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A MULTIDISCIPLINARY BIOCHEMICAL AND GENETIC APPROACH FOR THE DIAGNOSIS OF CONGENITAL ADRENAL HYPERPLASIA: A PEDIATRIC CASE

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***ABSTRACT* – Objective:** This study aimed to demonstrate the potential of integrating genetic and biochemical approaches for the rapid and definitive diagnosis of congenital adrenal hyperplasia.

Case presentation: We report the case of a 5-year-old boy presenting with early puberty and significantly advanced bone age. Due to a strong suspicion of congenital adrenal hyperplasia, the patient underwent diagnostic evaluation incorporating both biochemical and genetic analyses.

Results: Biochemical analysis using ultra-performance liquid chromatography–tandem mass spectrometry revealed a marked accumulation of 17-hydroxyprogesterone, with the steroid profile confirming a pattern characteristic of 21-hydroxylase deficiency. Sanger sequencing identified the pathogenic variant c.515T>A (exon 4) in a heterozygous state, while multiplex ligation-dependent probe amplification detected a heterozygous deletion of exons 1, 3, 4, 6 and 7 in the *CYP21A2* gene. Parental segregation analysis showed that the c.515T>A variant was inherited from the father, whereas the large heterozygous deletion originated from the mother.

Conclusions: A multidisciplinary approach integrating biochemical and genetic analyses enables a definitive and early diagnosis of congenital adrenal hyperplasia, facilitating appropriate treatment and preventing serious or fatal complications associated with the classic form of the disease.

Keywords: Congenital Adrenal Hyperplasia, 17-hydroxyprogesterone, Deletion.

List of abbreviations: 4-A: 4-androstene-3,17-dione; 11-DC: 11-deoxycortisol; 17-OHP: 17-hydroxyprogesterone; 21-OHD: 21-hydroxylase deficiency; ACTH: adrenocorticotropic hormone; CAH: Congenital Adrenal Hyperplasia; CORT: cortisol; DBS: dried blood spot; LC-MS/MS: liquid chromatography with tandem mass spectrometry; MLPA: multiplex ligation-dependent probe amplification; SV: Simple Virilizing; SW: Salt-Wasting; UPLC-MS/MS: ultra-performance liquid chromatography with tandem mass spectrometry.

INTRODUCTION

Congenital adrenal hyperplasia (CAH) is a monogenic autosomal recessive disorder caused by defective adrenal steroid hormone synthesis due to enzyme deficiencies or malfunctions in the steroidogenic pathway[1].

Steroid 21-hydroxylase deficiency (21-OHD) is the most common cause of CAH, accounting for at least 90% of cases[2,3]; however, other enzymes in the same biochemical pathway may also be implicated (Table 1)[4].

Table 1. Biochemical pathways involved in different forms of CAH.

Percentage of CAH	Deficient Enzyme	Substrate	Product	Androgen	Mineralo-corticoid
Unknown	Steroidogenic acute regulatory protein	–	Mediates cholesterol transport across the mitochondrial membrane	Deficiency	Deficiency
Unknown	3β-hydroxysteroid-dehydrogenase	Pregnenolone,17-OH pregnenolone, Dehydroepian-drosterone	Progesterone, 17-OHP,Δ -androstenedione	Deficiency	Deficiency
Unknown	17α-hydroxylase	Pregnenolone Progesterone	17-OH pregnenolone 17-OH (17-OHP)	Deficiency	Excess
>90%	21-hydroxylase	Progesterone 17-hydroxy progesterone	Deoxycorticosterone 11-deoxycortisol	Excess	Deficiency
5%	11β-hydroxylase	Deoxycorticosterone	Corticosterone	Excess	Excess

Abbreviations – 17-OH: 17-hydroxy; 11-DC: 11-deoxycortisol; 17-OHP: 17-hydroxyprogesterone; 21-OHD: 21-hydroxylase deficiency. Source: Nimkarn et al[5]. Reproduced with permission from GeneReviews® (© 1993-2025 University of Washington).

The *CYP21A2* gene encodes the 21-hydroxylase enzyme, and point mutations, deletions/duplications, or gene conversions involving its highly homologous pseudogene (*CYP21A1P*) can result in enzyme deficiency or dysfunction, leading to CAH with variable clinical phenotypes (**Table S1**).

The disease exhibits a wide spectrum of clinical severity (Table 2). The classic form presents prenatally with severe enzyme deficiency and is further classified into the salt-wasting (SW) and simple virilizing (SV)

Table 2. Mean levels and range of response of paternal engagement.

