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Novel non-classic *CYP21A2* variants, including combined alleles, identified in patients with congenital adrenal hyperplasia

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ABSTRACT

Objective: Congenital adrenal hyperplasia (CAH) is an inborn error of metabolism and a common disorder of sex development where > 90% of all cases are due to 21-hydroxylase deficiency. Novel and rare pathogenic variants account for 5% of all clinical cases. Here, we sought to investigate the functional and structural effects of four novel (p.Val358Ile, p.Arg369Gln, p.Asp377Tyr, and p.Leu461Pro) and three combinations of *CYP21A2* variants (i.e. one allele containing two variants p.[Ile172Asn;Val358Ile], p.[Val281Leu;Arg369Gln], or p.[Asp377Tyr;Leu461Pro]) identified in patients with CAH.

Methods: All variants were reconstructed by *in vitro* site-directed mutagenesis, the proteins were transiently expressed in COS-1 cells and enzyme activities directed toward the two natural substrates (17-hydroxyprogesterone and progesterone) were determined. In parallel, *in silico* prediction of the pathogenicity of the variants based on the human CYP21 X-ray structure was performed.

Results: The novel variants, p.Val358Ile, p.Arg369Gln, p.Asp377Tyr, and p.Leu461Pro exhibited residual enzymatic activities within the range of non-classic (NC) CAH variants (40–82%). An additive effect on the reduction of enzymatic activity (1–17%) was observed when two variants were expressed together, as identified in several patients, resulting in either NC or more severe phenotypes. *In silico* predictions were in line with the *in vitro* data except for p.Leu461Pro.

Conclusions: Altogether, the combination of clinical data, *in silico* prediction, and data from *in vitro* studies are important for establishing a correct genotype and phenotype correlation in patients with CAH.

1. Introduction

Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency (21OHD) is one of the most common inborn errors of metabolism [1]. The clinical manifestations of 21OHD (OMIM # 201910) are presented as a continuum of phenotypes from mild, late onset disease to severe neonatal forms. In the salt-wasting form (SW-CAH) the biosynthesis of both cortisol and aldosterone is affected and the child will die in a salt losing crisis if replacement therapy is not initiated within the first weeks of life. In the simple-virilising form (SV-CAH), the aldosterone production is sufficient to avoid salt loss during basal conditions. Girls affected with these classic forms of CAH (SW and SV) are

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exposed to high levels of adrenal androgens already *in utero* and are born with virilized external genitalia. The mild, non-classic phenotype (NC-CAH) is associated with signs of androgen excess from late childhood, but it may also escape diagnosis until adult age. A child may present with accelerated growth and precocious pseudo puberty, while women often seek medical attention due to hirsutism and menstrual irregularities [2–4].

The worldwide incidence of classic 21OHD is 1:10,000–1:16,000 live births, while NC 21OHD is much more prevalent (1:500) [4,5]. The *CYP21A2* gene locus has a complicated structure where the functional *CYP21A2* gene and the inactive pseudogene are adjacent to and alternating with the *C4B* and *C4A* genes. One *C4* gene and one *CYP21* gene are part of a repeated module (RCCX) located on 6p21.3 [3,6]. Ten common pathogenic variants are identified in 95% of all 21OHD cases and the less severe allele dictates the phenotype [7]. These variants are due to gene conversion or recombination events between the active gene and the pseudogene. In the rest of the cases novel variants specific for a single family or population are identified.

For 21OHD there is a good correlation between *in vitro* studies on mutated enzyme function and *in vivo* disease severity [8–10]. To improve genetic counselling and clinical management, functional analysis of recombinant CYP21 protein are proposed as a complement to disease classification [11,12], especially for novel or rare pathogenic variants where large groups of patients are not available for clinical investigations. In rare cases, one allele may harbour two or more missense variants [9,13,14]. In these situations, and if one of the variants is novel, it may be difficult to predict the exact phenotype. Functional investigations of such alleles will contribute to the prediction of the severity of the disease and the pathogenicity of the novel variants *per se*.

