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MUTATION UPDATE

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CYP21A2 mutation update: Comprehensive analysis of databases and published genetic variants

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Abstract

Congenital adrenal hyperplasia (CAH) is a group of autosomal recessive disorders of adrenal steroidogenesis. Disorders in steroid 21-hydroxylation account for over 95% of patients with CAH. Clinically, the 21-hydroxylase deficiency has been classified in a broad spectrum of clinical forms, ranging from severe or classical, to mild late onset or non-classical.

Known allelic variants in the disease causing *CYP21A2* gene are spread among different sources. Until recently, most variants reported have been identified in the clinical setting, which presumably bias described variants to pathogenic ones, as those found in the CYPAlleles database. Nevertheless, a large number of variants are being described in massive genome projects, many of which are found in dbSNP, but lack functional implications and/or their phenotypic effect. In this work, we gathered a total of 1,340 GVs in the *CYP21A2* gene, from which 899 variants were unique and 230 have an effect on human health, and compiled all this information in an integrated database. We also connected *CYP21A2* sequence information to phenotypic effects for all available mutations, including double mutants in cis. Data compiled in the present work could help physicians in the genetic counseling of families affected with 21-hydroxylase deficiency.

KEYWORDS

21-hydroxylase deficiency, congenital adrenal hyperplasia, *CYP21A2*, genetic variants, genotype–phenotype correlation

1 | BACKGROUND

Congenital adrenal hyperplasia (CAH; MIM# 201910, E.C.1.14.14.16) is a group of autosomal recessive disorders of adrenal steroidogenesis. Disorders of steroid 21-hydroxylation account for over 95% of patients with CAH. Clinically, the 21-hydroxylase deficiency has been classified in a broad spectrum of clinical forms, ranging from severe or classical, to mild late onset or non-classical (NC). Classical CAH includes the salt-wasting (SW) and simple virilizing (SV) forms, both of early onset. Females with classical CAH are typically born with ambiguous genitalia, while patients with NC CAH exhibit clinical manifestations of hyperandrogenism (Miller, 1994; New, White, Pang, Dupont, & Speiser, 1989; White & Speiser, 2000).

The 21-hydroxylase enzyme consists of 495 amino acids with a molecular weight of 52 kDa (Higashi, Yoshioka, Yamane, Gotoh, & Fujii-Kuriyama, 1986; Nebert et al., 1991). 21-Hydroxylase displays endoplasmatic reticulum localization, reducing molecular oxygen and hydrolyzing two natural substrates: 17-hydroxyprogesterone and progesterone (White & Speiser, 2000).

The gene encoding 21-hydroxylase enzyme, *CYP21A2* (MIM# 613815; GenBank ID 1589), spans 3.35 kb of the short arm of chromosome 6 (6p21.3) and consists of 10 exons and a 1,488 bp open

reading frame (Higashi et al., 1986; White et al., 1986). It is located within the human leukocyte antigen complex, in the so-called RCCX module. Approximately two thirds of the chromosomes analyzed have a duplicated RCCX module that includes a genomic DNA segment composed of the pseudogenes *STK19* (*RP2*), *CYP21A1P*, *TNXA*, and a second active copy of the *C4* (long or short) gene (Blanchong et al., 2000; Koppens et al., 1992). The active gene *CYP21A2* and its pseudogene *CYP21A1P* present 98% sequence identity; they differ in approximately 65 nucleotides. Due to the high degree of sequence identity, most of the disease-causing mutations described in 21-hydroxylase deficiency are likely to be the consequence of non-homologous recombination or gene conversion events (Donohoue et al., 1986; Higashi, Tanae, Inoue, & Fujii-Kuriyama, 1988). Nevertheless, an increasing number of novel or rare mutations have been found in disease-causing alleles during the last three decades (https://www.cypalleles.ki.se/cyp21.htm). Mutations in the *CYP21A2* gene cause varying degrees of 21-hydroxylase activity loss. In vitro studies revealed that mutations leading to a complete inactivation of 21-hydroxylase are usually associated with the SW phenotype. Mutations that reduce enzyme activity close to 2% cause the SV phenotype, whereas those with a residual enzymatic activity in the range of 20% to 60% result in the mild NC CAH phenotype. In addition, a great number of patients are compound heterozygotes carrying different *CYP21A2* mutations on each allele, and their phenotypes depend on the milder gene defect (Speiser & White, 2003).

The most comprehensive publicly available database (DB) regarding the clinical effects of genetics variants (GVs) in the *CYP21A2* gene is CYPAlleles, which contains 169 GVs, but has not been updated since March 2011. On the other hand, a large number of variants are being described in massive genome projects, many of which are found in dbSNP, but lack functional implications and/or their phenotypic effect.

With the aim of providing health professionals useful information for 21-hydroxylase deficiency, we have compiled and curated data from different sources to build up a DB of the GVs of the *CYP21A2* gene and their biological effects.

2 | *CYP21A2* GENETIC VARIANTS AND DATABASES

We compiled the information regarding GVs for the *CYP21A2* gene from six publicly available DBs: CYPAlleles (https://www.cypalleles.ki.se/cyp21.htm), NCBI's dbSNP (https://www.ncbi.nlm.nih.gov/SNP/) (Sherry et al., 2001), NHLBI-ESP's EVS (https://evs.gs.washington.edu/EVS/), ExPASy's SwissVar (https://swissvar.expasy.org/cgi-bin/swissvar/home) (Mottaz, David, Veuthey, & Yip, 2010), GWAS Central (www.gwascentral.org) (Beck, Hastings, Gollapudi, Free, & Brookes, 2014), and ExAC (https://exac.broadinstitute.org/) (Lek et al., 2016), which is mostly included in dbSNP. The ease with which data were retrieved from the different DBs, depended on the options offered by each one of them (Figure 1A): dbSNP was easily accessed and consulted through Biopython's "Entrez" package (Cook et al., 2009), while EVS, SwissVar, GWAS Central, and ExAC offered a link to download the information as a CSV file. CYPAlleles required manual copying of the GVs from the HTML table, as it does not offer any other way to download the information.

In order to integrate the information from the different sources into a single DB, we first had to establish a unique identifier. Among the DBs we explored, "rs#" and HGVS are the most frequently used identifiers. The "rs#" accounts for positions in a gene, so a single one can contain multiple GVs, not allowing a one-to-one unambiguous identification. In addition, not every GV has an "rs#" value. An alternative way of identifying GVs is by referring to the base or amino-acid substitution and a position in a determined scaffold (chromosome, contig, mRNA, CDS, etc.). HGVS offers a system to name variants at DNA, RNA, and protein levels in a very precise way (Dunnen et al., 2016), but the exact change and position refers to a certain scaffold, and not every DB uses the same one. In addition, in some occasions, the same GV has a different nomenclature, as not all DBs or the bibliography follow the same naming recommendations.

