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The Spectrum of Genetic Defects in Congenital Adrenal Hyperplasia in the Population of Cyprus: A Retrospective Analysis

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ABSTRACT

Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency (21-OHD) is caused by mutations in the *CYP21A2* gene. The study refers to CAH patients of Greek-Cypriot ancestry between years 2007 and 2018. One hundred and twenty patients with various degrees of CAH were categorized and genotyped. The patients were categorized in 4 mutation groups based on their clinical and biochemical findings. The majority of patients (85.0 %) belonged to the non-classic (NC)-CAH form and the disorder was more often diagnosed in females (71.7 %). The most severe classic salt-wasting (SW) form was identified in 11 neonates (9.2 %). Seven (5.8 %) children were also identified with the simple virilizing (SV) form and a median presentation age of 5 years [interquartile range (IQR) 3.2–6.5]. In the 240 nonrelated alleles, the most frequent mutation was p.Val281Leu (60.0 %) followed by c.655 A/C > G (IVS2–13A/C > G) (8.8 %), p.Pro453Ser (5.8 %), DelEx1–3 (4.6 %), p.Val304Met (4.6 %), and p.Gln318stop (4.2 %). Other less frequent mutations including rare deletions were also identified. Following our recent report that the true carrier frequency of *CYP21A2* in Greek-Cypriots is 1:10, this study reports that the CAH prevalence is predicted around 1.7 cases per 10 000 people. Therefore, the up-to-date 120 CAH patients identified by our group make only the 6.9 % of the ones estimated (approximately 1750) to exist in the Greek Cypriot population. The compiled data from a coherent population such as the Greek-Cypriot could be valuable for the antenatal diagnosis, management and genetic counselling of the existing and prospect families with CAH.

Introduction

Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency (21-OHD) is the most common monogenic autosomal recessive disorder and results from molecular defects in the *CYP21A2* gene [1]. The disorder has a broad spectrum of clinical phenotypes that depend on the patients' underlying *CYP21A2* genotype [1, 2]. The deficiency is evident during foetal development and leads to varying degrees of prenatal virilization of the external genitalia in affected girls. The elucidation of the genetic background of CAH has been influential in the diagnosis and the classification of the disease [1, 3]. Nowadays, the disorder is classified into the severe or moderate forms that lead to the classic or non-classic (NC)-CAH form, respectively. Worldwide the estimated incidence of the classic form is 1:10 000 to 1:15 000, while the NC-CAH occurs in a frequency of 1:500 to 1:100 live births [4–7].

Several studies of the general population estimated the carrier incidence in the general population to be 1:25–1:10 [8–10]. To date, more than 200 mutations in the *CYP21A2* gene differing in incidence and severity have been described, however, only 10 of them account for about 95 % of the disease-causing alleles [1, 11].

A number of studies demonstrated strong association between genotype and phenotype and over the most recent years mutation detection rate led to the identification of a large number of *CYP21A2* defects [12, 13]. Since 2007 our group has extensively studied the genetic implication of *CYP21A2* in Cypriot patients with CAH. These included the true carrier frequency in the general population of Cyprus [10], the genotype-phenotype association with the various forms of CAH [14–18], the impact of heterozygosity in female patients with hyperandrogenemia [19, 20], and the implication of variants in the untranslated 5′UTR region of the *CYP21A2* gene [21]. In the present update, we present the molecular genetic features of the disease in patients of Cypriot descent over the last 11 years. Consequently, our purpose is to generate a beneficial tool for clinicians and geneticists necessary for the genetic counselling, accurate diagnosis and management of patients with 21-hydroxylase deficiency.