In this report, we describe a detailed genotype-phenotype evaluation of seven patients with 21OHD including functional analysis of four novel amino acid substitutions (p.Val358Ile, p.Arg369Gln, p.Asp377Tyr, and p.Leu461Pro) and three combinations of pathogenic variants (i.e. one allele containing two variants p.[Ile172Asn;Val358Ile], p.[Val281Leu;Arg369Gln] or p.[Asp377Tyr;Leu461Pro]). In parallel, we also expressed three common pathogenic variants associated with classic and NC-CAH as reference (p.Ile172Asn, p.Val281Leu and p.Pro482Ser). Using this approach, we determined the pathogenicity of the novel amino acid substitutions *per se* and of the combined alleles. In addition, we performed computational modelling of mutated proteins based on the human CYP21 X-ray structure [15].

2. Material and methods

2.1. Patients

Patient 1, a boy, presented with failure to thrive and vomiting during his first month of life. He was admitted to hospital with malnutrition and severe dehydration accompanied with hyponatremia (112 mmol/L) and metabolic acidosis at 2.5 months of age. The 17OHP level was extremely high (> 900 nmol/L) and he was diagnosed with SW-CAH.

Patient 2, a girl, was born at gestational week 40 (birth weight, 3930 g, karyotype 46, XX) with ambiguous genitalia. She had elevated 17OHP (534 nmol/L), mild hyponatremia (134 mmol/L), and hyperkalemia (5.0 mmol/L) and was put on treatment with hydrocortisone and fludrocortisone due to SW-CAH. She went through a vaginoplasty at the age of 4 months and had menarche at the age of 12 y, reaching a final height of 171.9 cm (mean target height 174 cm).

Patient 3, a girl, was born at gestational week 42 (birth weight, 4205 g). She presented with precocious pubarche at the age of 5.8 years and her bone age was slightly advanced (BA 7.8 years at age 6.5 y). An ACTH-stimulation test was performed (250 μg Synacthen) and revealed the diagnosis of NC-CAH (17OHP increased from 7.7 nmol/L to 54 nmol/L). She reached a final height of 157.3 cm (mean target height, 163.5 cm) and at age 15 y she had still not been put on treatment with hydrocortisone.

Patient 4, a girl, was born at gestational week 42 (birth weight, 3460 g). She was identified in the neonatal screening program for 21OHD but since she did not have genital ambiguity treatment was not initiated. At age 3.5 years, her bone age was advanced with 1.5 years and an ACTH stimulation test confirmed the diagnosis of CAH (17OHP increased from 66 nmol/L to 273 nmol/L). She had menarche at the age of 11 years and reached a final height of 156 cm (mean target height 158.5 cm). During the last follow-up at age 12 years she still did not have any hydrocortisone treatment and she was not hirsute. She was assigned to have NC-CAH.

Patient 5 attracted medical attention at age 20 y when her daughter was investigated for CAH. The mother was found to be compound heterozygote for p.Arg356Trp (also present in the daughter who had classic CAH) and the novel variant p.Val358Ile (not inherited by her daughter). She denied any symptoms of menstrual irregularities but had acne and mild NC-CAH was suspected by the investigating physician.

Patient 6, a girl, was born at gestational week 40 (birth weight, 3060 g). She attracted medical attention at the age of 15.8 years due to primary amenorrhea (pubertal stage B1, PH 4), short stature, and mild clitoral hypertrophy. 17OHP increased from 84 to 333 mmol/L after stimulation with 250 μg Synacthen. She was diagnosed with NC-CAH and put on treatment with hydrocortisone. Her pubertal development advanced and she had menarche at age 16.4 years. She did not reach her target height potential (158.5 cm) with a final height of 150 cm.

Patient 7, a boy, was investigated after his sister was diagnosed with NC-CAH (patient 6). At 9.9 years of age, his only symptom was advanced bone age (BA 13 years) in addition to elevated basal 17OHP (141 nmol/L). The child was put on treatment with hydrocortisone and, at 15 years of age, he had reached a final height of 167.3 cm (mean target height 171.5 cm).

Patient 8, a girl, was admitted for investigation at age 5 due to precocious pubarche and advanced bone age. The stimulated 17OHP level was > 150 nmol/L and she was considered to have NC-CAH.