Enzyme activity	Phenotype	CYP21A2 pathogenic variant
0%	Severe (classic)	• Whole-gene deletion (null variant) • Large-gene conversion • p.Gly111ValfsTer21 • p.[Ile237Asn;Val238Glu;Met240Lys] • p.Leu308PhefsTer6 • p.Gln319Ter • p.Arg357Trp
<1%1		• c.293-13A>G • c.293C>G
2–11%		• p.Ile173Asn
~20–50%	Mild (non-classic)	• p.Pro31Leu • p.Val282Leu • p.Pro454Ser

Source: Nimkarn et al5. Reproduced with permission from GeneReviews® (© 1993-2025 University of Washington).

subtypes. The most severe SW form affects approximately 25% of individuals and is characterized by adrenal insufficiency, often leading to hypovolemic shock, hyponatremia, hyperkalemia, metabolic acidosis, and, in some cases, hypoglycemia. The less severe SV form accounts for ≥75% of affected individuals and is typically associated with hyperandrogenism, premature adrenarche/puberty, apocrine odor, clitoromegaly, rapid growth and accelerated skeletal maturation, which may compromise final height[1,5].

The non-classic (NC) form has a postnatal onset and is associated with the mildest enzyme deficiency. The most common reason for medical consultation in these patients is late-onset hyperandrogenism, which typically manifests during the peri-pubertal stage or adulthood. In adolescence, frequent clinical concerns include acne, hirsutism, and oligomenorrhea, a condition that may be clinically indistinguishable from polycystic ovary syndrome. Some affected individuals also exhibit polycystic ovarian morphology[6].

The initial diagnosis of CAH is typically based on plasma 17-hydroxyprogesterone (17-OHP) levels. Measurement techniques include immunoassays and liquid chromatography–tandem mass spectrometry (LC-MS/MS). In borderline cases, an adrenocorticotropic hormone (ACTH) stimulation test (250 µg intramuscularly or intravenously) is recommended to confirm the diagnosis. In our laboratory, for LC-MS/MS plasma measurements, 17-OHP reference range in pediatric population are 0.03–2.65 ng/mL.

When classic CAH is suspected, additional adrenal steroids, including cortisol, aldosterone, androstenedione, and dehydroepiandrosterone sulfate, should be assessed. The mineralocorticoid axis should also be evaluated by measuring plasma renin and plasma electrolytes. Genetic testing is strongly recommended for all cases in which biochemical findings suggest CAH and is particularly valuable for confirming the diagnosis in complex cases[6].

For molecular analysis, Sanger sequencing combined with multiplex ligation-dependent probe amplification (MLPA) is commonly used in many laboratories to detect single nucleotide variations, small insertions/deletions (indels), and large deletions/duplications in the *CYP21A2* gene[7,8].

The combination of biochemical and genetic analyses is essential for an accurate and rapid diagnosis of CAH. In this study, we report a case with a high suspicion of CAH to demonstrate the potential of integrating genetic and biochemical approaches for a definitive and early diagnosis. Furthermore, parental segregation analysis proved useful in refining the patient's genotype and determining the specific CAH subtype.

MATERIALS AND METHODS

Genetic and biochemical analyses were performed using peripheral blood and dried blood spot (DBS) samples collected from the patient and parents at the Pediatric Clinic of *SS. Annunziata* Hospital in Chieti, Italy.

DBS samples were analyzed using an ultra-performance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS) system to evaluate blood levels of cortisol (CORT), 21-deoxycortisol, 11-deoxycortisol (11-DC), 4-androstene-3, 17-dione (4-A), and 17-OHP[9]. Additionally, the plasma steroid profile was quantified by UPLC-MS/MS, measuring CORT, 11-DC, 4-A, 17-OHP, testosterone, progesterone, corticosterone, and aldosterone using the CHS™ MSMS Steroids Kit (Revvity®, Turku, Finland). The details for quantitative determination in the plasma samples of steroid profile by UPLC-MS/MS are fully described in **Supplementary Materials** and specifically reported in **Tables S2–S3**. The diagnosis of CAH was confirmed through genetic analysis. Genomic DNA was extracted from peripheral blood using the MagPurix instrument and the Whole Blood DNA Extraction Kit (Zinexts Life Science Corp., Ref: ZP01001, New Taipei City, Taiwan) following the manufacturer's protocol. Sanger sequencing and MLPA were employed to identify point mutations within the *CYP21A2* gene and large rearrangements involving *CYP21A2* and its pseudogene, *CYP21A1P*. Sanger sequencing was conducted using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA) and the SeqStudio™ Genetic Analyzer System (Thermo Fisher Scientific, Waltham, MA, USA). MLPA was performed using the SALSA® MLPA® probemix P050-D1 CAH kit (MRC-Holland, Amsterdam, The Netherlands) and analyzed on a 3500 Series Genetic Analyzer (Applied Biosystems, Foster City, MA, USA). Copy number variations in *CYP21A2* exons 1, 3, 4, 6 and 7 were assessed using Coffalyser® software (MRC-Holland, Amsterdam, The Netherlands).

