogenital sinus [9]; the uterus and fallopian tubes develop normally as anti-müllerian hormone (AMH) is not secreted. As these clinical manifestations affect both SW and SV forms, it is not possible to distinguish them based only on the degree of virilization alone of an affected female at birth. Affected males have usually normal genitalia but can present with scrotal

hyperpigmentation. Both females and males with classic SV CAH develop signs of androgen excess including precocious pubarche, acne, rapid linear growth, and advanced bone age; in these cases, glucocorticoid replacement therapy should be started. Untreated males have progressive penile enlargement and small testis and untreated females have clitoral enlargement, hirsutism, male pattern baldness, menstrual abnormalities, and infertility. Untreated patients with 21OHD CAH usually grow fast in prepubertal years and present with increased bone maturation. However, short adult stature is common resulting from premature epiphyseal fusion [10].

The blockade on 21OH function may be more severe and adrenal aldosterone secretion not enough for sodium reabsorption by the distal renal tubules, resulting in salt wasting characterized by cortisol deficiency and androgen excess. Newborns with SW-CAH caused by 21OHD are at risk of life-threatening salt-wasting crises in the first four weeks of life, which manifest by: poor feeding, vomiting, weight loss, dehydration, hypotension, hyponatremia and hyperkalemic metabolic acidosis. Affected newborn males, if not detected at newborn screening programs, are at higher risk for a salt-wasting adrenal crisis, because of their normal male genitalia [11]. Conversely, the atypical genitalia of females with the SW form usually allows early diagnosis and treatment.

Individuals with the NC form of 21-OHD CAH have moderate enzyme deficiency and present with symptoms of androgen excess postnatally. Females with the NC form are not virilized at birth and may present with precocious pubarche, hirsutism, acne, frontal baldness, delayed menarche, oligomenorrhea, and infertility. Hirsutism is the most common clinical manifestation affecting approximately 60 % of adult women with NC-CAH. Approximately 10 % of women have hirsutism and menstrual irregularities whilst approximately 10 % of women present with only a menstrual disorder. NC-CAH was identified in 2.2–10 % of women with hyperandrogenism [12–14]. The fertility rate among untreated women is reported to be 50 % [15]. Males with NC-CAH may have early beard growth and an enlarged penis with relatively small testes, however typically there is no evidence of impaired gonadal function, and adult male may present testicular adrenal rest tumors [16, 17].

Clinical diagnosis of CAH comprehends newborn screening that is commonly used in many countries. Newborn screening for CAH employs immunoassays to measure 17OHP in dried blood spots on filter paper cards. If the newborn is suspected of having CAH, referral to Paediatric endocrinology is warranted and serum 17OHP should be measured by liquid chromatography–tandem mass spectrometry [1]. A cosyntropin stimulation test should be performed to confirm diagnosis prior to initiation of corticosteroid treatment. In symptomatic individuals post infancy, an early-morning baseline serum 17OHP measurement by liquid chromatography–tandem mass spectrometry is recommended. Regarding individuals with borderline 17OHP levels, a complete adrenocortical profile after an ACTH stimulation test, to differentiate 21OHD from other enzyme defects, is recommended [1].

In those individuals where a clinical diagnosis of CAH was achieved, genotyping of CYP21A2 gene is advised when stimulation tests are equivocal or could not be accurately performed due to ongoing therapeutic administration. Beyond those cases, genetic testing should also be done in clinically and biochemically well

characterized C-CAH forms. In families at risk of the classical form of CAH, genotyping of the entire gene of at least one parent should be performed for an appropriate familial genetic counselling.

Genetic diagnosis of 21OHD

CYP21A2 gene is responsible for encoding the 21OH enzyme, member of the cytochrome P450 (Cytochrome P450 family 21 subfamily A member 2) superfamily of enzymes. The cytochrome P450 proteins are mono-oxygenases which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. The *CYP21A2* protein is exclusively expressed on the adrenal gland [18]. This protein localizes to the endoplasmic reticulum and hydroxylates steroids at the 21 position. Its activity is required for the synthesis of steroid hormones including cortisol and aldosterone. A related functionally inactive pseudogene (*CYP21A1P*) is located near the *CYP21A2* gene. This gene which is adjacent and alternate to the active gene and the active gene are located in a small region on chromosome 6p21.3, within the human leukocyte antigen (HLA) major histocompatibility complex (MHC) class III. Due to the high degree of homology between functional gene and inactive pseudogene, almost 98 % on coding regions and 96 % on non-coding regions, gene conversion events involving these genes are thought to be responsible for many cases of steroid 21OHD. Approximately 95 % of known pathogenic variants are due to recombination events between the inactive and active genes, resulting in gene conversions. However, pathogenic variants may also occur by new causal mutagenic events in about 5 % of the cases [19, 20]. Both, functional *CYP21A2* and inactive pseudogene *CYP21A1P* together with their neighbouring genes *tenascin TNXA/B*, complement *C4A/B*, and the serine/threonine nuclear protein kinase *RP*, plus small truncated sections of *TNX* and *RP*, form a genetic unit designated as RCCX module [21]. Due to conversion events between the active and inactive genes, copy number variations on RCCX locus have been described. Two RCCX modules represent the standard with three or four modules being rarer cases [21, 22]. A trimodular haplotype, described in less than 2 % of the general population and carrying one copy of the *CYP21A1P* pseudogene and two copies of the *CYP21A2* gene, with the latter harbouring the pathogenic variant p.Gln319*, may complicate the genetic analysis [21]. However, in healthy Spanish population, a duplicated *CYP21A2* gene, in which one copy is affected with p.Gln319*, was described in more than 7 % of the population [22].

The majority of children with SW-CAH present with a combination of two severe pathogenic variants, whereas individuals with the NC form may be compound heterozygous, having one severe and one mild pathogenic variant or may be homozygous with two mild pathogenic variants. So far, more than 300 pathogenic variants have been described (<http://www.hgmd.cf.ac.uk>). Although the majority of patients are compound heterozygous, it should be noted that approximately 20 % of the alleles in the NC-CAH form remain without any identifiable pathogenic variant, emphasizing the importance to investigate further on *CYP21A2* regulatory regions [23].

CYP21A2 gene analysis

CAH patients with clinical requirements for genetic analysis were initially screened for the ten most common disease-causing path-

ogenic variants by Polymerase Chain Reaction-Sequence Specific Primer (PCR-SSP) for p.Gly111Valfs*21, p.Ile173Asn and exon 6 cluster p.Ile236Asn, p.Val237Glu, p.Met239Lys, Restriction Fragment Length Polymorphism (RFLP) for large deletion/conversions extending to about 30Kb with breakpoints between exons 3 and 8 the *CYP21A2* promoter region and direct DNA sequencing for p.Pro311Leu, c.293-13 A/C > G, p.Val282Leu, p.Leu307Phefs*5, p.Gln319* and p.Arg357Trp (► Fig. 1a). Those variants represented about 70–75 % of CAH alleles [24–26]. This screening strategy proved to be expensive and labour intensive and the detection of two pathogenic variants did not exclude the presence of further pathogenic variants in regions not analysed. Recently, novel aspects related to the complete study of functional *CYP21A2* gene appeared and have been replacing the original screening strategy. The high homology between gene and pseudogene, and the presence of a great number of SNPs in the functional gene makes it difficult to design specific primers for the genetic diagnosis. In our current strategy, the functional gene is amplified by PCR into two partially overlapping fragments, respectively with 1517 and 2214 base-pairs (bp), avoiding the co-amplification of the pseudogene *CYP21A1P* [27]. The PCR products may be purified and sequenced with internal primers covering the entire 10 exons and intron-exon boundaries of *CYP21A2* [28] (► Fig. 1b). The use of proof reading Taq-polymerase is recommended in order to minimize the risk of allele drop-out that should be taken into account when complete homozygosity after sequencing is present [28].