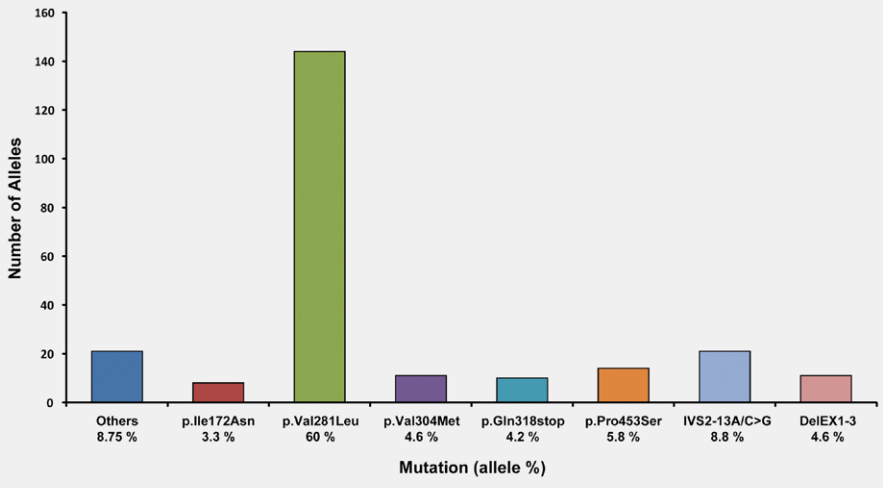
Patients and Methods

Clinical diagnosis, biochemical and genetic screening

One hundred and twenty Cypriot patients with various degrees of CAH were diagnosed with the disorder over a period of 11 years. The patients were categorized into the most severe salt-wasting (SW) form, the severe simple virilizing (SV) form and the mild NC-CAH form based on their clinical, biochemical and genetic findings. More specifically, patients with the SW form were initially allocated to this form based on clinical and biochemical findings of renal salt wasting [virilization, vomiting, failure to thrive, hyponatremia, hyperkalemia, high plasma renin activity (PRA), and significantly high 17-OH-Progesterone > 75 nmol/l] in the first month of their lives. The second group of patients categorized as having SV form also exhibited severe clinical symptoms of CAH without electrolyte imbalance (varying degree of virilization without any clinical evidence of salt loss, acceleration of growth velocity and bone age advancement, high 17-OH-Progesterone > 75 nmol/l, and normal or elevated PRA). On the contrary, patients with hyperandrogenism either in prepubertal or in peripubertal ages (premature pubic hair development, bone age advancement, severe acne and/or hirsutism, with or without menstrual irregularity, and complete lack of virilization with elevated 17-OHP levels) were allocated to the mild NC-CAH form. Genetic testing through Sanger sequencing and MLPA of the *CYP21A2* gene was performed according to a cascade strategy as previously described [10, 15].

Long range PCR for the detection *CYP21A2* rearrangements

Long range PCR was performed as previously described [22] for the detection of *CYP21A2* rearrangements. Briefly, PCR reactions were performed in 20 µl final volume using 50 ng of genomic DNA with final concentrations of 1X PrimeSTAR GXL Buffer, 125 nM of each primer, 200 µM dNTPs and 0.625 U of PrimeSTAR GXL DNA Polymerase (Takara Bio Inc., Shiga, Japan). The following PCR touch-down program was used: 98 °C for 3 min; 22 cycles of 98 °C for 10 s, 68 °C for 30 s (reduced 0.5 °C every cycle), and 68 °C for 6 min followed by



▶ **Fig. 1** Chart showing the percentage of mutations across the 120 CAH patients.

25 cycles of 98 °C for 10 s, 57 °C for 30 s, and 68 °C for 6 min with a final extension at 68 °C for 10 min, and a hold at 4 °C. Products were visualized on a 0.9 % agarose gel stained with RedSafe (iNtRON Biotechnology Inc., Seongnam, Korea). For the long range PCR the primers used were the ones used by Greene et al. [22]:

CYP21A2-F: 5′-CTTGCTTCTTGATGGGTGAT-3′
CYP21A2-R: 5′-AGGCGCTCGCTATGAGGTGAC-3′
CYP21P-F: 5′-TCCCCAATCCTTACTTTTTGTC-3′
CYP21P-R: 5′-GGACACAGAAACTCCAGGTGGGAGT-3′
CYP21A2 Ex3F: 5′-CTTGGGAGACTACTCCCTGC-3′
CYP21A2 Ex6R: 5′-CCTCAGCTGCATCTCCACGATGTGA-3′
CYP21P Ex3F: 5′-ACCTGTCGTTGGTCTCTGCTC-3′
CYP21P Ex6R: 5′-CCTCAGCTGCATCTCCACGATGTGA-3′

▶ **Table 1** Genotypes grouped according to predicted severity of involved mutations and phenotypes in 120 patients.