This case report does not require an Ethics Committee approval, as per national law. Written informed consents were obtained from parents to perform procedures and describe the case in the literature.

Case Presentation

We report the case of a 5-year-old male proband presenting with early puberty (P3G3, testicular volume: 3 mL) and significantly advanced bone age of 10 years, as assessed by the Greulich-Pyle method. At the time of his initial evaluation, the proband's height was 125.2 cm, weight 35 kg, and BMI 22.3 kg/m². To assess the hypothalamic-pituitary-gonadal axis, a luteinizing hormone-releasing hormone stimulation test was performed, revealing follicle-stimulating hormone and luteinizing hormone (LH) levels of 2.4 mUI/mL and 4.3 mUI/mL at 60 minutes, respectively, with an LH/follicle-stimulating hormone ratio favoring LH (**Table S4**). Serum 17β-estradiol was undetectable (<10 pg/mL), while testosterone and prolactin levels were 1.19 ng/mL and 21.6 ng/mL, respectively. Further endocrine evaluation showed elevated ACTH and dehydroepiandrosterone sulfate levels (52.4 pg/mL and 58 pg/mL, respectively), both exceeding the normal range for age and sex. Additionally, ACTH stimulation test revealed markedly increased baseline 17-OHP levels (>20 ng/mL) and a decrease in cortisol, with no significant increment post-stimulation (**Table S5**). Plasma renin activity was also elevated (168.3 µU/mL). A complete blood count, as well as liver, renal, and thyroid function tests, were within normal limits for age. These findings strongly suggested CAH, consistent with the non-classic form of 21-OHD.

Biochemical and genetic analyses were conducted in our laboratory. The biochemical evaluation revealed a marked accumulation of 17-OHP, and the steroid profile confirmed the diagnosis of CAH with a pattern characteristic of 21-OHD. The DBS and plasma steroid concentrations of the patient are presented in Tables 3 and 4, respectively. For molecular analysis, Sanger sequencing identified the pathogenic variant c.515T>A in exon 4 of the *CYP21A2* gene in a homozygous state, suggesting an SV form of CAH (Figure 1A). MLPA analysis detected a heterozygous deletion of exons 1, 3, 4, 6, and 7 in *CYP21A2* (Figure 1B).

Table 3. Dried blood spot steroid concentrations of analytes and the related cut-offs to which our laboratory refers, based on a neonatal population.

Analyte	Value	Cut-off
17-OHP	84.39 ng/mL	3.60 ng/mL
11-deoxycortisol	0.21 ng/mL	7.65 ng/mL
21-deoxycortisol	8.91 ng/mL	1.80 ng/mL
4-A	3.52 ng/mL	–
Cortisol	10.51 ng/mL	–
(17OHP+4A)/cortisol	8.63 ng/mL	1.00 ng/mL

Abbreviations – 4-A: 4-androstene-3,17-dione; 11-DC: 11-deoxycortisol; 17-OHP: 17-hydroxyprogesterone.

Table 4. Plasma steroid concentrations of analytes and the related cut-offs to which our laboratory refers, based on a pediatric population.

Analyte	Value	Cut-off
17-OHP	149.96 ng/mL	0.03–2.65 ng/mL
11-deoxycortisol	0.32 ng/mL	0.10–1.56 ng/mL
4-A	4.18 ng/mL	0.06–2.60ng/mL
Cortisol	22.45 ng/mL	10–330 ng/mL
Testosterone	1.37 ng/mL	0.03–9.70 ng/mL
Progesterone	4.88 ng/mL	0.07–12.94 ng/mL
Corticosterone	1.78 ng/mL	0.80–18.60 ng/mL
Aldosterone	0.30 ng/mL	0.05–0.90 ng/mL

Abbreviations – 4-A: 4-androstene-3,17-dione; 11-DC: 11-deoxycortisol; 17-OHP: 17-hydroxyprogesterone.