Multiplex Ligation dependent Probe Amplification (MLPA) analysis

Screening for deletions and duplications of the *CYP21A2* gene and *CYP21A1P* pseudogene is performed by MLPA (► Fig. 1c), using the P050-CAH Kit (MRC-Holland) [29]. This technique has the advantage of being easy to set up, inexpensive and not subject to subjective analysis between laboratories worldwide. Although the technique is widely used, the test is not CE-certified but complies with ISO 13485 certification for Quality Management System. Southern Blot (SB) analysis is the original method used to detect large gene deletions and conversions. This method used at least two restriction enzymes (TaqI and BglII) to differentiate between deletions and conversions. Compared to SB, MLPA is less time consuming, requires less DNA concentration and allows the manipulation of several samples simultaneously [11, 30, 31]. MLPA allows the confirmation of point pathogenic variants detected by Sanger sequencing and avoids misinterpretation of a pathogenic genotype. It is important to be aware of the high frequency of duplicated alleles in association with p.Gln319* variant and MLPA may help to clarify *CYP21A2*-gene copy number [21, 31]. In case p.Gln319* is present and 2 copies of *CYP21A2* genes were detected, the allele bearing p.Gln319* represents a CAH allele. When three copies of *CYP21A2* alleles are detected, a familial study should be performed in order to detect the localization of p.Gln319* variant. If the allele bearing the p.Gln319* variant is the same carrying the duplicated gene it does not represent a CAH allele, but rather a normal functional allele [32]. Such findings are of extreme importance both for a correct genotype report and for an appropriate genetic counselling.

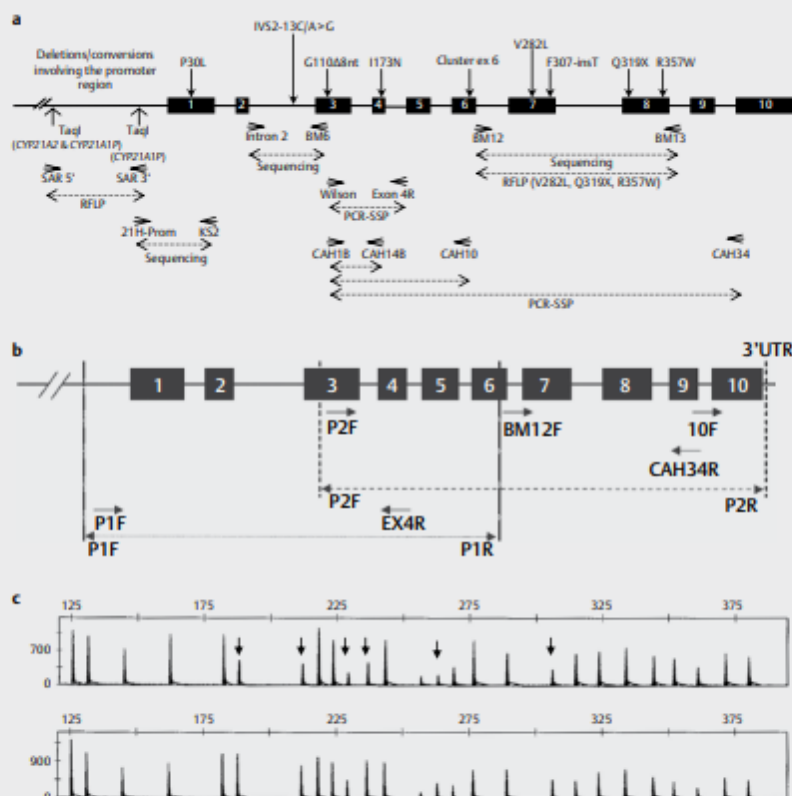


Fig. 1 **a** Schematic representation of the structure of the *CYP21A2* gene and the methodology used for screening of 10 most common variants causing 21OHD [26]. Numbered black boxes represent *CYP21A2* exons. Horizontal arrows show the position of primers used for the six different PCR amplifications; Double arrows represent PCR amplified fragments. Restriction site positions for *TaqI* restriction enzyme in *CYP21A2* and *CYP21A1P* are also represented. **b** Schematic representation of *CYP21A2* gene structure and strategy for complete gene sequencing [28]. Numbered black boxes represent *CYP21A2* exons. Dot line (...) represent the first PCR - P1 - amplification product (1517 bp) and dash line (- - -) represent the second PCR - P2 - amplification product (2214 bp). Arrows represent the primers used in 6 different sequencing reactions: P1F (5'-TCGGTGGGAGGGTACCT-GAAG-3') and Exon4R (5'-TCGTCCTGCCAGAAAAGGAG-3') for P1 fragment and P2F (5'-TGTCTTGGGAGACTACTCC-3'), BM12F (5'-GAGGGATCA-CATCGTGGAGAT-3'), CAH34R (5'-CTCTGGAGTTCTGCCTGGCTCCAGG-3') and 10F (5'-AGAGGCCACATGAGTTCTGG-3') for P2 fragment). **c** Electropherogram of a MLPA result. Upper panel show a sample with a heterozygous deletion in all probes for *CYP21A2* gene. Arrows represent the half diminish intensity probes. Lower show a normal control sample.

CYP21A2 genotype interpretation and reporting

Pathogenic variants on *CYP21A2* gene can present differently according to the gene transcript used. A very common polymorphism described on exon 1 of *CYP21A2* gene can affect the numbering of nucleotides and consequently, the codons numbering. Here, the authors recommended the nomenclature according to ENSEMBL transcript CYP21A2-221:ENST00000418967.6 as a reference sequence and HGVS nomenclature [33].

CYP21A2 genotyping in CAH index cases should be accompanied by a brief clinical description and biochemical 17OHP levels. Additional information, such as ACTH-values, ethnicity and familial history are useful in order to allow the correct interpretation of results. The description of two pathogenic variants in CAH patients should be followed by familial segregation studies in order to determine whether the variants are in *trans* or in *cis* and allow a genotype-phenotype correlation. Worldwide genotype-phenotype correlation is estimated to be around 80–90% [34, 35]. In a very re-

cent study on Portuguese paediatric cohort the global genotype-phenotype correlation was 92.4% particularly in groups of SW and NC patients [36]. Usually, a better correlation between phenotype-genotype is achieved on SW and NC forms, where genotype can predict the clinical severity of the disease [37]. Particular attention should be taken in SV form, where a large phenotypic variability is usually noted, and sometimes even in the supposed clearly correlated cases, the genotype-phenotype discordance exists [38, 39]. In summary, the lack of a genotype-phenotype correlation in some cases reinforces the necessity to extend the analysis to less studied regions that may direct gene regulation.

Conflict of Interest

The authors declare that they have no conflict of interest.