Group	Genotype	No of patients	Phenotype		
			SW	SV	NC
Null	p.Phe306insT + p.Val281Leu/p.Phe306insT + p.Val281Leu	1	1		
	IVS2–13A/C > G/p.Gln318stop	1	1		
	DelEX1–3/DelEx1–3	1	1		
	30 kb del/30 kb del	1	1		
	DelEx1–3/p.Gln318stop	1	1		
A	IVS2–13A/C > G/IVS2–13A/C > G	5	4	1	
	IVS2–13A/C > G/DelEx1–3	1	1		
	Partial conv with CYP21P:-4C > T, 92 C > T, 118 T > C, 138 A > C/DelEx1–3	1	1		
	IVS2–13A/C > G/Large del	1	1		
B	p.Ile172Asn/p.Ile172Asn	3		3	
	p.Ile172Asn/del CYP21A2	1		1	
C	p.Pro30Leu/p.Val281Leu	1		1	
	p.Pro30Leu/p.Pro30Leu	1		1	
	p.Val281Leu/p.Val281Leu	50			50
	p.Val281Leu/p.Pro453Ser	11			11
	p.Val281Leu/p.Val304Met	7			7
	p.Val281Leu/p.Gln318stop	5			5
	p.Val281Leu/p.Pro482Ser	3			3
	IVS2–13A/C > G/p.Val281Leu	7		3	4
	p.Val281Leu/p.Met283Val	1			1
	DelEX1–3/p.Val281Leu	3			3
	DelEX1–3/p.Val304Met	3			3
	p.Gln318stop/p.Pro453Ser	1			1
	p.Val304Met/p.Gln318stop	1			1
	p.Gln318stop/p.Pro482Ser	1			1
	p.Ile172Asn/p.Val281Leu	1			1
	IVS2–13A/C > G/p.Met283Val	1			1
	p.Pro453Ser/p.Pro453Ser	1			1
	p.Ile236Asn; p.Val237Glu; p.Met239Lys; p.Leu307frameshift/p.Val281Leu	2			2
	p.Val281Leu/30 kb del	2	1	1	
	p.Ile236Asn; p.Val237Glu; p.Met239Lys (Cluster E6)/p.Val281Leu	1			1
Total		120	13	11	96

Null group: Patients with mutations resulting in an enzyme with no activity. Group A: Patients who were homozygous or compound heterozygous for the IVS2–13A/C > G mutation which results in an enzyme with minimal residual activity. Group B: Mutations where the residual enzyme activity in in vitro expression experiments is about 2 %. Group C: Mutations that result in 30 to 60 % enzyme activity. SW: Salt-wasting; SV: Simple virilizing; NC: Non-classical.

▶ **Table 2** The type of the molecular defects with clinical and biochemical data in the patients with classic CAH.