We developed an algorithm (Supp. Methods) to relativize the GVs positions to that of the scaffold GRCh38.p7 (chromosome 6), that is used by the dbSNP DB, limiting the analysis to the region comprising 2,000 bp upstream the *CYP21A2* translation start-site, and only 480 bp downstream its 3′ UTR, due to its overlap with the 3′ UTR of the *TNXB* gene. The algorithm was implemented in the Python 2.7 programming language (https://www.python.org), and utilizes an alignment of the DNA sequences used as reference by the different DBs (Figure 1A). SwissVar DB, that has only amino acid sequences, was fed last to the algorithm so it could use the previously stored information to assign the genetic substitutions to the amino acid variants, when possible. The final "Integrated DB" has the compiled information under four different IDs (the genomic and cDNA position + substitution and, if available, the amino acid position+substitution and the "rs#") (Supp. Table S1). In addition, when available, this DB also contains information regarding the effect of each GV on human health ("allele associated phenotype" in Supp. Table S1), classified considering the combined information of the reported phenotype in patients, the mutation/s present in the homologous allele and/or the in vitro activity.

A total of 1,248 GVs were compiled from the different DBs, comprising 827 unique GVs (Figure 1B and Supp. Table S2). In addition, 71 GVs not present in the six DBs analyzed were found among the 133 publications reviewed for the present work. Furthermore, we included a novel GV (GenBank accession number: MF401543); found in an individual from our cohort (see Supp. Methods for details). All the GVs with a known effect on human health were uploaded to ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/).

It is interesting to note that dbSNP and CYPAlleles altogether account for 99.3% of all GVs from the six analyzed DBs, with only ExAC and SwissVar contributing 1 and 5 unique GVs, respectively (Figure 1B). Although dbSNP is the DB that contributed the most GVs ($N = 727$), these only account for 41.4% of the 169 GVs gathered from CYPAlleles (Supp. Table S2).

The number of genetic variants reported for *CYP21A2* had grown exponentially during the past years thanks to new sequencing technologies and human sequencing projects (Figure 1C). In fact, since CYPAlleles DB was last updated in 2011, ~600 new GVs

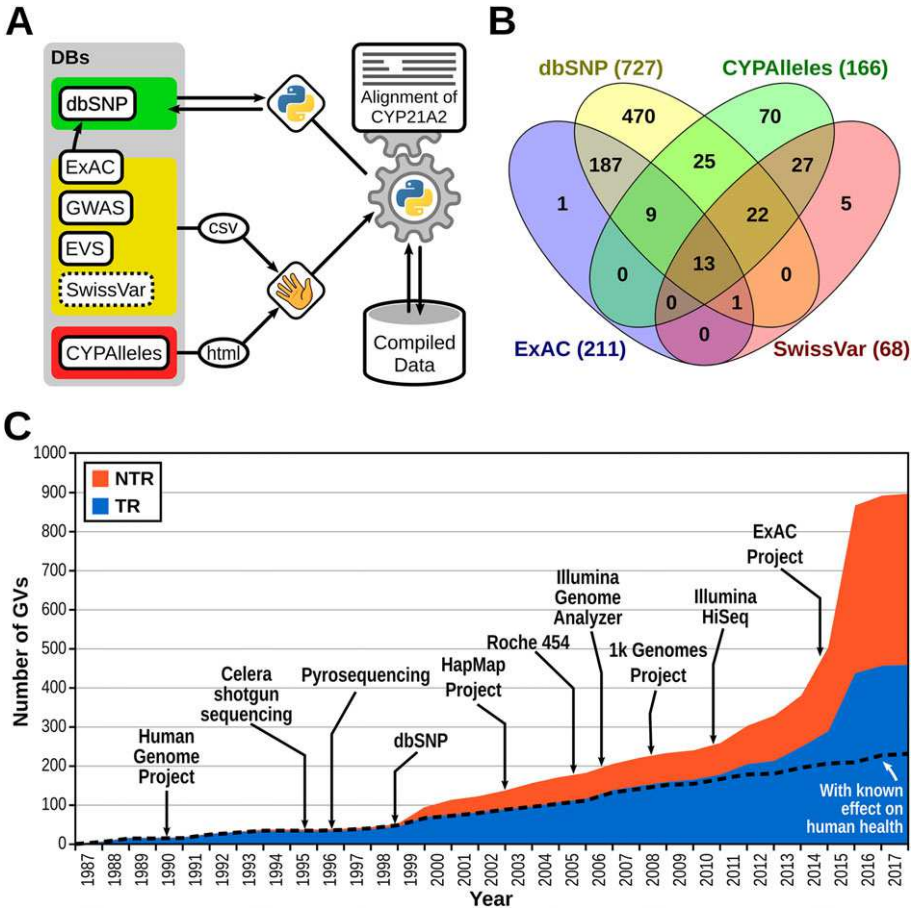

FIGURE 1 Compilation of genetic variants. A: The pipeline used to compile the different GVs from the DBs consulted in this work is shown as a flowchart. On the left, the DBs have been classified according to the ease to download its GVs, as easy (green) when they offer a way to interact with them through scripting, medium (yellow) when there is a way to manually download the data, and hard (red) when the data have to be retrieved from the HTML. To integrate the information from the different DBs, each GV was relativized to the same scaffold using scripting and the alignment of the different sequences used by each DB (cogs on the right). SwissVar, a protein DB, is shown in dotted line. The "hand" icon stands for "by hand," while the two interlaced, blue and yellow snakes icon, is the Python programming language logo (emoji by https://www.emojidex.com). B: The contribution of the four DBs that contain all the unique GVs found through the pipeline described in (**A**) is shown as a Venn diagram. The diagram was generated with Venny 2.1 (Oliveros 2007–2015). C: The cumulative number of GVs from translated (blue) and non-translated (orange) regions, reported over the years since *CYP21A2* was first described, is shown in, along with important milestones regarding sequencing technologies and human sequencing projects. The number of GVs with a reported effect on human health is shown superimposed as a dashed line

have been reported, including ~60 GVs with an effect on human health.

3 | MUTATION TYPES IN THE *CYP21A2* GENE

From the total of 899 unique variants, 460 were found affecting the translated region (TR), and 439 the non-translated region (NTR, including the 5′ and 3′ near gene sequences, UTRs and introns) (Supp. Table S1). The distribution of all GVs with an effect on human health, either pathogenic ($N = 212$) or benign ($N = 18$), is displayed in Figure 2 (Supp. Figure S1 depicts the same GVs but noted either by their "c." (NTR) or "p." (exonic) descriptor according to the M12792.1 reference sequence of Higashi et al., 1986).