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PAPER VI

Differences in hormonal levels between heterozygous *CYP21A2* pathogenic variant carriers, non-carriers, and females with non-classic congenital hyperplasia

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Differences in hormonal levels between heterozygous *CYP21A2* pathogenic variant carriers, non-carriers, and females with non-classic congenital hyperplasia

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ABSTRACT

Objective: *CYP21A2* mutation heterozygote carriers seem to have an increased risk of hyperandrogenism. However, the clinical relevance of the heterozygote carrier status and the reliability of hormonal testing in discriminating a carrier from a non-carrier are puzzling questions. We aimed to characterize a population of Portuguese females suspected of having non-classic congenital adrenal hyperplasia (NC-CAH) due to clinical and biochemical criteria and who have undergone *CYP21A2* molecular analysis. **Subjects and methods:** Retrospectively, we have analyzed the clinical records of 131 females (32 girls aged 3-9 and 99 adolescents and premenopausal women aged 13-49) who underwent complete *CYP21A2* molecular analysis due to suspicion of NC-CAH. We divided included participants into three groups according to the *CYP21A2* molecular analysis: NC-CAH females (46), heterozygous carriers (49), and wild type (36). We then compared clinical signs and symptoms as well as biochemical and molecular data between carriers and NC-CAH individuals and between carriers and wild type females. We measured 17OHP by electrochemiluminescence immunoassay. **Results:** Clinical features were similar between groups. Heterozygous carriers presented higher basal and post-cosyntropin 17-hydroxyprogesterone (17OHP) than wild type individuals ($p < 0.05$) and lower basal and stimulated 17OHP levels than NC-CAH patients ($p < 0.05$). We discovered a considerable overlap between 17OHP levels among groups. The most common pathogenic variant we identified was p.Val282Leu. **Conclusion:** In this population of hyperandrogenic women and children, heterozygous carriers showed higher basal and stimulated 17OHP than non-carriers although normal basal and stimulated 17OHP responses do not exclude heterozygosity for *CYP21A2* pathogenic variants. In this study, only the molecular analysis presented good sensitivity in identifying heterozygotes.

Keywords

Heterozygous carrier; 17-hydroxyprogesterone; precocious pubarche; hyperandrogenism

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INTRODUCTION

Congenital adrenal hyperplasia (CAH) entails a group of inborn errors of adrenal steroidogenesis. Deficiency of 21-hydroxylase (21OHD) accounts

for 95% of the CAH cases. It results in defective conversion of 17-hydroxyprogesterone (17OHP) to 11-deoxycortisol and leads to hypocortisolism and hyperandrogenism due to the accumulation of precursor



steroid hormones. It has a broad spectrum of clinical forms, ranging from classic salt wasting, classic simple virilizing and non-classic (NC). The NC female patients generally present premature pubarche in midchildhood or hirsutism, severe acne, oligomenorrhea, or infertility in adolescence and adulthood.

Non-classic congenital adrenal hyperplasia (NC-CAH) affects around 1 in 100 to 1 in 1,000 individuals (1), and the frequency of heterozygote carriers is approximately 1 in 55 in the European population (2).

Pathogenic variants in the *CYP21A2* gene cause various degrees of dysfunction in enzymatic activity, and a strong genotype-phenotype correlation emerges, especially in the disease's most severe forms (3). Genetically, CAH is an autosomal recessive disorder; therefore, an affected individual must present with at least two biallelic pathogenic variants, and the phenotype is conferred by the less deleterious variant (4).

The clinical relevance of the heterozygote carrier status and the reliability of hormonal testing in discriminating a carrier from a non-carrier are still puzzling questions. A heterozygous carrier's normal allele should encode a *CYP21A2* protein with almost complete activity, which ensures a normal physiological function. However, heterozygous carriers of *CYP21A2* pathogenic variants seem to have an increased risk of hyperandrogenism, which may manifest as premature pubarche, accelerated growth, apocrine body odor, and seborrhea in childhood (5-8) and hirsutism, oligomenorrhea, acne, and infertility in adolescence and adulthood (9-12).

In cohorts of hyperandrogenic women and children, the frequency of the carrier status varies based on various inclusion criteria and ranges from 8% to 35% (7,12-15). Although some studies have shown a higher prevalence of *CYP21A2* carriers in hyperandrogenic individuals than in the general population (16,17), others have shown no such difference (13,18-20).

We aimed to characterize a population of 131 Portuguese females, aged 3-49, suspected of having NC-CAH due to clinical and biochemical criteria, who have undergone *CYP21A2* molecular analysis. In this population of 131 females, we aimed to compare 49 heterozygous carriers of *CYP21A2* pathogenic variants with 46 NC-CAH individuals (2 pathogenic variants identified) and 36 subjects without any pathogenic variant on *CYP21A2*.

SUBJECTS AND METHODS

Population

We have analyzed a population of 131 females referred to our hospital's endocrinology and pediatric endocrinology clinics in the past 10 years who underwent *CYP21A2* molecular analysis due to suspected NC-CAH. This population included 32 (24%) girls aged 3-9 and referred to our clinics due to precocious pubarche as well as 99 (76%) adolescents and premenopausal women aged 13-49 referred due to hirsutism, oligomenorrhea, severe acne, alopecia, and/or infertility.

We performed *CYP21A2* molecular analysis for all these participants. Criteria for the molecular analysis in our center were basal 17 OHP > 2 ng/mL and post-ACTH > 10 ng/mL, familial history of CAH plus symptoms of hyperandrogenism, or severe hyperandrogenic signs or symptoms.

Participants fulfilled clinical and biochemical criteria, and we divided them into three groups according to the *CYP21A2* molecular analysis. Group I included 46 females (12 girls, 34 women) with a diagnosis of NC-CAH, which was defined as normal genitalia at birth and clinical signs of hyperandrogenism that appeared later in childhood (pubarche, overgrowth, apocrine body odor) and adolescence (hirsutism, acne, oligomenorrhea, infertility), together with a genetic analysis showing two pathogenic variants in both *CYP21A2* alleles (homozygous or compound heterozygous). Group II included 49 heterozygous carriers of the *CYP21A2* pathogenic variant (10 girls, 39 women) with clinical features overlapping NC-CAH but having a single disease-causing CAH *CYP21A2* pathogenic variant in one allele. Group III included 36 females (10 girls, 26 women) with features overlapping NC-CAH but without any *CYP21A2* pathogenic variant identified.

Clinical assessment

An endocrinologist or a pediatrician evaluated all patients enrolled in the study at referral, and clinical signs and symptoms were abstracted from clinical records. Premature pubarche was defined as the development of pubic hair before the age of 8 in girls and 9 in boys (21). Hirsutism was defined as a Ferriman-Gallwey score ≥ 8 (22) and oligomenorrhea as menstrual cycles lasting 35-90 days.

Biochemical analysis

We measured dehydroepiandrosterone sulphate (DHEAS), 17OHP, androstenedione, and total testosterone by electrochemiluminescence immunoassay on the Elecsys 2010 Modular Analytics E170 analyzer (Roche Diagnostics). In post-pubertal women, we conducted the analyses in the follicular phase, before 9 am. We performed the stimulation test with administration of 250 µg synthetic cosyntropin (Synacthen®) and measured 17OHP at 0 and at 60 min post-cosyntropin.