	Genotype	Form	Sex	Age of diagnosis	Clinical phenotype	Hyponatremia Hyperkalemia	17-OHP nmol/l basal	ACTH <60 pg/ml	Renin PRA* ng/ml/h (0.2–2.8)
1	p.Pro30Leu/p.Pro30Leu	SV	F	6.5 years	Premature pubarche clitoromegaly	No	>75.7	76.4	0.4
2	IVS2–13A/C>G/IVS2–13A/C>G	SW	F	neonate	Prader 3	Yes	>75.7	1450	10.3
3	IVS2–13A/C>G/IVS2–13A/C>G	SW	F	neonate	Prader 3	Yes	>75.7	1355	9.4
4	IVS2–13A/C>G/IVS2–13A/C>G	SW	M	neonate	Adrenal crisis	Yes	>75.7	>2100	11.4
5	IVS2–13A/C>G/IVS2–13A/C>G	SW	M	neonate	Adrenal crisis	Yes	>75.7	>2100	10.7
6	IVS2–13A/C>G/IVS2–13A/C>G	SV	M	5.5 years	GnRH independent Precocious Puberty	No	43.7	282	1.23
7	Partial conv with CYP21P:-4C>T, 92C>T, 118T>C, 138A>C/delEx 1_3	SV	M	6.5 years	GnRH independent Precocious Puberty	No	>75.7	N/A	N/A
8	IVS2–13A/C>G/del Exons 1_3	SW	M	neonate	Adrenal crisis	Yes	>75.7	2352	9.8
9	IVS2–13A/C>G/Large del	SW	F	neonate	Ambiguous genitalia Prader 5	Yes	>75.7	103	3.1
10	IVS2–13A/C>G/p.Gln318stop	SW	F	neonate	Ambiguous genitalia	Yes	>75.7	N/A	32.3
11	p.Phe306insT + p.Val281Leu/p.Phe306insT + p.Val281Leu	SW	F	neonate	Prader 4	Yes	>75.7	>2100	12
12	30 kb del/30 kb del	SW	M	neonate	Adrenal crisis	Yes	>75.7	>2100	8.5
13	del Exons 1_3/del Exons 1_3	SW	M	neonate	Adrenal crisis	Yes	>75.7	>2100	10.5
14	del Exons 1_3/p.Gln318stop	SW	M	neonate	Adrenal crisis	Yes	>75.7	1680	11.3
15	p.Ile172Asn/del of *CYP21A2*	SV	M	neonate	Ambiguous genitalia	No	>75.7	569	4.7
16	p.Ile172Asn/p.Ile172Asn	SV	F	4.5 years	Ambiguous genitalia	No	>75.7	392	8.2
17	p.Ile172Asn/p.Ile172Asn	SV	M	5.0 years	Ambiguous genitalia	No	>75.7	38	4.7
18	p.Ile172Asn/p.Ile172Asn	SV	M	3.2 years	Ambiguous genitalia	No	>75.7	122	7.5

* PRA: Plasma renin activity.

Ethical approval

All procedures performed in studies involving human participants 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. Informed consent was obtained from all individual participants in the study.

Categorization in mutation groups

The *CYP21A2* disease-causing mutations were divided into 4 mutation groups according to a previous description [1]. The null group contained alleles with mutations resulting in an enzyme with no activity (classical known mutations: DelEx1–3, p.Phe306insT, and p.Gln318stop) [23–26]. Group A contained patients who were homozygous for the IVS2–13A/C>G splice-site mutation or were compound heterozygous with IVS2–13A/C>G and mutations from null group. The IVS2–13A/C>G splice site mutation is also known to result in an enzyme with minimal residual activity [27, 28]. Group B included patients homozygous for a less severe (p.Ile172Asn) or compound heterozygous with mutations from null or A group mutations, where the residual enzyme activity in in vitro expression experiments is about 2% [29]. Patients in the null, A, and B groups were described in detail in a recent publication by our group [18].

Group C contained patients who were homozygous for p.Val281Leu, or compound heterozygotes with p.Pro30Leu, DelEx1–3, p.Gln318stop, p.Pro482Ser, p.Val304Met, p.Met283Val, cluster E6, 30 kb del, and p.Pro453Ser. These mutations result in 30 to 60% enzyme activity [23, 30–32]. Genotypes categorized in groups null and A were predicted to result in SW CAH. Those in group B were expected to manifest as a SV phenotype, and those in group C as NC-CAH.

Statistical analyses

Using a binomial distribution approximation and the results of our lab from testing of healthy individuals in Phedonos et al. [10], we calculated the point estimate of the prevalence (and the exact 95% Confidence Interval) in the Cypriot population of 11 *CYP21A2* mutations identified in 45 patients.