From the 460 variants affecting TR, 401 were single nucleotide substitutions. Out of these, 281 (~70%) were missense mutations, which also represent the majority of the GVs found to have an effect on human health (153 out of the 230 GVs; Supp. Table S1 and Figure 2), and as expected, they were related to all clinical forms of the disease. The degree of severity of each of the mutants depends on the nature of the amino-acid change and/or the position in the protein (Robins, Carlsson, Sunnerhagen, Wedell, & Persson, 2006; White & Speiser, 2000).

Nonsense ($N = 28$) and frameshift ($N = 32$) mutations may cause either a premature stop codon or the addition of a considerable number of amino acids to the carboxy-terminal end of the protein (Figure 2 and Supp. Table S1). From the total of 60 nonsense and frameshift GVs found, 52 were reported in the clinic, and all of them are related to the classical form of the disease, mainly the SW form.

Only 12 GVs cause an in frame change (deletions, duplications, and indels), from which eight have been identified in the clinic, causing diverse effects on the phenotype: p.L10del, p.(W22_P58dup), p.P46L (c.137_138delinsTG), p.(D68_V71dup), p.S102N

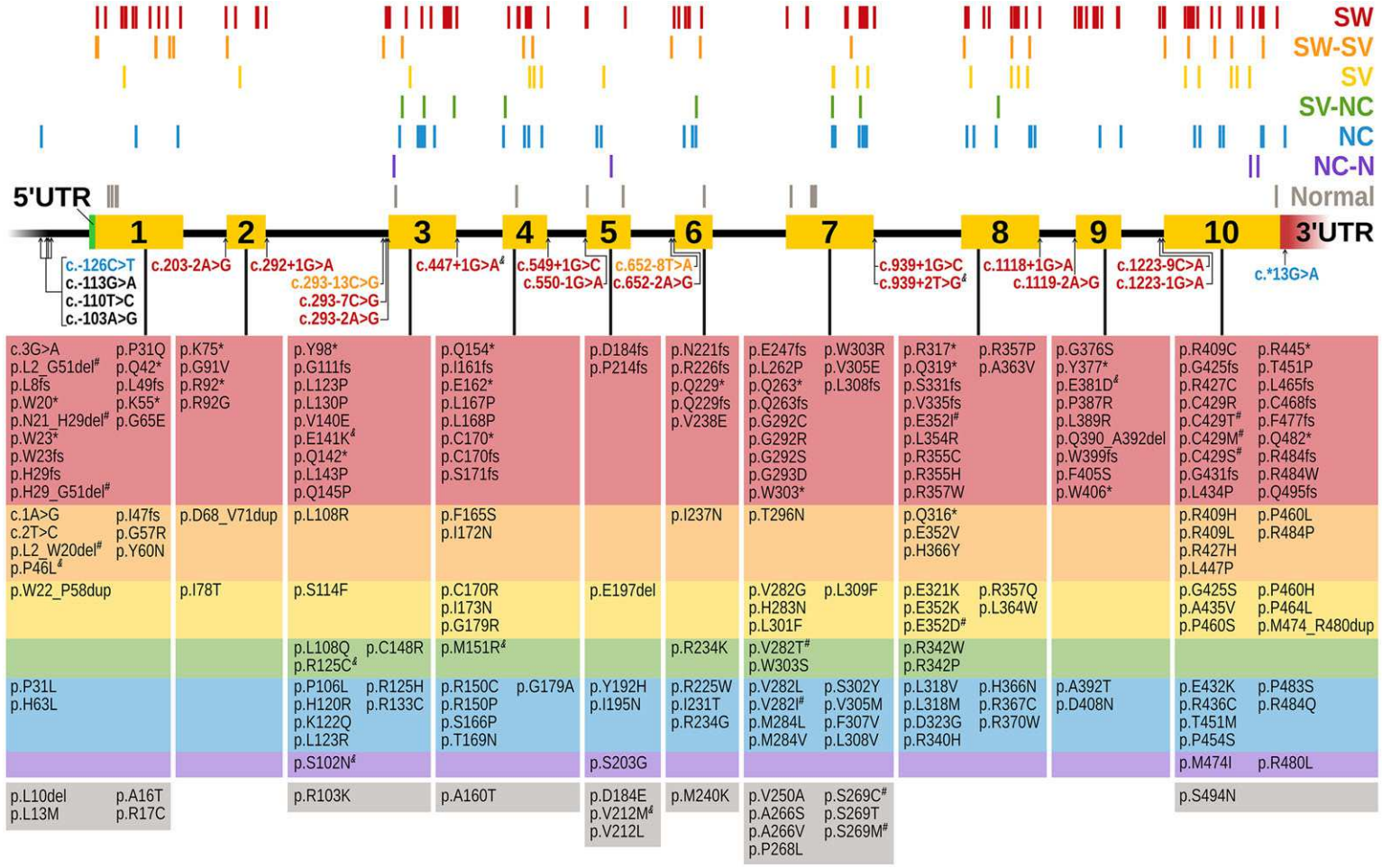

FIGURE 2 Distribution of GVs with known effect over the *CYP21A2* gene. The positions of all GVs with a reported or deduced effect on human health, both pathogenic (SW, SV, NC, and intermediate forms) and benign (normal), are shown. The *CYP21A2* gene is represented in the middle, depicting the 5′ UTR (green) and the 3′ UTR (red), the 10 exons (numbered yellow boxes) and 9 introns (lines in between). On the upper region, every line represents the position of each GV with a given effect, while on the bottom, the same GVs are noted either by their "c." (coding DNA) or "p." (protein) descriptor, according to the GRCh37.p13/GRCh38.p7 NC_000006.12 reference sequence from GRCh38.p7 (RefSeq for cDNA: NM_000500.7; Protein: NP_000491.4). If two different GVs cause the same effect on the protein sequence, the "p." identifier is noted only once. The same color scheme is followed in both regions. The four GVs that conform the promoter conversion (Chang and Chung, 1995) are shown grouped. [&]Marks the nine GVs where there is uncertainty regarding its effect on human health (phenotypic effect accompanied by "?" in Suppl. Table S1); [#]Marks the 13 GVs that were artificially introduced and their in vitro activity tested, but were not found in patients

(c.304_305delinsAA); p.E197del, p.Q390_A392del, p.(M474_R480 dup).

Finally, we compiled 439 GVs located in the NTR, 19 of which have a reported effect on human health (Figure 2 and Supp. Table S1). The intronic regions contain 290 GVs, from which 15 affect the consensus splicing sites. Regarding the 5′ and 3′ UTRs and near regions, only two GVs with known effect on the phenotype have been described: from the four GVs that produce the promoter conversion (c.[-126C > T;-113G > A;-110T > C;-103A > G]) c.-126C > T has been observed alone causing the NC form of the disease (Araújo et al., 2007), and the c.*13C > T, also related to the NC form (Cargill et al., 1999; Menabò et al., 2012) (Figure 2).