Molecular analysis

We selectively amplified functional *CYP21A2* gene sequencing by polymerase chain reaction (PCR) into two partially overlapping fragments, avoiding the co-amplification of the pseudogene *CYP21A1P* (23,24). Using the complete gene sequencing strategy, on the promoter region, approximately 130bp upstream, we sequenced the start codon by Sanger sequencing. We sequenced all coding sequences and intron-exon boundaries with internal primers that covered the entire *CYP21A2* gene. We performed both restriction fragment length polymorphism (RFLP) for large deletion/conversions involving the *CYP21A2* promoter region and MLPA for detection of large rearrangements (23,24). In all cases where it was mandatory, we performed parental segregation studies to determine whether the variants were in cis or in trans.

We determined nomenclature of *CYP21A2* variants according to the Ensembl transcript *CYP21A2-002*: ENST00000418967.6 (NM_000500.7 from *National Center for Biotechnology Information*).

Statistical analysis

We performed statistical analysis using SPSS® v.20. We applied nonparametric tests to compare independent samples and used chi-square tests to determine the association between categorical variables. We established comparisons between Group II (carriers) and Group I (NC-CAH patients) and between Group II (carriers) and Group III (wild type individuals). We regarded P values below 0.05 as statistically significant.

Ethics approval

We conducted all procedures in this study in accordance with the national committee for data protection and with the 1964 Helsinki declaration or comparable ethical

standards. The local Ethics Committee approved this study. We obtained informed consent from the participants.

RESULTS

Table 1 summarizes the 131 included females' ages at referral, clinical phenotypes at presentation, genotypes, and biochemical data.

The participants' median age at referral was 6.3 years in children and 27.9 years in adolescents and adults. We found no significant differences between groups.

Children presented mostly with premature pubarche (97%), and adolescents and adults presented with hirsutism (76%) and oligomenorrhea (11%). We found no significant differences in presentation between NC-CAH patients (Group I), heterozygous carriers (Group II), and wild type individuals (Group III).

We found statistically significant differences in basal and stimulated 17OHP levels in both the children and the adolescents/adult populations between carriers (Group II) and NC-CAH patients (Group I) and between carriers (Group II) and wild type subjects (Group III).

In children, heterozygous carriers showed higher basal and cosyntropin-stimulated 17OHP than wild type girls (basal 17OHP: median 3.3 vs. 2.0 ng/mL; $p < 0.05$ stimulated 17OHP: median 11.9 vs. 7.7 ng/mL; $p < 0.05$) and lower basal and stimulated 17OHP than NC-CAH patients (basal 17OHP: median 3.3 vs. 16.7 ng/mL; $p < 0.05$; stimulated 17OHP: median 11.9 vs. 55.1 ng/mL; $p < 0.05$, respectively).

In adolescents and adults, heterozygous carriers showed higher basal and cosyntropin-stimulated 17OHP than wild type women (basal 17OHP: median 3.7 vs. 1.7 ng/mL; $p < 0.05$; stimulated 17OHP: median 10.8 vs. 5.5 ng/mL; $p < 0.05$) and lower basal and stimulated 17OHP than NC-CAH patients (basal 17OHP: median 3.7 vs. 7.4 ng/mL; $p < 0.05$; stimulated 17OHP: median 10.8 vs. 16.5 ng/mL; $p < 0.05$).

Figure 1 shows basal and post-ACTH 17OHP levels according to groups, for the whole sample. Differences in basal and post-ACTH 17OHP levels between children and adolescent/adult women were non-significant (medians, respectively, 4.8 vs. 3.4 ng/mL ($p = 0.278$) and 12.5 vs. 13.0 ng/mL ($p = 0.409$)).

We found no statistically significant differences in DHEAS, androstenedione, or total testosterone levels between groups in either children or adolescent and adult women.

Table 1. Comparison between age at referral, phenotype at presentation, genotype, and biochemical data of the 131 included females, by age and genotype groups

	All	Group I (+/+)	Group II (+/-)	Group III (-/-)	p
All					
Number of individuals, n (%)	131 (100%)	46 (35%)	49 (37%)	36 (28%)	
Age at referral, years (median, min-max)	22.6 (3.3-49.6)	23.0 (5.1-49.6)	22.6 (3.3-48.3)	22.5 (3.7-46.0)	NS†
Childhood hyperandrogenism*					
Number of individuals (%)	32 (100%)	12 (38%)	10 (31%)	10 (31%)	
Age at referral, years (median, min-max)	6.3 (3.3-9.2)	6.9 (5.1-9.6)	5.5 (3.3-7.3)	6.6 (3.7-8.2)	NS†
Phenotype at presentation (%)					
Premature pubarche	97%	92%	100%	100%	NS*
Tall stature	3%	8%			
Genotype (number of individuals)					
p.Val282Leu/p.Val282Leu		7 (58%)			
p.Val282Leu/del/conv promotor CYP21A2		2 (17%)			
p.Val282Leu/p.Gly111ValfsTer21		1 (8%)			
p.Val282Leu/p.Pro454Ser		1 (8%)			
p.Val282Leu/p.R444X		1 (8%)			
p.Val282Leu/-			9 (90%)		
c.290-13A/C>G/-			1 (10%)		
Biochemical data					
Basal 17OHP, ng/mL (median, min-max)	4.8 (0.4-54.3)	16.7 (1.4-54.3)	3.3 (0.9-9.0)	2.0 (0.4-1.3)	<0.05†
Pos-ACTH 17OHP, ng/mL (median, min-max)	12.5 (4.4-79.0)	55.1 (17.3-79.0)	11.9 (6.2-13.9)	7.7 (4.4-11.1)	<0.05†
DHEAS, µg/dL (median, min-max)	86.3 (40.0-178.2)	125.4 (46.0-178.2)	77.5 (46.9-141.8)	74.2 (40.0-150.3)	NS†
Androstenedione, ng/mL (median, min-max)	1.0 (0.0-2.9)	1.5 (0.8-2.9)	0.5 (0.0-1.8)	0.4 (0.03-1.2)	NS†
Total testosterone, ng/mL (median, min-max)	0.1 (0.0-0.3)	0.1 (0.1-0.3)	0.1 (0.1-0.2)	0.0 (0.0-0.2)	NS†
Adolescence or adulthood hyperandrogenism**					
Number of individuals (%)	99 (100%)	34 (34%)	39 (39%)	26 (26%)	
Age at referral, years (median, min-max)	27.9 (13.2-49.6)	28.2 (13.2-49.6)	27.3 (14.2-48.3)	27.7 (13.5-46.0)	NS†
Phenotype at presentation (%)					
Hirsutism	76%	63%	91%	82%	NS*
Oligomenorrhea	11%	19%	0%	9%	
Severe acne	5%	6%	9%	0%	
Alopecia	5%	6%	0%	9%	
Infertility	3%	6%	0%	0%	
Genotype, n (%)					
p.Val282Leu/p.Val282Leu		17 (50%)			
p.Val282Leu/del/conv promotor CYP21A2		17 (50%)			
p.Val282Leu/-			39 (100%)		
Biochemical data					
Basal 17OHP, ng/mL (median, min-max)	3.4 (0.1-30.6)	7.4 (0.6-30.6)	3.7 (0.6-15.7)	1.7 (0.1-5.7)	<0.05†
Pos-ACTH 17OHP, ng/mL (median, min-max)	13.0 (2.7-21.9)	16.5 (13.0-21.9)	10.8 (4.2-13.0)	5.5 (2.7-9.5)	<0.05†
DHEAS, µg/dL (median, min-max)	198.2 (0.7-598.1)	209.3 (0.7-399.4)	138.2 (12.3-598.1)	204.8 (2.1-536.0)	NS†
Androstenedione, ng/mL (median, min-max)	3.2 (0.2-8.8)	3.8 (0.2-8.8)	2.7 (0.2-7.3)	2.2 (0.3-8.4)	NS†
Total testosterone, ng/mL (median, min-max)	0.4 (0.03-134.0)	0.5 (0.0-1.0)	0.6 (0.1-2.1)	0.3 (0.0-134.0)	NS†

Group I (+/+) included females with a diagnosis of NC-CAH (hyperandrogenism and genetic analysis showing 2 pathogenic variants in the CYP21A2). Group II (+/-) included heterozygous carriers of CYP21A2 pathogenic variant. Group III (-/-) included females with features overlapping NC-CAH but without any CYP21A2 pathogenic variant identified.