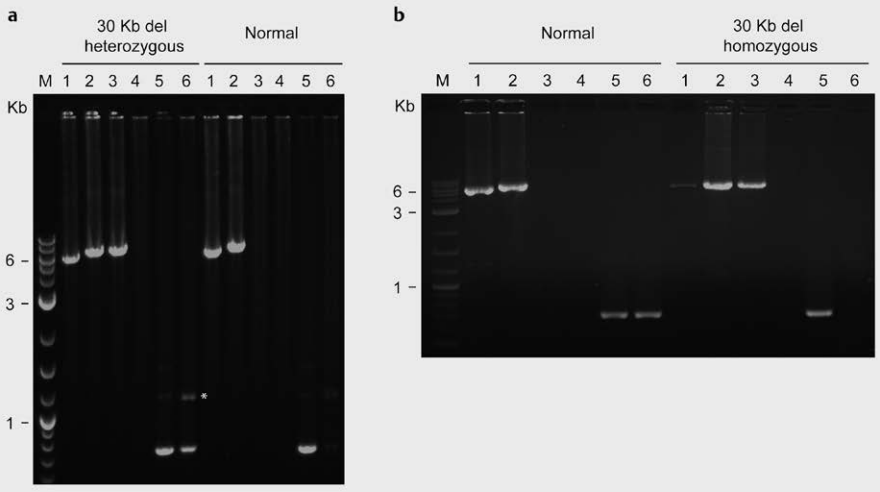


Figure Legend

M. 2-Log DNA Ladder

1. CYP21A2 gene (5.6 Kb)
2. CYP21P pseudogene (6.1 Kb)
3. CYP21AP/CYP21A2 chimeric gene - 30 Kb deletion (6.2 Kb)
4. CYP21A2/CYP21P rearrangement product (5.5 Kb)
5. CYP21A2 Ex3F - CYP21A2 Ex6R (control, ~0.8 Kb)
6. CYP21P Ex3F - CYP21P Ex6R (control, ~0.8 Kb)

▶ **Fig. 2** Long-range PCR in patients carrying the DelEX1–3 deletion. Examples of patients carrying the 30 Kb deletion in **a** heterozygous and in **b** homozygous state. M: 2-Log DNA Ladder; 1: Amplicon corresponding to the *CYP21A2* gene (primers: CYP21A2-F, CYP21A2-R); 2: Amplicon corresponding to the *CYP21A1P* pseudogene (primers: CYP21P-F, CYP21P-R); 3: Amplicon corresponding to the *CYP21A1P/CYP21A2* chimeric gene – 30 Kb deletion (primers: CYP21P-F, CYP21A2-R); 4: Amplicon corresponding to the *CYP21A2/CYP21A1P* rearrangement product (primers: CYP21A2-F, CYP21P-R); 5: Amplicon corresponding to *CYP21A2* Ex3F–*CYP21A2* Ex6R control reaction (primers: CYP21A2 Ex3F–CYP21A2 Ex6R); 6: Amplicon corresponding to *CYP21P* Ex3F–*CYP21P* Ex6R control reaction (CYP21P Ex3F–CYP21P Ex6R). The normal controls used in **a** and **b** are not the same individuals and that justifies the difference in banding formation as a result of the variability of the pseudogene observed in different individuals. Asterisk indicates a non-specific band.

Results

One hundred and twenty patients with CAH were categorized in four mutation groups (null, A, B, and C) based on genotype/phenotype correlations (▶ **Fig. 1**, ▶ **Table 1**). More specifically, mutations allocated in the null and Group A result in no and minimal residual enzymatic activity, respectively [23–26]. Mutations allocated to Group B usually exhibit residual enzymatic activity of about 2 % [27–29]. Finally, the mutations allocated to Group C typically result in 30–60 % enzymatic activity [29]. All 5 patients who belonged in the null group with no enzymatic activity manifested the full clinical picture of the SW form as expected. Eight out of nine patients in mutation group A exhibited the classical SW form and one presented the SV form. All four patients in group B had the SV form in accordance with the genotype p.Ile172Asn [18]. The majority of patients (85.0 %) categorized as group C exhibited the NC-CAH form with predominance in females (71.7 %) (▶ **Table 1**). The most severe form of CAH, the classic SW, was identified in 11 neonates (9.2 %) (▶ **Table 2**). Seven (5.8 %) children were also identified with the SV form at a median presentation age of 5 years (interquartile range (IQR) 3.2–6.5). The clinical presentation at diagnosis was considerably different between the SW and SV group, and also between males and females of the SW group. All 5 females with SW CAH presented with various degrees of external genitalia virilization accompanied by hyponatremia and hyperkalemia. All males with the SW CAH presented with adrenal crisis and all had hyponatremia, hyperkalemia, dehydration, and hypovolemic shock. The children belonging to the SV group had no electrolyte imbalance. Notably two girls and two boys from the SV group were born with ambiguous genitalia. The remaining three children from the same group exhibited GnRH independent precocious puberty (▶ **Table 2**).