4 | *CYP21A2* GENETIC VARIANTS: GENOTYPE–PHENOTYPE CORRELATION

As shown in Figure 2, the distribution of GVs with an effect on human health is in general homogeneous along the TR, although exon 2 and 5 show a diminished ratio of the GVs *per* exon length (data not shown).

The majority of the GVs encode for the classical form of the disease (156 out of the 230 GVs), while at least 43 mutants are related to the mild NC form. Although many of the GVs can be linked to a particular form of the disease, there are some GVs that have been found occupying an intermediate position, associated with two forms, establishing a gradient along the severity of the phenotypic effects observed.

There are 122 variants whose enzymatic residual activity (ERA) has been tested in vitro (Supp. Table S1). Most of the GVs related to the classical form show an ERA below 10% (62 out of 65, 95.4%). Among these, the ERAs of the 91.9% of GVs associated with the SW phenotype are below 2% (34 out of 37). About 90% of GVs related to the NC phenotype show an ERA between 10% and 78% (27 out of 30).

There are 27 GVs where, although coming from the clinic, their severity could not be ascertained (Table 1). These GVs lack a reported in vitro activity and either no information of the patient phenotype or the mutation on the homologous allele is provided in the bibliography, the phenotype is explained by mutations present on the homologous allele (mostly NC patients with mild substitutions), they were found in carrier individuals or the GV is located in cis with another mutation.

All GVs (as point mutations) found in the clinic that have not been uploaded or updated in CYPAlleles are shown in Table 2, where 64 new GVs occur in the TR, 12 new GVs affect the introns, two the promoter region, and one the 3′ UTR. Also included in Table 2 are 17 updated GVs that were present in CYPAlleles, but new information regarding their effect and/or functionality has been published.

5 | DOUBLE MUTANTS IN CIS

A number of 21-hydroxylase deficient alleles have been reported with two or more mutations occurring in cis. Table 3 discloses 43 double mutants in cis not present in CYPAlleles retrieved from the bibliography (not included in this table are micro-conversions that often produce neighboring mutations and the so-called chimeras that may contain several mutations). Only three of these double mutants have been tested in vitro, nevertheless most of them present an associated effect on human health, mostly classical CAH (35 out of the 43), with 27 associated to the SW form of the disease.

6 | FUTURE PROSPECTS AND CONCLUSIONS

The 21-hydroxylase deficiency is the most common cause among patients with CAH. The classical form has an overall incidence of 1:15,000 live births (Pang and Shook, 1997; Therell, 2001; Van der Kamp and Wit, 2004), while NC CAH is one of the most common autosomal recessive disorders in humans and affects approximately one in 1,000 individuals, more frequent in certain ethnic groups such as Jews of Eastern Europe, Hispanics and Yugoslavs (Speiser et al., 1985).

In the three decades passed since *CYP21A2* (previously called P450c21B, CYP21B, or CYP21) was first described, many GVs have been reported. Until recently, most variants reported in the *CYP21A2* gene were identified in the clinic, as those found in the CYPAlleles DB. However, a large number of GVs are being described in massive genome projects, many of which are found in dbSNP, but lack functional implications and/or their phenotypic effect. Moreover, some of the GVs are not even deposited in a DB, with the consequence that the information is spread in several sources and in some circumstances hard to be identified.

With the aim to join all this information, make it widely accessible and linking the effects of GVs to their phenotypic outcomes, we have gathered all the information regarding GVs for the *CYP21A2* gene from six publicly available DBs and from publications and compiled it in an integrated DB.

Even though an effort was made toward the design and implementation of tools to automate the collection and integration of GVs' information, it was not possible to consider every case, and manual curation was necessary in many cases. That was particularly true for information regarding the effect of GVs on human health, absent in most of the DBs explored, highlighting the importance of DBs aimed at gathering clinical information, like ClinVar (Landrum et al., 2016) and LOVD (Fokkema et al., 2011).

In addition, when compiling the information into the integrated DB, the HGVS naming recommendations were applied to all GVs (Dunnen et al., 2016). Through this process, and especially when applying the 3′ naming rule, we found cases where the same GV was reported as different changes. Also, some frameshifts descriptions were fixed to start with the first new amino acid.

21-Hydroxylase deficiency is an autosomal recessive disorder with the underlying phenotype related to the residual activity of the milder allele. Although there is in general a good genotype–phenotype correlation (Dain et al., 2002; Krone, Braun, Roscher, Knorr, & Schwarz,

TABLE 1 *CYP21A2* GVs found in the clinic with uncertain severity

g.(GRCh38.p7)	c.(GRCh38.p7)	p.(GRCh38.p7)	Patient´s phenotype	Homologous allele	References
g.32038423A > C	c.1A > C	p.?	Unknown	Unknown	Tardy and Morel, 2007a
g.32038538A > T	c.116A > T	p.(H39L)	Unknown	Unknown	Tardy, 2006
g.32038551del	c.129del	p.(D44fs)	Unknown	Unknown	Zeng et al., 2004
g.32038565A > G	c.143A > G	p.(Y48C)	Unknown	Unknown	Tardy et al., 2007
g.32038727G > T	c.208G > T	p.(V70L)	SV	p.[(Q319*);R357W]; [H63L;(V70L)][a]	Wang et al., 2016
g.32038816G > A	c.292+5G > A	p.?	SW	p.[?];[?;V282L][b]	Friães et al., 2006
g.(32039142C > A)	c.(341C > A)	p.(S114Y)	NC	p.V282L	New et al., 2013
g.32039199G > A	c.398G > A	p.(R133H)	NC	p.[V282L];[(R113H);V282L][a]	Bruque et al., 2016
g.32039812G > A	c.715G > A	p.(E239K)	Unknown	Unknown	Kirac et al., 2014
g.32040056G > C	c.790G > C	p.(G264R)	Normal	WT	This report[d]
g.(32040183T > A)	c.(917T > A)	p.(V306D)	Unknown	Unknown	Haider et al., 2013
g.(32040416G > T)	c.(950G > T)	p.(R317L)	NC	p.V282L	New et al., 2013
g.(32040431T > C)	c.(965T > C)	p.(L322P)	Unknown	Unknown	Haider et al., 2013
g.32040473C > T	c.1007C > T	p.(P336L)	NC	p.[?];[V282L; (P336L)][a,c]	Bruque et al., 2016
g.32040477C > G	c.1011C > G	p.(Y337*)	NC	p.V282L	Bernal González et al., 2006
g.32040566G > A	c.1100G > A	p.(R367H)	Unknown	Unknown	Haider et al., 2013
g.(32040693G > A)	c.(1144G > A)	p.(G382S)	Unknown	Unknown	Haider et al., 2013
g.32040709C > T	c.1160C > T	p.(P387L)	Unknown	Unknown	Haider et al., 2013
g.32040713C > G	c.1164C > G	p.(N388K)	NC	p.V282L	Wasniewska et al., 2009
g.(32040764C > R)	c.(1215C > R)	p.(F405L)	NC	p.V282L	New et al., 2013
g.32040772G > C	c.1222+1G > C	p.?	Unknown	Unknown	Krone et al., 2013
g.32040919_32040927del	c.1273_1281del	p.(G425_R427del)	NC	p.P31L	New et al., 2013
g.32040926G > C	c.1280G > C	p.(R427P)	NC	p.V282L	Finkielstain et al., 2011
g.32040944C > T	c.1298C > T	p.(P433L)	NC	p.P454S	Carvalho et al., 2012
g.(32040980G > C)	c.(1334G > C)	p.(R445P)	Unknown	Unknown	Haider et al., 2013
g.32041027_32041044del	c.1381_1398del	p.(S461_P466del)	Unknown	p.V282L	Bidet et al., 2009
g.32041091A > C	c.1445A > C	p.(Q482P)	SW	p.[?];[P483S;(Q482P)][a]	Di Pasquale et al., 2005