Clinical data are summarized together with the molecular data in Table 1.

2.2. Genotyping

Genotyping of the *CYP21A2* gene was performed at Universidade Estadual de Campinas, Brazil, and at the University of Bologna, Italy; as previously published [16,17]. Sequencing electropherograms were analyzed against the reference sequence NM_000500.6, but removing 3 base pairs of the CTG repeat at the beginning of the gene thus keeping the numbering of the variants as previously stated in the literature (e.g. p.Pro30Leu and not p.Pro31Leu). Segregation analyses were performed in all subjects by sequencing parental samples and MLPA was used for determination of gene copy numbers (MRC-Holland P050 MLPA kit, Amsterdam, Netherlands). The Coffalyser software was used for data analysis.

2.3. Functional studies

The general description for construction of vectors for expression studies of mutated CYP21A2 protein in COS-1 cells has been presented previously [9,18]. In those cases where two variants were identified on the same allele both variants were introduced into the mutagenesis vector (pALTER) and thereafter subcloned to the expression vector (pCMV4).

After construction of the expression vectors harbouring the different variants, *in vitro* expression experiments were performed. COS-1 cells (1×10^5) seeded in 12 well plates were transfected with 0.5 μg of the pCMV4 vector (containing the wild type CYP21 or the mutant CYP21 or in its native form as mock transfection as negative control) as well as 0.2 μg of the β-galactosidase vector to ensure equal transfection

Table 1
Summary of clinical data and genotypes. Novel variants that were expressed *in vitro* are marked in bold.

Patient No.	Sex	Age at diagnosis	Symptoms at diagnosis	17OHP (nmol/L)[a] basal/stimulated	Genotype [allele1];[allele2]	Phenotype	Country of origin
1	M	2.5 months	Severe dehydration, metabolic acidosis. Na 112 mmol/L; K 7.7 mmol/L.	> 900/NA	p.[Ile172Asn;**Val358Ile**];c.[290-13A/C > G]	SW	Brazil
2	F	At birth	Ambiguous genitalia. 46, XX. Na 134 mmol/L; K 5.0 mmol/L.	534/NA	p.[Ile172Asn;**Val358Ile**];p.[Leu480fs]	SW	Italy
3	F	5.8 years	Precocious pubarche, axillary hair, advanced BA (7.8 years at 6.5 years).	7.7/54	p.[Ile172Asn;**Val358Ile**];c.[[a]13G > A]	NC	Italy
4	F	At birth	Positive neonatal screening for 21OHD. No genital ambiguity.	67/273	p.[Ile172Asn;**Val358Ile**];p.[Val281Leu]	NC	Italy
5	F	20 years	Acne.	NA	**p.[Val358Ile]**;p.[Arg356Trp]	Mild NC	Brazil
6[b]	F	15.8 years	Primary amenorrhea, short stature, mild clitoral hypertrophy.	84/333	p.[Ile172Asn];p.[Val281Leu;**Arg369Gln**]	NC	Italy
7[b]	M	9 years	Advanced BA (13 years at 9.9 years).	141/269	p.[Ile172Asn];p.[Val281Leu;**Arg369Gln**]	NC	Italy
8	F	5 years	Precocious pubarche, accelerated growth and advanced BA (8.6 years at 6.3 years).	14/ > 150	**p.[Asp377Tyr;Leu461Pro]**;p.[Val281Leu]	NC	Brazil

[a] 250 μg Synacthen stimulation test; cut-off level 17-OHP basal: 6 nmol/L; at 60 min: 30 nmol/L (colorimetric enzyme immunoassay).
[b] Siblings. NA, not available; SW, salt wasting; NC, non-classic; BA, bone age.

efficiency, for more details see [13,19].