*The "childhood hyperandrogenism" group includes 32 girls aged 3-9 years-old at referral due to precocious pubarche or tall stature.

**The "adolescence or adulthood hyperandrogenism" included 99 adolescents and older women aged 13-69 years at referral due to infertility, oligomenorrhea, severe acne, alopecia, and/or hirsutism.

†Non-parametric tests (Group II compared to I and III). *Pearson Chi-square.

NS: non-significant (p > 0.05); 17OHP: 17-hydroxyprogesterone; DHEAS: dehydroepiandrosterone sulphate.

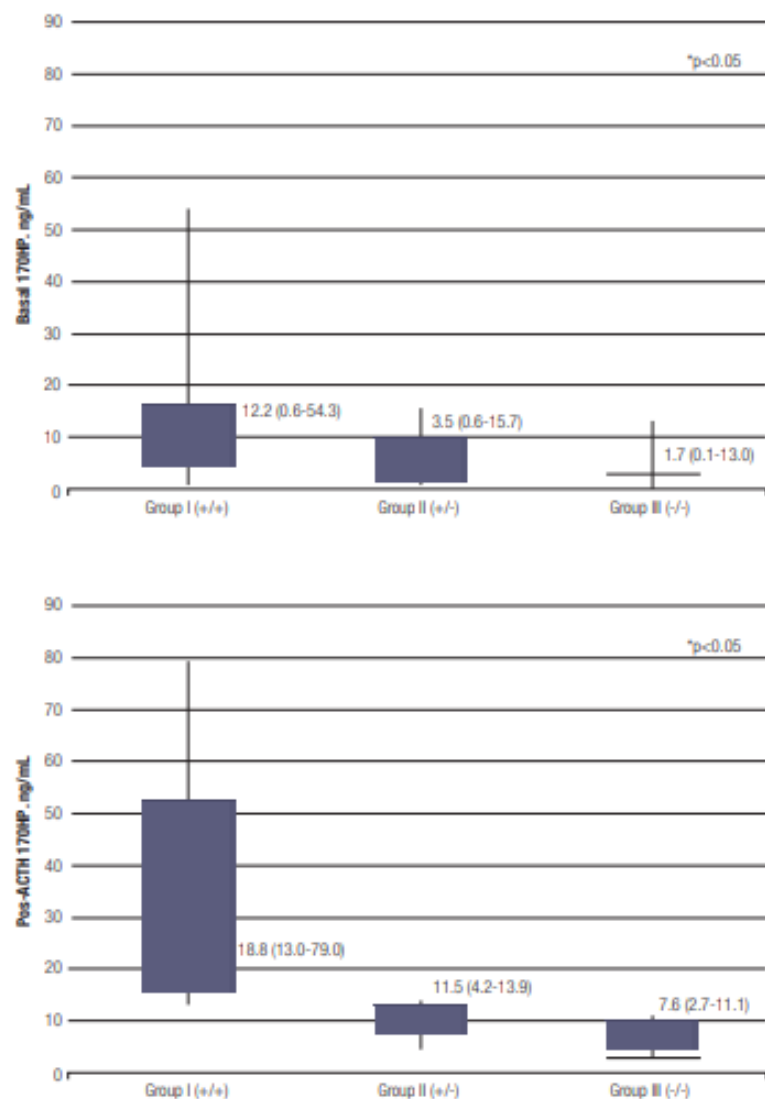


Figure 1. Basal (1A) and pos-ACTH (1B) 17OHP levels (ng/mL) according to groups (median (min-max)). Group I (+/+) included females with NC-CAH (hyperandrogenism and genetic analysis showing 2 pathogenic variants in the *CYP21A2*). Group II (+/-) included heterozygous carriers. Group III (-/-) included wild type females. *Nonparametric tests were applied to compare Group II (carriers) and Group I (NC-CAH patients), and Group II (carriers) and Group III (wild type individuals) ($p < 0.001$ for all the comparisons).

Regarding genotypes, the majority of the NC-CAH patients (Group I) were homozygous for the p.Val282Leu pathogenic variant (58% of the children and 50% of the adults) or compound heterozygous for the p.Val282Leu/del/conv promotor *CYP21A2* pathogenic variants (17% of the children and 50% of the adults). As for the heterozygous carriers (Group

II), the majority were simple heterozygous for the p.Val282Leu variant (90% of the children and 100% of the adults).

Clinical features did not significantly differ between patients homozygous for the p.Val282Leu variant and patients with the p.Val282Leu/del/conv genotype. Median basal and stimulated 17OHP were higher in

homozygous than in compound heterozygous patients (respectively, 12.3 vs. 6.9 ng/mL ($p = 0.367$), and 17.8 vs. 19.0 ng/mL ($p = 0.141$)).

DISCUSSION

We have analyzed a population of 131 females suspected of having NC-CAH due to clinical and biochemical criteria, including 46 NC-CAH individuals, 49 heterozygous carriers, and 36 wild type subjects. We have explored our data in two groups (32 children and 99 adolescents and women), as we realize that young girls with premature pubarche are clinically different from adolescents and women with hirsutism, oligomenorrhea, or infertility. However, we found similar results regardless of the age group, including similar signs and symptoms and various 17OHP levels between groups.

Heterozygous carriers presented higher basal and ACTH-stimulated 17OHP levels than wild type subjects and lower basal and stimulated 17OHP levels than NC-CAH patients. In accordance with our data, some previous studies showed higher basal and/or stimulated 17OHP levels in heterozygous *CYP21A2* pathogenic variant carriers than in non-carriers (5,9,10,16,17,25–28). Other studies, though, showed no significant differences in hormone levels between heterozygous carriers and the general population (12,14,18,19). Conflicting results may have arisen due to differences in the participants' selection criteria.

In our population, 17OHP concentrations exhibit significant overlap between carriers and the remaining two groups. In heterozygous carriers and wild type individuals, we found post-ACTH 17OHP levels > 3 ng/mL, a cutoff proposed for the identification of carrier status (28,29), and even some > 10 ng/mL, the cutoff usually applied for the NC-CAH diagnosis (30). Therefore, we state that these groups cannot be distinctly separated according to biochemical criteria and underline that the detection of the carrier status should be based on molecular genotype analysis.