The type of the molecular defects, clinical and biochemical data of the patients with classic CAH are shown in ▶ **Table 2**. The splice site mutation IVS2–13A/C > G in homozygosity was the most frequently detected genotype. Five out of eighteen patients with the classic SW form of CAH were found in homozygosity for the severe causing IVS2–13A/C > G splice mutant. The remaining thirteen patients had a combination of compound heterozygote genotypes belonging to the null and group A mutations (▶ **Table 2**). One patient affected with the SW form was associated with the rare genotype p.Phe306insT + p.Val281Leu/p.Phe306insT + p.Val281Leu. The same genotype was detected both on the paternal and the maternal alleles and upon review of the family consanguinity was noticed.

Using MLPA analysis and long range PCR (▶ **Fig. 2**) several deletions (DelEx1–3, del CYP21A2, Large del, 30 kb del) and a partial

conversion (Partial conv with CYP21P:-4C > T, 92 C > T, 118 T > C, 138 A > C) were identified. The DelEx1–3 was identified as the second most severe frequent defect and was detected in homozygosity or in the compound heterozygosity state in eleven patients with various degrees of severity (▶ **Fig. 1**; ▶ **Table 1** and ▶ **Table 2**).

In total, 17 different variants were identified and consisted of (a) eight (47.1 %) missense mutations, (b) one (5.9 %) nonsense mutation, (c) one (5.9 %) splicing mutation, (d) one (5.9 %) frameshift mutation, (e) one (5.9 %) deletion/insertion, (f) one (5.9 %) partial conversion, and finally (g) four (23.5 %) large deletions (▶ **Table 3**). The overall frequency of the identified molecular defects detected in our patients is also depicted in ▶ **Table 3**. In the 240 nonrelated alleles, the most frequent mutation was found to be p.Val281Leu (60.0 %) followed by the IVS2–13A/C > G (8.8 %), p.Pro453Ser (5.8 %), DelEx1–3 (4.6 %), p.Val304Met (4.6 %), p.Gln318stop (4.6 %), and p.Ile172Asn (3.3 %) (▶ **Fig. 1**). For the mutation p.Gln318stop, MLPA analyses verified that the patients of the present study carrying it are not associated with the rare haplotype of a duplicated *CYP21A2* gene with the mutation on one of the genes [33]. A series of 10 other less frequent and mostly severe mutations were identified and are also depicted in ▶ **Table 3**.

In ▶ **Table 4**, a binomial distribution approximation is used, in combination with the results from testing of 300 healthy individuals in Phedonos et al. [10]. The point estimate of the prevalence (and the exact 95 % Confidence Interval) for 11 mutations identified in 45 CAH patients is calculated. Given that none of the 300 healthy individuals tested had the mutation, the estimate of the prevalence is 0 % with a 95 % exact confidence interval between 0 % and 1.22 % (▶ **Table 4**).

▶ **Table 3** Mutation frequency of 240 affected alleles from 120 unrelated patients with 21-OHD.

Mutation *	Number of alleles	% of alleles	Reported frequencies (%) by Phedonos et al. 2013 in the Cypriot population
p.Pro30Leu	3	1.3	0
p.Ile172Asn	8	3.3	0
p.Val281Leu	144	60	4.33
p.Met283Val	2	0.8	0.17
p.Val304Met	11	4.6	0.83
p.Gln318stop	10	4.2	2.5
p.Pro453Ser	14	5.8	1.33
p.Pro482Ser	4	1.7	0.67
IVS2–13A/C > G (c.655 A/C > G)	21	8.8	0
DelEX1–3	11	4.6	0
p.Phe306insT + p.Val281Leu *	2	0.8	0
Partial conv with CYP21P:-4C > T, 92 C > T, 118 T > C, 138 A > C	1	0.4	0
del CYP21A2	1	0.4	0
Large del	1	0.4	0
p.Ile236Asn; p.Val237Glu; p.Met239Lys; p.Leu307frameshift	2	0.8	0
p.Ile236Asn; p.Val237Glu; p.Met239Lys (Cluster E6)	1	0.4	0
30 kb del	4	1.7	0
Total	240	100 %	9.83 %

* Mutation p.Phe306insT + pVal281Leu fall under the category of multiple mutations because they are found in *cis* on the same allele.