For each GV, the position and substitution for the genomic DNA, the coding DNA, and the protein sequence level are shown (RefSeq for DNA: NC_000006.12; cDNA: NM_000500.7; Protein: NP_000491.4). When available, the mutation found on the homologous allele is also shown. SW, salt wasting; SV, simple virilizing; NC, non-classical; WT, wild type.

[a]These patients have a mutation in cis and therefore the complete genotype was included.

[b]This patient presented a partial conversion.

[c]This patient presented a conversion or a deletion in the homologous allele.

[d]This was studied to exclude a carrier condition.

2000; Finkelstein et al., 2011; Marino et al., 2011), exceptions have been found (New et al., 2013). Moreover, although 21-hydroxylase deficiency is typically classified into three different forms, the fact that some GVs associate with more than one phenotypic effect (as shown in this work) is in line with the idea that the disease represents a continuous phenotypic spectrum. Indeed, several factors may be responsible for the genotype–phenotype variability in patients with CAH. Extra-adrenal 21-hydroxylase activity have been described (Gomes et al., 2009), and it was suggested that genes related to the fetal androgen synthesis could modulate the degree of external genitalia virilization (Kaupert et al., 2013).

Due to the proximity and the high degree of sequence identity between the gene *CYP21A2* and its pseudogene *CYP21A1P*, most of the patients displayed the 10 most frequent pseudogene-derived mutations. Nevertheless, the great majority of the disease-causing mutations found in the *CYP21A2* gene listed in the present work and depicted in Figure 2 are rare ones and were found in a single family or at most in a reduced number of patients. It must be noted, that there may be variations in the measured activities in vitro depending on the assay technology used (ex vivo systems of COS1/COS7 cells, yeast co-expression system, bacterial expression systems, etc.) and that only the percentage of enzyme conversion is provided; biochemical parameters such as K_m or V_{max} are not reported. In that sense, when more than one activity was reported for the same mutation, the most accurate, newest, and better related to patient's phenotype was preferred to incorporate in the integrated DB. Therefore, the compiled data are intended to be used only as a guideline when providing professional genetic counseling.

In the post-genomic and personalized medicine era, a large amount of genetic information is expected to accumulate. A comprehensive

TABLE 2 Compilation of GVs (as point mutations) found in the clinic that have not been uploaded or updated in CYPAlleles

Rare variants						
				In vitro activity		
g.(GRCh38.p7)	c.(GRCh38.p7)	p.(GRCh38.p7)	Allele associated phenotype	17OHP	P	References
g.32038424T > C	c.2T > C	p.?	SW-SV	ND	ND	Kirac et al., 2014; Toraman et al., 2013
g.32038459C > A	c.37C > A	p.L13M	Normal	99 ± 1	100 ± 1	De Paula Michelatto et al., 2016
g.32038471C > T	c.49C > T	p.R17C	Normal	95 ± 3	81 ± 3	De Paula Michelatto et al., 2016
g.32038546C > T	c.124C > T	p.(Q42*)	SW	ND	ND	Marino et al., 2011
g.32038559_32038560 delinsTG	c.137_138delinsTG	p.P46L	SW-SV?	105 ± 10.6	ND	Brønstad et al., 2014
g.32038793A > G	c.274A > G	p.(R92G)	SW	ND	ND	Wang et al., 2016
g.(32038793A > T)	c.(274A > T)	p.(R92*)	SW	ND	ND	New et al., 2013
g.32039105_32039106 delinsAA	c.304_305delinsAA	p.S102N	NC-Normal?	94 ± 3	74 ± 2	De Paula Michelatto et al., 2016
g.32039124T > A	c.323T > A	p.(L108Q)	SV-NC	ND	ND	Bruque et al., 2016
g.(32039142C > A)	c.(341C > T)	p.(S114Y)		ND	ND	New et al., 2013
g.32039142C > T	c.341C > A	p.S114F	SV	4 ± 1	4 ± 2	De Paula Michelatto et al., 2016; Haider et al., 2013
g.32039169T > C	c.368T > C	p.L123P	SW	1.42 ± 2.13	−1.86 ± 5.19	Massimi et al., 2014
g.32039169T > G	c.368T > G	p.(L123R)	NC	ND	ND	Bruque et al., 2016
g.32039174C > T	c.373C > T	p.R125C	SV-NC?	ND	16 ± 0.6	Krone et al., 2013
g.32039190T > C	c.389T > C	p.(L130P)	SW	ND	ND	Milacic et al., 2015
g.32039222G > A	c.421G > A	p.E141K	SW?	11.30 ± 2.4	ND	Brønstad et al., 2014
g.32039225C > T	c.424C > T	p.(Q142*)	SW	ND	ND	Krone et al., 2013
g.32039235A > C	c.434A > C	p.(Q145P)	SW	ND	ND	Wang et al., 2016
g.32039357G > C	c.449G > C	p.R150P	NC	23.4 ± 1.7	16.9 ± 2	Chu et al., 2013
g.32039360T > G	c.452T > G	p.M151R	SV-NC?	17.66 ± 1.87	4.57 ± 1.87	Massimi et al., 2014
g.32039368C > T	c.460C > T	p.(Q154*)	SW	ND	ND	Wang et al., 2016
g.32039386G > A	c.478G > A	p.A160T	Normal	126.6 ± 29.9	ND	Brønstad et al., 2014
g.32039392G > T	c.484G > T	p.E162*	SW	0.29 ± 0.11	0.18 ± 0	Massimi et al., 2014
g.32039402T > C	c.494T > C	p.(F165S)	CL	ND	ND	Wang et al., 2016
g.32039404T > C	c.496T > C	p.(S166P)	NC	ND	ND	Milacic et al., 2015
g.32039570T > C	c.574T > C	p.Y192H	NC	37.1 ± 7	25.8 ± 9	Concolino et al., 2012
g.32039603A > G	c.607A > G	p.S203G	NC-Normal	85 ± 2	81 ± 3	De Paula Michelatto et al., 2016