The experiments were performed for each mutant CYP21A2 using 0.5 μCi of 3H-labeled substrates and 2.0 μM unlabelled, either 17-hydroxyprogesterone (17OHP) or progesterone (Amersham Biosciences, Sweden), and 4 mM of reduced nicotinamide adenine dinucleotide phosphate. Cells were incubated for 40 min and four independent experiments were performed for each substrate. After incubation, steroids were extracted with ethylacetate and vacuumed to dryness, re-suspended in ethanol and subjected to thin-layer chromatography (chloroform:ethylacetate), and substrates and products were quantified by liquid scintillography (for more details see [13,19]). The cells were further harvested by trypsination and the ratio of β-galactosidase activity to total protein content was measured to verify equal transfection efficiency between the different constructs. Enzyme activities are expressed as a percentage of conversion, using total protein content to normalize the samples and considering the apparent specific activity of the wild type protein as 100%. For more details, descriptions of the *in vitro* CYP21A2 expression assays have been covered previously [13,19].

2.4. Western blot

Western blot analysis was performed using rabbit polyclonal antibodies against human CYP21A2 as the primary antibody (SAB1300942, Sigma-Aldrich, USA) and anti-rabbit IgG (GE Healthcare Life Sciences) as the secondary antibody; see Robins et al. for details [19].

2.5. Structural evaluation

The resolved three-dimensional structure of human CYP21, pdb id: 4y8w [15], was used as a starting point to evaluate the effect of the novel variants on the protein structure. The ICM molecular modelling software (version 3.8–1, Molsoft LLC, La Jolla, CA, USA) was used to perform structural calculations regarding the structures of each variant, expanding on the strategy previously described [20,21]. First, all missing intra-structural loops were added to the 4y8w structure and the structure was optimized using energy minimization. A model of each variant was generated by modifying the corresponding amino acid residue, followed by multiple steps of energy minimization. The energy minimization was initially carried out with strong backbone restraints, which were gradually relaxed and, finally, a global minimization without backbone restraints was performed. Each variant was then evaluated based on energy and distances to the steroid and heme, as well as using the ICM built-in function for evaluating stability changes from variants by calculating the free energy changes.

The BLAST web interface (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify mammalian proteins similar to the human CYP21A2 enzyme. The search resulted in CYP21 protein sequences from 81 mammals. The sequences with the strongest similarity to the human CYP21A2 protein, one sequence per species, were retrieved from the database, aligned using the L-INS-i approach of MAFFT [22] and, for each position, the sequence identity was calculated, counting a gap as a mismatch.

3. Results

We have determined *in vitro* the impact on enzyme function for three novel *CYP21A2* mutant alleles carrying simultaneously two missense variants, p.[Ile172Asn;Val358Ile], p.[Val281Leu;Arg369Gln], or p.[Asp377Tyr;Leu461Pro], respectively. Four of the investigated variants were novel and were identified in individuals who had been diagnosed with CAH. The p.Val358Ile variant was, in addition, identified alone on one allele and in *trans* with the common p.Arg356Trp variant in a woman who denied subjective symptoms but was suspected of having very mild CAH due to presence of acne at the age of 20 years (patient 5). For a detailed description of the patient phenotypes and genotypes, see Table 1. Each of the novel missense changes (p.Val358Ile, p.Arg369Gln, p.Asp377Tyr, and p.Leu461Pro) was also expressed individually and in parallel with three common variants known to cause classic or NC-CAH (reference variants: p.Ile172Asn, p.Val281Leu, and p.Pro482Ser). A schematic overview of the gene

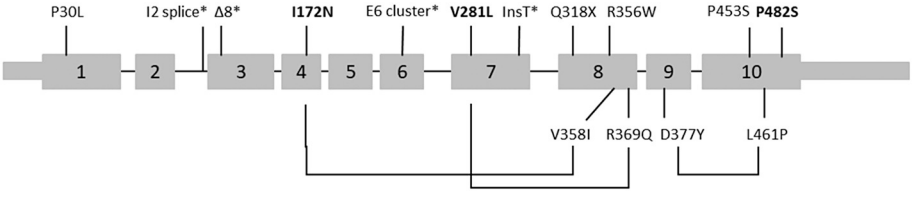

Fig. 1. A schematic overview of the localization in the *CYP21A2* gene of the studied novel variants (lower part of the figure) and of the ten common *CYP21A2* pathogenic variants (upper part of the figure). The reference variants used in the study are depicted in bold text (upper part of the figure).
*I2 splice, c.293-13C > G/A; Δ8, c.332_339del GAGACTAC; E6 cluster, p.(I236N;V237E;M239K); InsT, c.923dupT.