Discussion

Our data characterize an extensive description of the diverse clinical forms of CAH over time. From 2007 to 2018, 120 patients with various degrees of CAH were categorized and genotyped at the Molecular Genetics, Function and Therapy (MGFT) department of the Cyprus Institute of Neurology and Genetics. Calculations of the CAH prevalence in Cyprus were based on a recent report by our group regarding the estimated true *CYP21A2* carrier frequency (1:10) [10] in combination with the recent reported population of 701 000 Greek Cypriots by the Cyprus statistical service (http://www.mof.gov.cy/mof/cystat/statistics.nsf/populationcondition_21main_puparchive_en/populationcondition_21main_puparchive_en?OpenForm&yr=2016). Consequently, the estimated CAH prevalence is predicted to be around 1.7 cases per 10 000 people and the current 120 CAH patients identified by our group make only the 6.9 % of the ones estimated (approximately 1750) to exist in the Greek Cypriot population. Therefore, a significant number of patients suffering from the milder form of the disorder remain undiagnosed in our population. Early screening in combination with *CYP21A2* genetic analyses, enables clinicians to manage severe cases in neonatal period promptly even before the appearance of any electrolyte imbalance and/or urgent adrenal crisis. Currently, novel strategies on the management of prenatal treatment of CAH using fetal sex determination and dexamethasone have also been described, but remain a subject of debate [34]. In pregnant women at risk for carrying a fetus affected with CAH it is recommended obtaining prenatal therapy only through protocols approved by Institutional Review Boards at centers capable of collecting outcomes from adequately large number of patients [34]. The aim of prenatal treatment with dexamethasone aims to diminish female genital virilization and its associated risk of social stigma [35], to avoid the need for reconstructive surgery, and to relieve the emotional suffering and anxiety of the parents associated with the birth of a child with atypical sexual development [36].

As expected, the great majority of patients (85.0 %) were identified with the mild NC-CAH form, which was more frequently diagnosed in females (71.7 %) who presented with various degrees of hyperandrogenemia. Thus, the female dominance among the late-diagnosed patients is mainly the reason for the higher ratio of female patients in our cohort. Usually, there is a time interval be-

▶ **Table 4** Estimate of the prevalence in the Greek-Cypriot population of 11 *CYP21A2* mutations identified in 45 patients.

Mutation	Number of patients with disease: Tested, (n Positive for mutation, %)	Point estimate of mutation prevalence in patients with disease (95% exact CI)	Number of individuals without disease: Tested (n Positive for mutation, %)	Point estimate of mutation prevalence in the Greek-Cypriot population (95% exact CI)
p.Pro30Leu	45 (2, *4.4*)	4.4 (*1.0*, *15.0*)	300 (*0*, *0.0*)	0.0 (*0.0*, *1.22*)
p.Phe306insT + p.Val281Leu	45 (*1*, *2.2*)	2.2 (*0.0*, *12.0*)	300 (*0*, *0.0*)	0.0 (*0.0*, *1.22*)
p.Ile172Asn	45 (5, *11.1*)	11.1 (*4.0*, *24.0*)	300 (*0*, *0.0*)	0.0 (*0.0*, *1.22*)
DelEX1–3	45 (13, *28.9*)	28.9 (*16.0*, *44.0*)	300 (*0*, *0.0*)	0.0 (*0.0*, *1.22*)
IVS2–13A/C>G (c.655A/C>G)	45 (17, *37.8*)	37.8 (*24.0*, *53.0*)	300 (*0*, *0.0*)	0.0 (*0.0*, *1.22*)
Partial conv with CYP21P:-4C>T, 92C>T, 118T>C, 138A>C	45 (*1*, *2.22*)	2.2 (*0.0*, *12.0*)	300 (*0*, *0.0*)	0.0 (*0.0*, *1.22*)
del CYP21A2	45 (*1*, *2.22*)	2.2 (*0.0*, *12.0*)	300 (*0*, *0.0*)	0.0 (*0.0*, *1.22*)
Large del	45 (*1*, *2.22*)	2.2 (*0.0*, *12.0*)	300 (*0*, *0.0*)	0.0 (*0.0*, *1.22*)
p.Ile236Asn; p.Val237Glu; p.Met239Lys; p.Leu307frameshift	45 (*2*, *4.44*)	4.4 (*1.0*, *15.0*)	300 (*0*, *0.0*)	0.0 (*0.0*, *1.22*)
p.Ile236Asn; p.Val237Glu; p.Met239Lys (Cluster E6)	45 (*1*, *2.22*)	2.2 (*0.0*, *12.0*)	300 (*0*, *0.0*)	0.0 (*0.0*, *1.22*)
30 kb del	45 (*4*, *8.9*)	8.9 (*0.0*, *12.0*)	300 (*0*, *0.0*)	0.0 (*0.0*, *1.22*)