(*Continues*)

TABLE 2 (Continued)

Rare variants						
				In vitro activity		
g.(GRCh38.p7)	c.(GRCh38.p7)	p.(GRCh38.p7)	Allele associated phenotype	17OHP	P	References
g.32039630G > A	c.634G > A	p.V212M	Normal?	99.5 ± 32.4	ND	Brønstad et al., 2014; Kirac et al., 2014
g.32039759del	c.662del	p.(N221fs)	SW	ND	ND	Girgis, Ajamian, & Metcalfe, 2013
g.32039773_32039774 del	c.676_677del	p.(R226fs)	SW	ND	ND	New et al., 2013
g.32039812G > A	c.715G > A	p.(E239K)		ND	ND	Kirac et al., 2014
g.32040053dup	c.787dup	p.(Q263fs)	SW	ND	ND	Finkielstain et al., 2011
g.32040056G > C	c.790G > C	p.(G264R)		ND	ND	This report
g.32040062G > T	c.796G > T	p.A266S	Normal	90 ± 9	104 ± 15	Barbaro et al., 2014
g.32040069C > T	c.803C > T	p.P268L	Normal	97 ± 1	87 ± 7	De Paula Michelatto et al., 2016
g.32040113C > A	c.847C > A	p.H283N	SV	1.6 ± 6	2.7 ± 5	Concolino et al., 2012
g.32040180T > A	c.914T > A	p.(V305E)	SW	ND	ND	Wang et al., 2016
g.(32040183T > A)	c.(917T > A)	p.(V306D)		ND	ND	Haider et al., 2013
g.32040185T > G	c.919T > G	p.(F307V)	NC	ND	ND	Haider et al., 2013; Khajuria, Walia, Bhansali, & Prasad, 2017
g.(32040188T > G)	c.(922T > G)	p.(L308V)	NC	ND	ND	New et al., 2013
g.(32040416G > T)	c.(950G > T)	p.(R317L)		ND	ND	New et al., 2013
g.(32040431T > C)	c.(965T > C)	p.(L322P)		ND	ND	Haider et al., 2013
g.32040469del	c.1003del	p.(V335fs)	SW	ND	ND	Krone et al., 2013
g.32040521A > T	c.1055A > T	p.(E352V)	SW-SV	ND	ND	Carvalho et al., 2016
g.32040562C > A	c.1096C > A	p.(H366N)	NC	ND	ND	Khajuria et al., 2017
g.32040566G > A	c.1100G > A	p.(R367H)		ND	ND	Haider et al., 2013
g.(32040693G > A)	c.(1144G > A)	p.(G382S)		ND	ND	Haider et al., 2013
g.32040709C > T	c.1160C > T	p.(P387L)		ND	ND	Haider et al., 2013
g.32040715T > G	c.1166T > G	p.L389R	SW	1.1 ± 0.6	ND	Brønsta et al., 2014
g.32040717_32040725 del	c.1168_1176del	p.Q390_A392del	SW	0 ± 0	<1 ± ND	De Paula Michelatto et al., 2016
g.32040764C > R	c.1215C > R	p.(F405L)		ND	ND	New et al., 2013
g.32040872G > A	c.1226G > A	p.(R409H)	SW-SV	ND	ND	Finkielstain et al., 2011
g.32040918_32040922 del	c.1272_1276del	p.(G425fs)	SW	ND	ND	Finkielstain et al., 2011
g.32040919_32040927 del	c.1273_1281del	p.(G425_R427del)		ND	ND	New et al., 2013
g.32040926G > C	c.1280G > C	p.(R427P)		ND	ND	Finkielstain et al., 2011
g.32040931T > C	c.1285T > C	p.(C429R)	SW	ND	ND	Wang et al., 2016
g.(32040947T > C)	c.(1301T > C)	p.(L434P)	SW	ND	ND	New et al., 2013
g.(32040980G > C)	c.(1334G > C)	p.(R445P)		ND	ND	Haider et al., 2013

(*Continues*)

TABLE 2 (Continued)

Rare variants						
				In vitro activity		
g.(GRCh38.p7)	c.(GRCh38.p7)	p.(GRCh38.p7)	Allele associated phenotype	17OHP	P	References
g.32040998C > T	c.1352C > T	p.T451M	NC	78 ± 6	43 ± 5	De Paula Michelatto et al., 2016
g.32041025C > T	c.1379C > T	p.(P460L)	SW-SV	ND	ND	Wang et al., 2016
g.32041039del	c.1393del	p.(L465fs)	SW	ND	ND	Wang et al., 2016
g.32041047dup	c.1401dup	p.(C468fs)	SW	ND	ND	Bruque et al., 2016
g.32041090C > T	c.1444C > T	p.Q482*	SW	2.98 ± 4.08	0.07 ± 0.35	Massimi et al., 2014
g.32041129dup	c.1483dup	p.(Q495fs)	SW	ND	ND	Kirac et al., 2014
GVs with updated information						
g.32039109G > A	c.308G > A	p.R103K	Normal	119.7 ± 22.5	ND	Rodrigues et al., 1987; Brønsta et al., 2014
g.32039198C > T	c.397C > T	p.R133C	NC	35.40 ± 7.4	15.5 ± 2.7 0	Minutolo et al., 2011; Taboas et al., 2014
g.32039220T > A	c.419T > A	p.V140E	SW	0.7 ± 1.3	0.5 ± 0.6	Barbaro et al., 2012; Robins et al., 2006
g.32039243T > C	c.442T > C	p.C148R	SV-NC	4.3 ± 0.9	3.6 ± 1.8	Barbaro et al., 2012; Robins et al., 2006
g.32039356C > T	c.448C > T	p.R150C	NC	35.8 ± 14.6	47.3 ± 12.9	Minutolo et al., 2011; Taboas et al., 2014
g.32039797A > G	c.700A > G	p.R234G	NC	8 ± 2	2 ± 1	Robins et al., 2006; Barbaro et al., 2014
g.32040116A > G	c.850A > G	p.M284V	NC	16.2 ± 9.3	19 ± 6.8	Minutolo et al., 2011; Taboas et al., 2014
g.32040140G > C	c.874G > C	p.G292R	SW	0.5 ± 0.7	0.7 ± 0.2	Barbaro et al., 2012; Stikkelbroeck et al., 2003
g.32040153C > A	c.887C > A	p.T296N	SW-SV	5.0 ± 1.6	0.8 ± 0.4	Barbaro et al., 2012; Robins et al., 2006
g.32040191C > T	c.925C > T	p.L309F	SV	0.2 ± 0.3	0.1 ± 0.3	Barbaro et al., 2012; Robins et al., 2006
g.32040490C > T	c.1024C > T	p.R342W	SV-NC	5 ± 0.4	4 ± 3	Gunn, Sherman, & Therrell, 1993; Barbaro et al., 2014
g.32040565C > T	c.1099C > T	p.R367C	NC	37 ± 7	28 ± 4	Robins et al., 2006; Barbaro et al., 2014
g.32040940G > A	c.1294G > A	p.E432K	NC	26.2 ± 3.8	24.2 ± 7.4	Dain et al., 2006; Taboas et al., 2014
g.32040952C > T	c.1306C > T	p.R436C	NC	ND	6.5 ± 0.9	Deneux et al., 2001; Krone et al., 2013
g.32040997A > C	c.1351A > C	p.T451P	SW	<1 ± ND	<1 ± ND	Baradaran-Heravi et al., 2007: Michelatto et al., 2016