Table 2
Summary of *in vitro* enzyme activities, *in silico* predictions of the pathogenicity of the variants and predictions of the phenotype.

Nucleotide change	Predicted amino acid change	Structural location	*In silico* prediction	*In vitro* enzyme activity % (SD)		Final prediction of phenotype
				17OHP	Prog	
c.515 T > A	**p.Ile172Asn**	**α-helix E**		**< 1**	**< 1**	**CL**
c.841G > T	**p.Val281Leu**	**α-helix I**		**33 (8)**	**26 (3)**	**NC**
c.1444C > T	**p.Pro482Ser**	**End of β9**		**82 (10)**	**54 (7)**	**Mild NC**
c.1072G > A	p.Val358Ile	Loop between α-helix K and β-sheet β4	Minor effect	72 (7)	34 (3)	Mild NC
c.1106G > A	p.Arg369Gln	Loop between β4 and β5 sheets	Minor effect	82 (6)	63 (4)	Mild NC
c.1129G > T	p.Asp377Tyr	β6	Minor effect	81 (6)	58 (4)	Mild NC
c.1382 T > C	p.Leu461Pro	Loop between β8 and β9 sheets	No effect	55 (8)	40 (2)	NC
c.515 T > A;c.1072G > A	p.[Ile172Asn;Val358Ile]		Severe effect	< 1	< 1	CL
c.841G > T;c.1106G > A	p.[Val281Leu;Arg369Gln]		Minor effect	10 (2)	9 (1)	NC
c.1129G > T;c.1382 T > C	p.[Asp377Tyr;Leu461Pro]		Minor effect	17 (4)	10 (2)	NC

Enzyme activity is expressed as % of wild type, mean of four independent experiments. NC, non-classic; CL, classic. Reference variants are in bold.

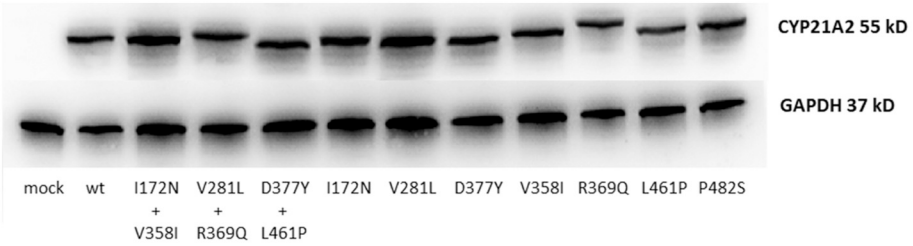

Fig. 2. Western blot results for all expressed constructs.

localization of all novel variants and the reference variants is depicted in Fig. 1 All single novel variants showed quite high residual enzymatic activities (55–80% for 17OHP and 30–60% for progesterone) when expressed individually (Table 2). They would thus give a mild NC phenotype with a severity in between the phenotypes of p.Val281Leu and p.Pro482Ser (Table 2). Constructs harbouring two variants, as found in the patients, showed lower activities ranging from classic to NC levels, depending on the combination (Table 2). Similar expression of mutant and wild type proteins was confirmed by Western blot (Fig. 2).

According to the *in silico* analysis, none of the single missense variants or combinations thereof should have a severe effect on protein structure except for the mutant allele harbouring the p.Ile172Asn variant. The results of the *in silico* predictions are summarized in Fig. 3 and Table 2. The predictions were concordant with the *in vitro* results, except for p.Leu461Pro. This variant was not predicted to be pathogenic but, in the *in vitro* studies, the enzyme activity was clearly reduced. P.Leu461Pro introduces a proline in a loop region, which might cause a disturbance due to the major structural difference between leucine and proline.