Admoni and cols. distinguished two groups of carriers, a group of symptomatic carriers presenting with premature pubarche, accelerated growth, and hirsutism and a group of asymptomatic family member carriers, and they stated that the symptomatic carriers had higher cosyntropin-stimulated 17OHP levels and a higher rate of the p.Val282Leu pathogenic variant than family member carriers (27). They suggested

that the subjects who carried the mild p.Val282Leu pathogenic variant had higher rates of androgen excess symptoms than the carriers of other severe pathogenic variants and speculated that the impairment of enzymatic activity in the symptomatic carriers results from this mutant allele's dominant-negative effect on the wild type in which the mutant enzyme may compete or interfere with the wild type for the conversion of 17OHP to 11-deoxycortisol (27,31). The monoallelic pathogenic variant affects the normal allele's function, leading to a slight reduction in the 21OH activity, increased androgens secretion, and subsequent clinical manifestations (31). Other studies have supported this observation and showed higher rates of either PCOS or hirsutism in p.Val282Leu carriers but not in other mutation carriers (9,27,32). In our population, the large majority of our carriers were simple heterozygous for the p.Val282Leu variant (90% of the children and 100% of the adults), which is the most frequent variant in the Portuguese population (3). This fact may explain our carriers' higher 17OHP levels compared to wild type subjects, and we may question whether we would see different results if our heterozygous subjects carried other pathogenic variants.

Possible explanations for the phenotypic heterogeneity among individuals carrying the same pathogenic variant include the activity of other genes encoding proteins with extra-adrenal 21OH activity, the presence of other still unidentified mutations, the activity of other genes encoding proteins with extra-adrenal 21OH activity, and interindividual variation in androgens' peripheral sensitivity and in the amount of protein produced (33).

In our population, we found no significant differences in signs and symptoms between NC-CAH patients, heterozygous carriers, and wild type individuals. We only included females in the genetic study for the *CYP21A2* gene and selected them based on their hormonal patterns and clinical features; therefore, the similarities between the three groups did not surprise us. Moreover, we obtained our data from clinical records, which we must interpret carefully. Our hyperandrogenic wild type girls probably presented premature adrenarche, a diagnosis of exclusion, while most of our post-menarche woman presented polycystic ovarian syndrome (PCOS). Premature adrenarche and PCOS are difficult to differentiate from NC-CAH and heterozygous carrier status based on clinical findings.

We recommend treatment of symptomatic heterozygote carriers for the symptoms of androgen excess with both pharmacological therapies, including glucocorticoids and cosmetic approaches (29).

It would have been interesting to compare 17OHP levels in individuals showing one hyperandrogenic sign or symptom *versus* individuals showing three or more hyperandrogenic signs or symptoms, both in the wild type and in the carriers group. However, due to this study's retrospective nature, we could not conduct this analysis.

Limitations include the small sample size, our study's retrospective design, and the fact that heterozygous carriers with monoallelic pathogenic variants were not studied for other point sequence variants within regulatory or epigenetic control regions in the CYP21 locus, mainly the analysis of the 3'untranslated region of the *CYP21A2* gene (3'UTR region), where it was recently reported that 3'UTR sequence variants in combination with other pathogenic variants may be associated with a mild form of the disease (34). More accurate biochemical predictors of the heterozygous carrier status, such as the serum 21-deoxycortisol (35,36) or the 17OHP/cortisol ratio (37), were not available in our population. We did not analyze data regarding 17OHP levels in women without hyperandrogenic manifestations.

In conclusion, we found no significant differences in clinical features between NC-CAH patients, heterozygous carriers, and wild type females. The most common pathogenic variant in the *CYP21A2* gene identified among NC-CAH patients and carriers was p.Val282Leu. Heterozygous carriers presented higher basal and post-cosyntropin 17OHP levels than wild type individuals and lower basal and stimulated 17OHP levels than NC-CAH patients. We discovered a considerable overlap between 17OHP levels among groups; therefore, normal basal and stimulated 17OHP responses do not exclude heterozygosity for *CYP21A2* pathogenic variants.

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IV. GENERAL DISCUSSION AND CONCLUSIONS

CAH is a common genetic disorder with a high clinical heterogeneity. Due to the different enzymes involved in the adrenal androgens' pathway, the clinical forms of the disease can be distinct, what may difficult the achievement of a diagnosis, especially for late onset and less severe forms. The molecular analysis is of major importance in diagnosis confirmation and provides a powerful and reliable tool for genetic counseling. Over the years, the development of comprehensive methodologic approaches has improved our ability to genotype patients. However, several pitfalls and challenges persist in the molecular diagnosis of CAH. The molecular genetic analysis allows the detection of several pathogenic variants, gene recombination and gene deletions or duplications, making the molecular diagnosis of CAH particularly challenging.

In the first study here described, we aimed to determine the *CYP21A2* pathogenic variant profile in a cohort of Portuguese Congenital Adrenal Hyperplasia patients, by assaying the 10 most frequent pathogenic variants in *CYP21A2* gene. Eighty-four CAH patients due to 21-OHD were enrolled in the study, as well as 54 relatives [parents (n = 44), siblings (n = 8) and offspring (n = 2)] of 29 patients. Clinical diagnosis criteria were solely based on phenotype and 17-OHP levels, before and after ACTH stimulation (White and Speiser, 2000). Of the 84 patients recruited, 10 were SW, 6 SV and 68 NC-CAH and the majority were females. Genetic analysis screening for the ten most common disease-causing pathogenic variants was performed using different molecular techniques: Polymerase Chain Reaction – Sequence Specific Primer (PCR-SSP) - 8 bp deletion in exon 3 (G110 Δ 8nt), p.Ile173Asn (I172N) in exon 4, cluster of three point pathogenic variants (L236N, V237E, M239K) on exon 6 (E6 cluster), Restriction Fragment Length Polymorphism (RFLP) – for large deletions or conversions events involving the promoter region of the *CYP21A2* gene (del / conv of promoter region), or direct DNA sequencing – P30L (p.Pro31Leu) on exon 1, intron 2 splicing pathogenic variant IVS2 A/C>G (c.293-13A/C > G), V281L (p.Val282Leu) and a T-insertion (p.Leu308fs) in exon 7, and p.Gln319* (Q318X) and p.Arg357Trp (R356W) on exon 8.

Along several years of molecular diagnosis of 21-OHD, the screening for the 10 most common pathogenic variants was the gold standard approach for all the laboratories performing CAH molecular diagnosis. Pathogenic variants were detected in 91.7% of the patients (77/84), and from these, 57.1% (48/84) presented 2 pathogenic variants, 15.5% (13/84) showed 3 pathogenic variants and 2.4% (2/84) presented 4 or more pathogenic

variants. The number of patients presenting just one variant correspond to 16.7% (14/84) and 8.3% (7/84) did not showed any pathogenic variant. In this cohort, the most frequent pathogenic variant was the NC variant, p.Val282Leu, in 41.7% of the patients, followed by del /conv of promoter region (28.3%). Frequency of several sequence variants were evaluated and for missense sequence variation p.Ala266Val (A265V) a properly screening study on the general population was performed.