(*Continues*)

TABLE 2 (Continued)

GVs with updated information						
				In vitro activity		
g.(GRCh38.p7)	c.(GRCh38.p7)	p.(GRCh38.p7)	Allele associated phenotype	17OHP	P	References
g.32041068G > T	c.1422G > T	p.M474I	NC-Normal	85 ± 7	66 ± 12	Barbaro et al., 2012; Robins et al., 2006
g.32041096C > T	c.1450C > T	p.R484W	SW	ND	2.9 ± 1.5	Jiang et al., 2012; Kharrat et al., 2004
GVs in promoter, introns, and 3´ UTR						
g.(GRCh38.p7)	c.(GRCh38.p7)	p.(GRCh38.p7)	Allele associated phenotype	Functional assay		References
g.32038320A > G	c.-103A > G	p.?		Yes		Chin et al., 1998
g.32038297C > T	c.-126C > T	p.?	NC	Yes		Araújo et al., 2007
g.32038812G > A	c.292+1G > A	p.?	SW	Yes		Lee et al 2001
g.32039087C > G	c.293-7C > G	p.?	SW	Yes		Rubtsov et al., 2011
g.32039249G > A	c.447+1G > A	p.?	SW	No		Raisingani et al., 2016
g.32039458G > C	c.549+1G > C	p.?	SW	No		Wang et al., 2016
g.32039545G > A	c.550-1G > A	p.?	SW	No		Concolino et al., 2017
g.32039741T > A	c.652-8T > A	p.?	CL	No		Concolino et al., 2017
g.32039747A > G	c.652-2A > G	p.?	SW	Yes		Taboas et al., 2014
g.32040585G > A	c.1118+1G > A	p.?	SW	No		Finkielstain et al., 2011
g.32040666A > G	c.1119-2A > G	p.?	SW	No		Concolino et al., 2017
g.32040772G > C	c.1222+1G > C	p.?		No		Krone et al., 2013
g.32040860C > A	c.1223-9C > A	p.?	SW	Yes		Katsumata, Shinagawa, Horikawa, & Fujikura, 2010
g.32040868G > A	c.1223-1G > A	p.?	SW	No		Finkielstain et al., 2011
g.32041147G > A	c.*13G > A	p.?	NC	Yes		Cargill et al., 1999; Menabò et al., 2012

For each GV, the position and substitution for the genomic DNA, the coding DNA, and the protein sequence level are shown (RefSeq for DNA: NC_000006.12; cDNA: NM_000500.7; Protein: NP_000491.4). When available, the allele associated phenotype and the in vitro activity (expressed as the percentage of activity relative to the wild-type enzyme) are also shown. ND, not determined; SW, salt wasting; SV, simple virilizing; CL, classic (when there is no certainty about the effect of a GV as either SW or SV); NC, non-classical; empty cells, GVs whose effect on the phenotype could not be deduced due to the lack of a reported in vitro activity and the patient phenotype, or because the phenotype is explained by mutations present in the homologous allele. ?, The question mark sign in the "Allele associated phenotype" column refers to GVs that have a published in vitro activity but there is uncertainty regarding its effect on human health due to the lack of information regarding the second allele, the patient phenotype is absent, or is not concordant with the in vitro activities, or the in vitro activities are inconclusive. 17OHP, 17-hydroxyprogesterone; P, progesterone.

repository of this information and a linkage to known mutations require both, specialized bioinformatics and clinical expertise to specific pathologies. Therefore, development of an efficient tool to compile and join this information, as well as trustworthy DBs, are highly important and could assist physicians in the near future connecting sequence information to their phenotypic effects.

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DISCLOSURE STATEMENT

The authors declare no conflict of interest.

ETHICAL APPROVAL

All the procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written informed consent was obtained from the individual involved in this work. The study was approved by the ethics committee of the Administración Nacional de Laboratorios e Institutos de Salud (ANLIS), Buenos Aires, Argentina.

TABLE 3 Compilation of double mutants in cis found in the clinic that have not been uploaded or updated in CYPAlleles