The variants p.Val358Ile, p.Arg369Gln, p.Asp377Tyr, and p.Leu461Pro are visualized in Fig. 3. In the case of p.Val358Ile, the residue change (valine to isoleucine) would not normally be expected to cause any major effect, but it is located near the active site (4 Å to heme, 6 Å to steroid), which it could potentially disturb. The variant p.Arg369Gln removes a positive charge, which may disturb interactions with helix B´. Tyrosine is a much larger amino acid residue than aspartate, which could explain the effect of p.Asp377Tyr, especially since it is located next to another tyrosine.

4. Discussion

Alleles with combinations of pathogenic variants in the *CYP21A2* gene and associated with CAH are relatively common compared to other autosomal recessive disorders. Pathogenic variants can have synergistic or additive effects regarding residual enzyme activity and when the combination includes a severe variant the prediction of the disease severity will obviously be severe. However, when mild or novel variants are involved the prediction of the phenotype is more complicated (see Table 3 for a summary).

Here we describe the evaluation of three double mutants, representing different types of combinations and their possible final effect. Each missense variant has also been expressed separately in order to establish the contribution of individual variants and to determine the associated phenotype if the variant would be identified alone in a *CYP21A2* allele.

The p.[Ile172Asn;p.Val358Ile] allele was identified in four independent patients of Brazilian and Italian origin (Table 1). p.Ile172Asn is a common severe variant with very low residual enzyme activity and the presence of another variant in the same allele can be expected to lead to even more aggravating effects. This difference is not detectable by the *in vitro* studies due to the extremely low activity (Table 2). However, patient 2, who has this combined allele and the null variant p.Leu480fs on the other allele, presents a SW phenotype suggesting a null activity.

Interestingly, the p.Val358Ile has also been identified alone in patient 5. This woman was identified due to being the mother of a patient with classic CAH (she passed the p.Arg356Trp variant to her daughter). She had acne and a mild NC phenotype cannot be ruled out. The residual *in vitro* activity, similar to the activity of p.Pro482Ser, is compatible with such a mild form of CAH (Table 2).

Val358 is highly conserved in mammals and is located in a loop near the region that interacts with the substrate. *In silico* prediction also indicated a minor effect. The variant is located at a CpG site, so it can be expected to be detected again in patients with CAH.

The p.Arg369Gln also occurs at a CpG site. This variant was identified *in cis* with the p.Val281Leu in two siblings presenting with NC-CAH (Table 1). Arg369 is located in a loop in an arginine-rich region where positively charged amino acids are important for the interaction with the redox partner. In other mammalian CYP21A2, a lysine is present at the corresponding position. Following an *in silico* evaluation, a minor effect on protein structure was expected. The functional studies showed a minor reduction in enzyme activity for the p.Arg369Gln mutant (82% and 63% toward 17OHP and progesterone), similar to the