fore these patients contribute to the frequency [37]. A considerable percentage of our CAH patients were also assorted to the classic form of CAH. Up-to-date 11 (9.2%) neonates were identified with the SW form and 7 (5.8%) children with the SV form. As evidenced from the current and previous studies, the clinical presentation was significantly different between the SW and SV groups and also between males and females in the SW group [14, 15]. The female neonates with SW presented various degrees of genital virilization accompanied by hyponatremia and hyperkalemia and/or hypovolemic shock whereas all males exhibited hyponatremia, hyperkalemia, and/or hypovolemic shock only. None of the children in the SV group had any electrolyte imbalance regardless of the fact that two girls and two boys of the same group were born with ambiguous genitalia. Additionally, 3 children belonging to the SV group exhibited GnRH independent precocious puberty.

At this time, more than 200 mutations in the *CYP21A2* gene have been reported in numerous studies and there is a good concordance between the clinical phenotype and the patient genotypic findings [13, 16, 17, 21, 37–40]. The genetic population profile of CAH and endocrinopathies in the Cypriot population is characterized by low consanguinity rates and by similarities to what is observed regarding these disorders in the Eastern Mediterranean countries [41, 42]. The first reported human activity in Cyprus dates back to around the 10th millennium BC and the island was settled by Mycenaean Greeks in the second millennium BC. Since then the island of Cyprus experienced extended periods of Greek rule under the Ptolemaic Egyptians and the Byzantines followed by occupation from several major powers, including the empires of the Assyrians, Egyptians, Persians, Arabs, Lusignans, Venetians, and the Ottomans [41]. Up to date, a total of 17 different variants have been found in the Greek-Cypriot population and topologically are dispersed throughout the entire coding sequence of the *CYP21A2* gene. As expected the most frequent defect among the tested 240 alleles of the present study was the mild p.Val281Leu (60.0%) followed by a series of 16 other less frequent mutations. Therefore, in a similar fashion with studies performed in countries around the Mediterranean basin, the majority of NC-CAH alleles (>90%) were either homozygous for the p.Val281Leu or compound heterozygous to a mild/severe mutation [16, 43–49]. The mutations DelEx1–3 and 30 kb del have been identified in several patients of the present study. DelEx1–3 could be the result of a chimeric gene (CH1) generated by the 30 kb deletion. Previous studies reported different chimeric *CYP21A1P*/*CYP21A2* genes in Taiwanese and Italian populations [50, 51]. At the present study for DelEx1–3, we observe in our patients that their categorization into one of the reported chimera types cannot be clarified with the methods currently used in our lab, that is, Long Range PCR and MLPA.

In conclusion, the identified *CYP21A2* mutations of the present study differ in prevalence and severity even though the number of reported cases needs to be larger for more accurate assessment of prevalence rates. The molecular analysis confirmed the diagnosis of the different forms of CAH. Additionally, genotype-phenotype correlation was exceptional in all patients. However, differences in phenotypic presentation may appear possibly attributed to undefined factors modifying 21-OH gene expression. Knowing the genetic defects will be valuable for the antenatal diagnosis, management and genetic counselling of the existing and prospect families.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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