Double mutants in cis in the *CYP21A2* translated region						
			Allele associated phenotype	In vitro activity		
g.(GRCh38.p7)	c.(GRCh38.p7)	p.(GRCh38.p7)		17OHP	P	References
g.[32038459C > A;32040421C > T]	c.[37C > A;955C > T]	p.[L13M;(Q319*)]	SW	ND	ND	De Paula Michelatto et al., 2016
g.[32038481G > A;32040110G > T]	c.[59G > A;844G > T]	p.[(W20*);V282L]	SW	ND	ND	Carvalho et al., 2016
g.[32038514C > T;32040110G > T]	c.[92C > T;844G > T]	p.[P31L;V282L]		ND	ND	New et al., 2013
g.[32038514C > T;32041006C > T]	c.[92C > T;1360C > T]	p.[P31L;P454S]	SW	ND	ND	Milacic et al., 2015
g.[32038610A > T;32038727G > T]	c.[188A > T;208G > T]	p.[H63L;(V70L)]	SV	ND	ND	Wang et al., 2016
g.[32039133_32039140del;32039807T > A; 32039810T > A32039816T > A]	c.[332_339del;710T > A; 713T > A;719T > A]	p.[(G111fs);I237N; V238E;M240K]	SW	ND	ND	New et al., 2013
g.[32039133_32039140del;32040110G > T]	c.[332_339del;844G > T]	p.[(G111fs);V282L]	SW	ND	ND	Finkielstain et al., 2011
g.[32039199G > A;32040110G > T]	c.[398G > A;844G > T]	p.[(R133H);V282L]		ND	ND	Bruque et al., 2016
g.[32039444G > C;32040110G > T]	c.[536G > C;844G > T]	p.[G179A;V282L]	CL	ND	ND	Lobato, Ordóñez-Sánchez, Tusié-Luna, & Meseguer, 1999; Nunez et al., 1999
g.[32039426T > A;32040189dup]	c.[518T > A;923dup]	p.[I173N;(F307fs)]	SW	ND	ND	Krone et al., 2000
g.[32039426T > A;32040535C > T]	c.[518T > A;1069C > T]	p.[173N;R357W]	SW	ND	ND	Krone et al., 2000
g.[32039426T > A;32040110G > T]	c.[518T > A;844G > T]	p.[I173N;V282L]	SW	ND	ND	Deneux et al., 2001; New et al., 2013
g.[32039426T > A;32040421C > T]	c.[518T > A;955C > T]	p.[I173N;(Q319*)]	SW	ND	ND	New et al., 2013
g.[32039630G > A;32040110G > T]	c.[634G > A;844G > T]	p.[V212M;V282L]	SV-NC	ND	ND	Brønstad et al., 2014
g.[32039807T > A;32039810T > A; 32039816T > A;32040421C > T]	c.[710T > A;713T > A; 719T > A; 955C > T]	p.[I237N;V238E;M240K; (Q319*)]	SW	ND	ND	Jiang et al., 2012
g.[32040110G > T;32040185T > G]	c.[844G > T;919T > G]	p.[V282L;(F307V)]	CL	ND	ND	New et al., 2013
g.[32040110G > T;32040421C > T]	c.[844G > T;955C > T]	p.[V282L;(Q319*)]	SW	ND	ND	Vrlazalová et al., 2010
g.[32040110G > T;.32040473C > T]	c.[844G > T;1007C > T]	p.[V282L;(P336L)]	NC	ND	ND	Bruque et al., 2016
g.[32040110G > T;32040535C > T]	c.[844G > T;1069C > T]	p.[V282L;R357W]	SW	ND	ND	Krone et al., 2000
g.[32040110G > T;32040872G > A]	c.[844G > T;1226G > A]	p.[V282L;(R409H)]	SW	ND	ND	New et al., 2013
g.[32040110G > T;32041006C > T]	c.[844G > T;1360C > T]	p.[V282L;P454S]	SW	ND	ND	Bidet et al., 2009; New et al., 2013
g.[32040110G > T;32041097G > A]	c.[844G > T;1451G > A]	p.[V282L;R484Q]		ND	ND	Bidet et al., 2009
g.[32040189dup;32040535C > T]	c.[923dup;1069C > T]	p.[(L307fs);R357W]	SW	ND	ND	Wang et al., 2016
g.[32040421C > T;32041096C > T]	c.[955C > T;1450C > T]	p.[(Q319*);R484W]	SW	ND	ND	Loidi et al., 2006
g.[32040434A > G;32040940G > A]	c.[968A > G;1294G > A]	p.[D323G;E432K]	SV	2.1 ± 1.1	5.6 ± 3.3	Minutolo et al., 2011; Taboas et al., 2014
g.[32040675G > A;32041006C > T]	c.[1126G > A;1360C > T]	p.[G376S;P454S]	SW	0 ± 1	0 ± 1	Lajic et al., 2002
g.[32040723G > A;32041025C > A]	c.[1174G > A;1379C > A]	p.[A392T;P460H]	SV	ND	ND	Jiang et al., 2012
g.[32040766G > A;32041006C > T]	c.[1217G > A;1360C > T]	p.[(W406*);P454S]	SW	ND	ND	New et al., 2013
g.[32041006C > T;32041097G > C]	c.[1360C > T;1451G > C]	p.[P454S;R484P]	SW	ND	ND	New et al., 2013

(*Continues*)

TABLE 3 (Continues)

Double mutants in cis including GVs in *CYP21A2* non-translated region					
g.(GRCh38.p7)	**c.(GRCh38.p7)**	**p.(GRCh38.p7)**	**Associated allele phenotype**	**Functional assay**	**References**
g.32033649A > G	c.[-4774A > G;1360C > T]	p.[?;P483S]		No	Fernández et al., 2015
g.[32038297C > T;32038310G > A; 32038313T > C;32038320A > G; 32039081C > G]	c.[-126C > T;-113G > A; -110T > C;-103A > G; 290-13C > G]	p.[?;?;?;?;?]	SW	No	Tardy et al., 2010
g.[32038297C > T;32038310G > A; 32038313T > C]	c.[-126C > T;-113G > A; -110T > C]	p.[?;?;?]		Yes	Araujo et al., 2007; Zhang et al., 2009
g.[32038310G > A;32038313T > C]	c.[-113G > A;-110T > C]	p.[?;?]	SV	No	New et al., 2013
g.[32038310G > A;32038313T > C; 32039081C > G]	c.[-113G > A;-110T > C; 290-13C > G]	p.[?;?;?]	CL	No	New et al., 2013
g.[g.32038419C > T;32040421C > T]	c.[-4C > T;955C > T]	p.[?;(Q319*)]	SW	No	Charfeddine., et al 2012
g.[32039081C > G;32040189dup]	c.[293-13C > G > G;923dup]	p.[?;(L307fs)]	SW	No	Loke, Lee, Lee, & Poh, 2001
g.[32039081C > G;32040535C > T]	c.[293-13C > G;1069C > T]	p.[?;R357W]	SW	No	Loke et al., 2001
g.[32039081C > G;32041006C > T]	c.[290-13C > G;1360C > T]	p.[?;P454S]	SW	No	Pinto et al., 2003
g.[32039081C > G;32039807T > A; 32039810T > A;32039816T > A]	c.[290-13C > G;710T > A; 713T > A;719T > A]	p.[?;I237N;V238E;M240K]	SW	No	New et al., 2013
g.[32039081C > G;32040871C > T]	c.[290-13C > G;1225C > T]	p.[?;R409C]	SW	No	Carvalho et al., 2016
g.[32039081C > G;32039172C > T]	c.[290-13C > G;371C > T]	p.[?;(T124I)]	SW	No	Wang et al., 2016
g.[32040110G > T;32041186C > T; 32041574C > T;32041577T > C]	c.[844G > T;*52C > T; *440C > T;*443T > C]	p.[V282L;?;?;?]	NC	No	Neocleous et al., 2017
g.[32040421C > T;32041146C > T; 32041186C > T]	c.[955C > T;*12C > T;*52C > T]	p.[(Q319*);?;?]	NC	No	Neocleous et al., 2017

For each double mutant, the position and substitution for the genomic DNA, the coding DNA, and the protein sequence level are shown (RefSeq for DNA: NC_000006.12; cDNA: NM_000500.7; Protein: NP_000491.4). When available, the allele associated phenotype and the in vitro activity (expressed as the percentage of activity relative to the wild-type enzyme) are also shown. ND, not determined; SW, salt wasting; SV, simple virilizing; CL, classic (when there is no certainty about the effect of a GV as either SW or SV); NC, non-classical; empty cells, GVs whose effect on the phenotype could not be deduced. 17OHP, 17-hydroxyprogesterone; P, progesterone. The three GVs that compose the common cluster E6 (p.[I237N;V238E;M240K]) are considered as a single mutation.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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