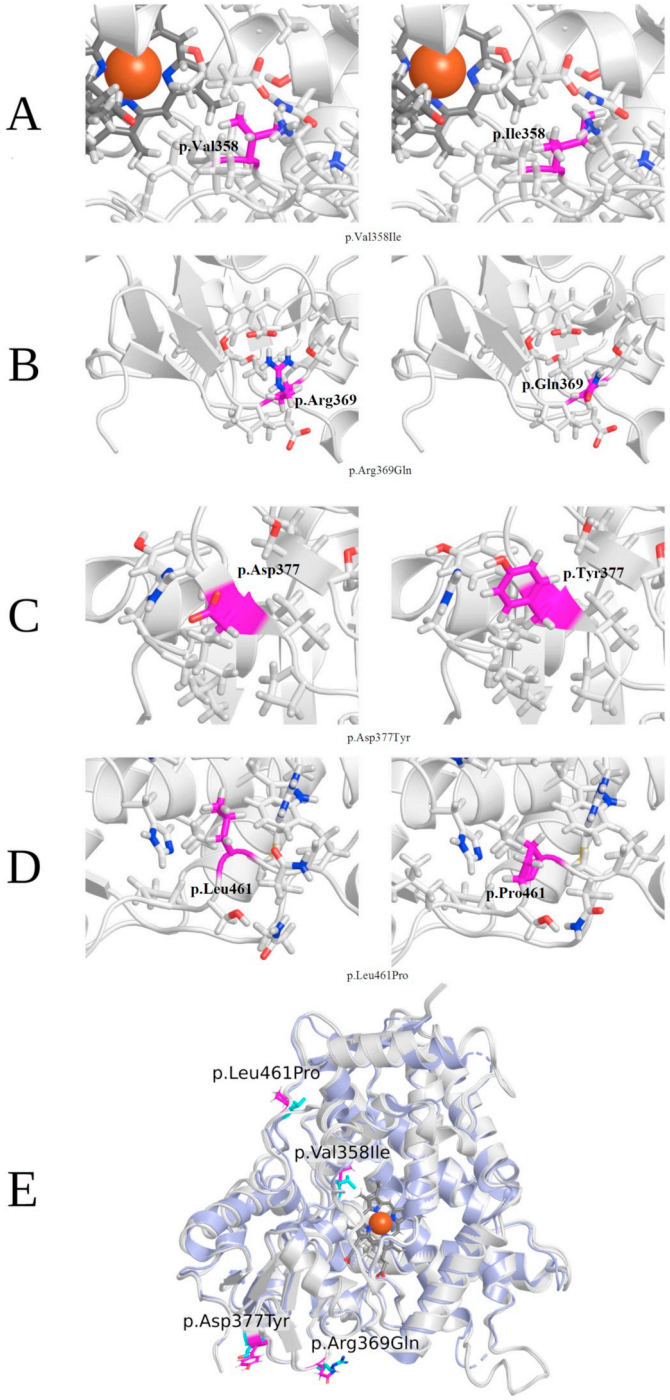

Fig. 3. Visualization of the structural changes caused by the novel variants mapped on the structure of CYP21A2. A-D: The wildtype structures are on the left, the mutated on the right. The bottom most picture (E) overlaps all mutated models with the wild type structure. Non-carbon residues use the standard coloring (*e.g.* oxygen is red, nitrogen is dark blue, hydrogen is white). Dark grey is the central heme group with the iron ion visualized as orange. Purple is the position of interest (the mutated position), while white represent other residues in the vicinity. A: p.Val358Ile, B: p.Arg369Gln, C: p.Asp377Tyr, D: p.Leu461Pro.
E: Positioning of the mutated residues in the structure of CYP21A2; white: the four models of the mutated CYP21A2 structures, blue: the wild type CYP21A2 structure, purple: the mutated residues, cyan: the wild type residues. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

p.Pro482Ser variant. In our assay, p.Val281Leu had an activity around 30%, and in combination with p.Arg369Gln it had an additive effect with a reduction in enzyme activity to around 10% (Table 2). This activity is sufficient to prevent prenatal virilisation of the affected female, but the patient presented with quite severe NC symptoms.

The common p.Val281Leu variant has been described in combination with three other rare variants, p.Val211Met, p.Ile230Thr, and p.Leu353Arg, respectively [14,23,24]. In the latter case, no functional studies were performed, but the variant was homozygous in a patient with classic CAH. The change p.Val211Met was only studied alone and a normal enzyme activity was reported [23], unfortunately a synergistic effect was not investigated. Tardy and colleagues reported an activity of approximately 60% for both p.Ile230Thr and p.Val281Leu, and with a reduction to around 9% when the combined allele was expressed [14]. In these two studies, the female patients carrying the combined alleles had a null variant on the other allele, and their phenotype is described as severe NC, similar to that of our patient 6. This shows that the gradient in phenotype correlates well with the gradient in enzyme activity.

The two novel variants p.Asp377Tyr and p.Pro461Leu were identified in *cis* in patient 8. Functional studies show a reduction in enzyme activity to values similar to the very mild p.Pro482Ser for p.Asp377Tyr. This variant is located in a β-hairpin loop between β1-3 and β1-4 and is part of an important region which conserves close contacts between the N-terminal loop and the β-hairpin loop. For p.Leu461Pro, *in silico* studies did not predict any negative effect on protein function, however, this variant presented with the lowest enzyme activity (55% toward 17OHP) of all four novel variants studied in this report. A possible explanation is that the Leu461Pro change may interfere with the recognition and binding of the substrate as Leu461 is situated next to the residues Cys467 and Gly468, which are part of the distal region binding 17OHP [25].