In this study, discrepancies were observed on patients' number with different forms of the disease. This fact results from the majority of patients referred to our Center were adult females diagnosed at Department of Endocrinology of Centro Hospitalar Universitário de São João, presenting signs and symptoms of androgen excess. We described a high frequency of p.Val282Leu what is in accordance with the previous data reported with a mean allelic frequency of 40-50 % reported for p.Val282Leu pathogenic variant in NC-CAH patients (Krone et al., 2000; Speiser et al., 2000; Deneux et al., 2001; Friães et al., 2006; Wilson et al., 2007). Furthermore, this result is also in agreement with a previous report showing a higher prevalence of this mild pathogenic variant in NC form of the disease in Southern Europe and North Africa (Kotaska et al., 2003). Unexpectedly, in our study a low frequency (5.6%) of c.293-13A/C>G was observed. Moreover, other studies (Wedell et al., 1994; Krone et al., 2000; Stikkelbroeck et al., 2003; Wilson et al., 2007) showed a higher frequency of this splice-site pathogenic variant, being one of the most common pathogenic variants in USA, Mexico, Germany and Japan (Ezquieta et al., 2002). Previous studies, including one describing a different Portuguese cohort, showed a low frequency for c.293-13A/C>G, but higher than here reported (Friães et al., 2006). Our results showed severe pathogenic variants being observed in about 67% of non-classic patients, similar to data from other studies in Spain and Italy in which the p.Ile173Asn was frequently found in NC patients (Ezquieta et al., 2002; Carrera et al., 1996). The two point pathogenic variants (p.Ile173Asn and p.Gln319*), seem to vary their frequency along with the North-South European axis and much lower than in North Europe (Wedell et al., 1994; Krone et al., 2000), which can explain the findings in our study. In our data, we verified that the frameshift severe pathogenic variant (p.Leu308fs) on exon 8 is more common in North of Portugal than in other North European population, but similar with other south European countries, Italy and Spain (Carrera et al., 1996; Ezquieta et al., 2002; Friães et al., 2006). In our cohort, a concordance of 75%

(63/84) between genotype and phenotype was obtained and a full concordance in patients carrying severe pathogenic variants in both alleles.

Additionally, the screening of a sequence variant, p.Ala266Val, in exon 7, in 105 controls revealed an allelic frequency of 1%. Results obtained from analysis of protein conservation, functional *in vitro* activity studies and computerized *CYP21A2* structural model studies pointed to the same results as ours. This sequence variant is a polymorphic variant rather a pathogenic variant (Bleicken et al., 2008). The 25% of patients in which just one or no pathogenic variant were identified are real potential candidates to carry other pathogenic variants in other region not studied with this methodology, what make a way to the here presented paper II.

After our first publication, and in transition to a distinct and comprehensive diagnosis strategy, a small case-study aimed to compare 5 patients, initially studied for screening of the 10-most common pathogenic variants, in which one or no pathogenic variants were identified. On this well clinical characterized patients, 3 presented NC-CAH and 2 classical CAH forms. In total, after the complete *CYP21A2* gene analysis, 5 more pathogenic variants were found. Two novel sequence variants were identified, p.(Pro432Leu) and p.(Ala434Glu) (Carvalho et al., 2010), and they were structurally analysed with ICM-Pro software for biochemical properties such as protein stability, accessibility to surface and hydrophobicity, in order to elucidate their effects on the *CYP21A2* protein. Bioinformatic data was very explicit for the 2 affected residues, Pro432 and Ala434, and analysis for conservation purposes to predict the severity of both pathogenic variants with PolyPhen-2 software, considered them as "probably damaging". The prediction of clinical severity for those novel variants, based on molecular modelling and sequence conservation parameters, was in accordance with the patient's clinical diagnosis.

On the paper III, the aim was a national wide study on the pediatric CAH population aiming to establish a genotype-phenotype correlation rate. To the best of our knowledge, this is one of the largest NC 21-OHD pediatric populations studies, where 212 unrelated pediatric CAH patients were recruited from 13 Portuguese pediatric endocrinology centers and classified accordingly clinical CAH form: SW, SV, or NC. Enrolled patients were genetic characterized and presented pathogenic variants in homozygosity or compound heterozygosity. Genetic analysis was performed using

distinct molecular methods, polymerase chain reaction (sequence specific primer, restriction fragment length polymorphism), direct Sanger sequencing and/or MLPA, based on each reference laboratory methodology. On the analysis genotypes were categorized into 4 groups (O, A, B, and C), according to their predicted enzymatic activity, to achieve the genotype–phenotype correlation. The cohort comprised 61 (29%) presented a SW form, 24 (11%) presenting a SV form and 127 (60%) evincing a NC form. From the enrolled patients, genetic analysis showed homozygosity in 46.7% of the patients and 53.3% revealed compound heterozygous patients, the majority of the NC CAH patients enrolled were females (80.3%) and only a small part of the patients, 16.5% (21/127), were diagnosed after the age of 10 years. Of the 424 unrelated alleles, 38 (8.2%) carried 2 or more pathogenic variants in *cis*, which were appropriately assigned to the paternal or maternal chromosomes after segregation paternal analysis. Due to molecular analysis, 20 different point pathogenic variants were identified, and large rearrangements (deletions and gene conversions) were found in 6.7% of the cases. The most common pathogenic variant detected was p.Val282Leu (41.3% of the 424 alleles analyzed). This pathogenic variant, together with c.293-13A/C>G, p.Ile173Asn, p.Leu-308Thr, p.Gln319*, and large deletions/conversions were responsible for 86.4% of the mutated alleles. Patients' stratification by disease subtype revealed that the most frequent pathogenic variants were c.293-13A/C>G in SW group (31.1%), p.Ile173Asn in SV group (46.9%), and p.Val282Leu in NC patients (69.5%). Reinforcing the high prevalence of p.Val282Leu in our population, the most common genotype was homozygosity for this particular pathogenic variant (33.0%, 70/212), all of whom presenting NC-CAH, except for a boy with an adrenal crisis in the second week of life, where a non-correlation between genotype and phenotype is evident. In other populations, the p.Val282Leu accounts for approximately 35–55% of NC-CAH (Krone et al., 2000). In accordance with reported in this study, a high frequency of this pathogenic variant was also found in Galicia (63.2%), that is in the northwest of the Iberian Peninsula and share with Portugal a very similar ethnic background (Loidi et al., 2006). The most prevalent genotype in our population was the homozygosity for p.Val282Leu, detected in 33% of the individuals, which has been previously reported in high frequency in the Ashkenazi Jewish population (New et al., 2013). Probably, this means that, genetically, the Ashkenazi jews are similar to the Sephardi jews (Iberian jews).

Moreover, 2 novel variants p.(Ile161Thr) and p.(Trp202Arg), were found on exons 4 and 5, respectively. Bioinformatic analysis help to predict the impact on the structure and function of the CYP21A2 protein. PolyPhen and Mutation Taster bioinformatics software's, predicted "damaging" and "disease causing," respectively, with a high pathogenicity score for the altered amino acid for both novel variants, although multiple alignments analysis showed that amino acids, Isoleucine, at codon 161, and Tryptophan, at codon 202, were fully conserved through species. The p.(Trp202Arg) variant was found in a female with atypical genitalia that presented a SW form and presented this novel pathogenic variant in one allele and a large conversion in the other allele. Based on clinical data and bioinformatic results, this novel variant may be associated with a very low enzymatic activity. The p.(Ile161Thr) variant was identified in a female with a normal genitalia in infancy that presented a premature pubarche at 5 years old and was classified as a NC CAH form. This particular patient presented this novel pathogenic variant in one allele and the missense pathogenic variant in exon 4, p.Ile173Asn, in the other allele. Based on that, we considered that this variant may be associated with a mild reduction of the 21-OH activity.

Overall, the genotype-phenotype correlation achieved was 92.4%, but on group B (associated with the SV form) the genotype-phenotype correlation was lower (80%) than in the other groups. This study constituted an important milestone on the CAH data in Portuguese patients, as this cohort was one of the largest on NC-CAH pediatric population in Portugal. This study also emphasized the high frequency of p.Val282Leu pathogenic variant and a high genotype-phenotype correlation in our population.