The p.[Asp377Tyr;Leu461Pro] mutant protein exhibited an even lower activity (10%) (Table 2), indicating an additive or possibly a synergistic effect of multiple pathogenic variants. This allele can be associated with a NC phenotype, which was not possible to predict since the patient had the Val281Leu variant on the other allele. The *in silico* prediction was expecting a minor effect, but it should also be noted that the method for prediction was designed primarily for single-residue variants and need to be improved for predicting the effect of more than one variant. It is therefore evident, that while *in silico* prediction is useful when evaluating the impact of a novel variant, the functional studies are needed to accurately characterize the pathogenicity of a variant in detail.

5. Conclusions

We studied the functional consequences of four novel NC *CYP21A2* missense variants and of three *CYP21A2* alleles harbouring two variants each. All novel amino acid changes had mild effects when expressed alone. *In vitro* studies suggest an additive effect between mild pathogenic variants when identified together on the same allele, leading to a more severe phenotype. The residual activities of the combined alleles ranged from 17% to almost null, thus being associated with NC and classic forms of CAH. Altogether, the combination of clinical data, *in silico* prediction, and data from *in vitro* studies is important to establish a correct genotype phenotype correlation.

Statement of ethics

The study was approved by the Ethics Committees of the Universidade Estadual de Campinas, São Paulo, Brazil and Karolinska Institutet, Stockholm Sweden.

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Table 3
Summary of studies on combined *CYP21A2* alleles.

Combined *CYP21A2* allele	Clinical phenotype	*In vitro* activity 17OHP/prog double mutant	*In vitro* activity 17OHP/prog variant 1	*In vitro* activity 17OHP/prog variant 2	Other allele	Reference
p.[Pro30Leu;His62Leu[a]]	SV	ND	ND	ND	Pro30Leu; His62Leu or deletion/conversion	[26]
p.[His62Leu[a];Val69Leu]	CL	ND	ND	ND	NA	[31]
p.[His62Leu[a];Pro453Ser]	SV	4.1%/2.3%	44.5%/20.7%	38.2%/22.4%	IVS2-13A/C > G or insT + Gln318X	[13]
p.[Pro105Leu;Pro453Ser]	NC	1%/7%	52%/64%	68%/46%	Deletion/Large gene conversion	[9]
p.[Ile172Asn;Arg435Cys]	CL	ND	ND	ND	NA	[31]
p.[Val211Met;Val281Leu]	NC/SV	ND	99,5% for 17OHP	np	Deletion	[23]
p.[Ile230Thr[a];Val281Leu]	NC/SV	9.4%/8.5%	63.1%/70.6%	65.6%/63.4%	Large gene conversion	[14]
p.[Val281Leu;Leu353Arg]	SW	ND	ND	ND	Val281Leu;Leu353Arg	[24]
p.[Asp332Gly[a];Glu431Lys]	NC	2.1%/5.6%	41.6%/32.4%	26.2%/24.2%	Val281Leu	[27]
p.[Arg339His;Pro453Ser]	NC	ND	50%/20%	50%/20%	NA	[28]
p.[Gly375Ser;Pro453Ser]	Het carrier	0%/0%	1,6%/0,7%	ND	WT	[29]
p.[Gln481Pro;Prp482Ser]	SW	ND	ND	ND	IVS2-13A/C > G	[30]
p.[Ile172asn;Val358Ile[a]]	SW	< 1%/ < 1%	< 1%/ < 1%	72%/34%	See Table 1	This study
p.[Val281Leu;Arg369Gln]	NC	10%/9%	33%/26%	82%/63%	See Table 1	This study
p.[Asp377Tyr;Leu461Pro]	NC	17%/10%	81%/58%	55%/40%	See Table 1	This study

ND, not determined; NA, not available.
[a] Rare missense variant that is also reported alone in one allele.

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Declaration of Competing Interest

All authors stated that they have no conflicts of interest related to this work.

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