After some publications on the same thematic, evolving different methodologies, we decided to make the point of situation on paper IV. In this paper the concern was highlight the importance of having a correct diagnosis for CAH due 21-OHD to improve a complete genetic characterization, as aspects of CAH patients, mainly on severe forms, as life-threatening crises, genital ambiguity with all its consequences of gender determination and reconstructive surgeries ultimately affecting normal sexuality and reproduction. For most patients, whom besides the infertility, cannot even attempt to conceive, genetic counselling is of major importance. Different aspects such as, prevalence according to ethnicity, molecular genetic testing approaches and CYP21A2 pathogenic variants and genotype-phenotype correlation data were extensively

discussed. Aspects that have been constitute a barrier and present some limitations on molecular diagnosis were recently discussed in order to obtain the widest possible consultation with practicing laboratories for CAH genotyping (Baumgartner-Parzer et al., 2020).

In continuation of previous studies published by our group, on paper V our aim was bringing off a mini review updating general aspects on the genetic analysis of *CYP21A2* pathogenic variants affecting CAH patients. Due to the diversity of pathogenic variants distribution worldwide and the high frequency of some of them on specific population, epidemiology and aetiology of 21-OHD were aspects addressed on this study. We reviewed the current situation and noticed the lack of official guidelines for molecular diagnosis, also genotyping interpretation and reporting. Almost simultaneously, Baumgartner-Parzer et al., 2020, on behalf of European Molecular Genetics Quality Network (EMQN), published the guidelines for best practice molecular genetic testing and reporting of 21-hydroxylase deficiency. Those recommendations were obtained after a European-wide external quality assessment scheme for CAH (due to 21-OH deficiency) promoted by EMQN, in 2009. This meeting joint about 60 laboratories from Europe, USA and Australia regularly participated in that scheme, in which several Researchers took part, including myself (Appendix I). Before the publication of Baumgartner-Parzer et al., 2020, we felt the lack of newly and updated information regarding to how to deal with a genetic analysis in CAH patients and how to report results accordingly. Therefore, we updated the molecular diagnosis strategy for *CYP21A2* analysis, highlighting the importance of a complete study rather to the outmoded screening for the 10-most common pathogenic variants. Sometimes, the genotype-phenotype correlation observed in literature, particularly in NC-CAH forms, is not complete and understandable, which reinforces the need to extend the genetic study for a full comprehensive analysis and focused on less denominative regions that could regulate gene expression.

The lack in correlation between genotype and phenotype remained a concerning on CAH studies due to 21-OHD. Beyond this, the percentage of suspected NC-CAH patients without a molecular diagnosis constituted a challenge and to address this question, on paper VI, we evaluated a group of 131 females suspecting of having NC-CAH due to clinical and biochemical findings, with a molecular analysis of *CYP21A2* gene. A cohort

of hyperandrogenic children (32 aged between 3-9 years old) and women (99 aged between 13-49 years old) was retrospectively studied in an attempt to determine differences on clinical and biochemical reports, between carrier heterozygous, NC-CAH and patients without pathogenic variants identified. Groups were constituted based on genetic molecular results and 49 patients presented as heterozygous carriers (one single pathogenic variant identified), 46 patients with two pathogenic variants in different alleles (homozygous or compound heterozygous) and 36 wild-type patients without any pathogenic variant identified in *CYP21A2* gene. Genetic analysis showed that the most common pathogenic variant in the *CYP21A2* gene among the recruited NC-CAH records was p.Val282Leu. Almost all of carriers were simple heterozygous for the p.Val282Leu variant (90% in the children and 100% in the adults groups). Clinically, the majority of young girls presented premature pubarche and adolescents and women manifested hirsutism, oligomenorrhea and/or infertility. No differences were detected regardless the age, signs and symptoms between groups, probably because inclusion criteria in the genetic study for the *CYP21A2* gene were based on their similar hormonal patterns and clinical features.

One of the most relevant aspects on this study was that heterozygous carriers presented higher basal and ACTH-stimulated 17-OHP levels than wild type individuals and lower basal and stimulated 17OHP levels compared with NC-CAH patients. This aspect was previously discussed by other groups that evidenced higher basal and/or stimulated 17-OHP levels in heterozygous *CYP21A2* carriers than in non-carrier's individuals (Guarnotta et al., 2020). Other groups also emphasized the existence of two types of carriers (symptomatic carriers, presenting premature pubarche, accelerated growth, and hirsutism and asymptomatic carriers, constituted by family members) wherein the symptomatic carriers revealed higher cosyntropin-stimulated 17-OHP levels and higher prevalence of the p.Val282Leu pathogenic variant than family member carriers (Admoni et al., 2006). Although this interesting disclosure, we found a considerable overlap between 17-OHP levels among groups which excludes the capacity to distinguish groups based only on biochemical criteria. This finding underlines the fact that the carrier status should be determined by molecular genotype analysis. Based on the results obtained on paper VI we considered that symptomatic heterozygous carriers should be carefully clinical followed, as 17-OHP may constitute a predictive marker for heterozygous

carriers. Nevertheless, other predictor markers (21-deoxycortisol and ratio 17-OHP/cortisol) should constitute a clue for hyperandrogenic carriers' identification (Miller, 2019; Guarnotta et al., 2020).

As general conclusions, this thesis highlight the importance of a global perspective of CAH in Portugal, through several papers that had been published. As mentioned previously, CAH is a common disorder with genetic and clinical heterogeneity and a particularly life-threatening in its most severe form. However, the molecular analysis of CAH is useful as a confirmation of clinical diagnosis and also an important tool in genetic counseling. Overtime, the development of broad methodologic approaches has improved the ability to achieve a correct patients' genotype. Nevertheless, several pitfalls and challenges remain in the molecular diagnosis of CAH along its distinct genotypes. Although the various forms of CAH represent monogenic disorders, the identification of *de novo* pathogenic variants and the presence of duplicated genes, makes the molecular diagnosis particularly challenging. Future research is needed to assist in the molecular pathogenesis of CAH that will contribute for a more effective management of affected patients and their family members across all age groups. Advances in genetics are remodeling medicine and personalized genetic information, that including CAH genotyping, will be a clue for individualized therapy.

V. REFERENCES

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VI. APPENDIX

**Book Chapter: Congenital Adrenal Hyperplasia - The Main Effect of
21-Hydroxylase Deficiency**

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***In: Adrenal Glands - The Current Stage and New Perspectives of Diseases and
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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: 11 β -hydroxylase (11 β -OH), 17 α -hydroxylase (17 α -OH), 3 β -hydroxysteroid dehydrogenase (3 β -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 adrenocorticotrophic 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 program-mess, 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 promoter 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 Croats, 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].

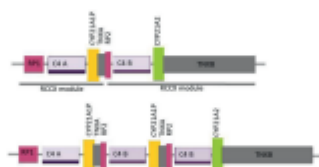


Figure 1. RCCX modules: Bimodular haplotype (upper) and a three modular haplotype with two modules harbouring the *CYP21A1P* pseudogene and one the *CYP21A2* gene.

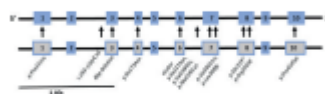


Figure 2.

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 suppresses 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-Classical 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 fetuses [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-keto-steroids, 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, namely ENST00000418967.6 [NM_000500 from National Center for Biotechnology Information].

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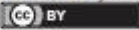
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