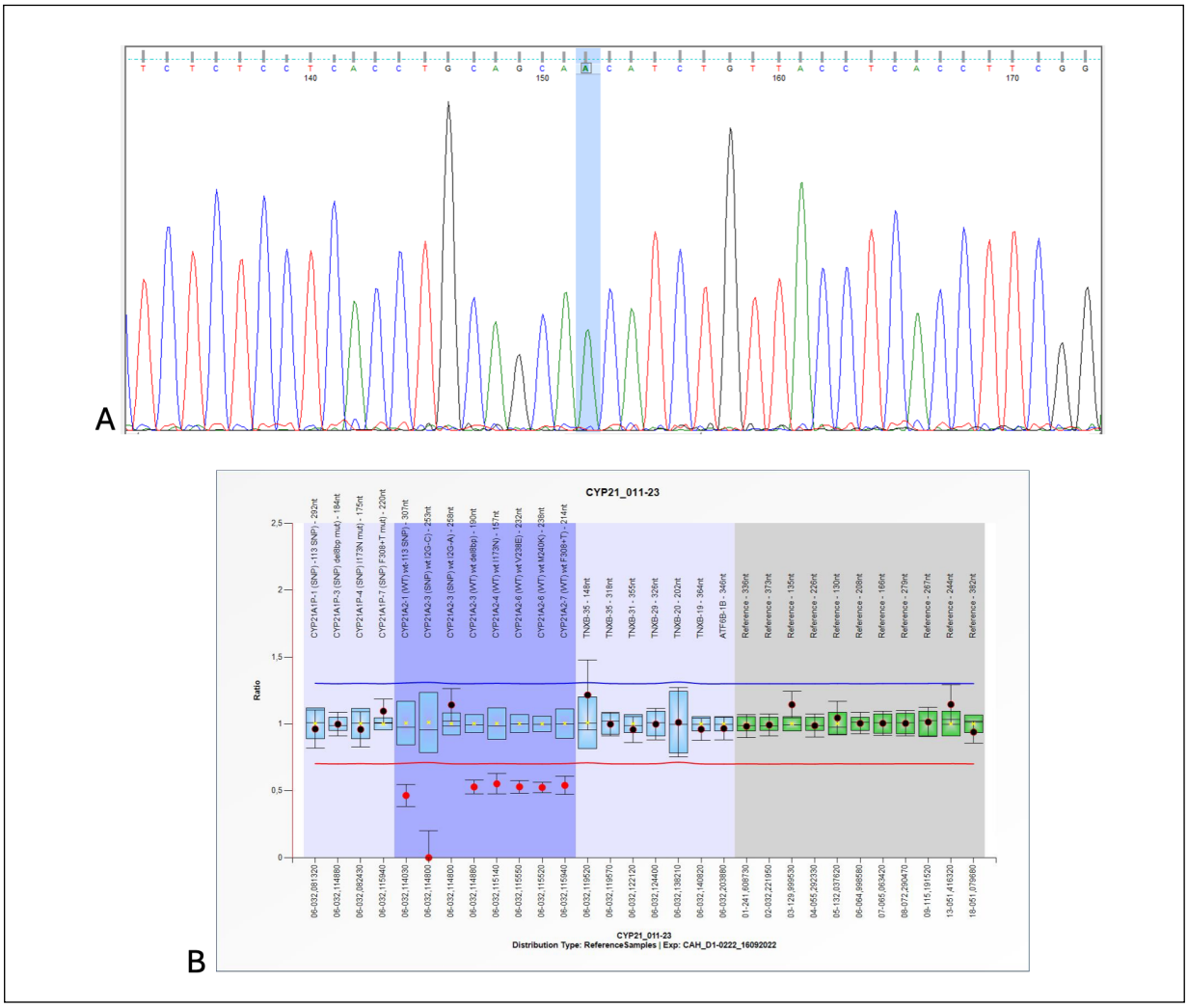

Figure 1. *A*, Sanger Sequencing showed the variant c.515T>A in exon 4 of the CYP21A2 gene in homozygous state in proband; *B*, MLPA analysis detected a heterozygous deletion of exons 1, 3, 4, 6, and 7 in CYP21A2 gene in proband. *Source*: Original.

To further characterize the proband's genotype, parental segregation analysis was performed. MLPA and gene sequencing of the parents revealed that the father carried the c.515T>A variant in a heterozygous state, while the mother harbored a large heterozygous deletion of exons 1, 3, 4, 6, and 7 in the *CYP21A2* gene (Figure 2A and 2B).

As a result, the proband's genotype was determined to be compound heterozygous for c.515T>A and large deletion (c.515T>A/del), consistent with the SV form of CAH.

DISCUSSION

Our patient presented with early puberty and significantly advanced bone age. Blood tests revealed elevated basal 17-OHP levels, which further increased after ACTH stimulation, a biochemical profile initially suggestive of the non-classic form of 21-OHD. Given the strong suspicion of CAH, additional biochemical and genetic investigations were conducted. DBS analysis demonstrated a marked accumulation of 17-OHP, and the steroid profile obtained via UPLC-MS/MS confirmed the diagnosis of 21-OHD, displaying a characteristic steroidogenic pattern of this congenital disorder. Genetic analysis identified a *CYP21A2* genotype (c.515T>A/del), consistent with the classic SV form of CAH. Sequencing analysis revealed that the proband was a compound heterozygote for a severe missense mutation (c.515T>A; p.Ile172Asn) inherited from the father and a large deletion (exons 1, 3, 4, 6 and 7) inherited from the mother. Initial-

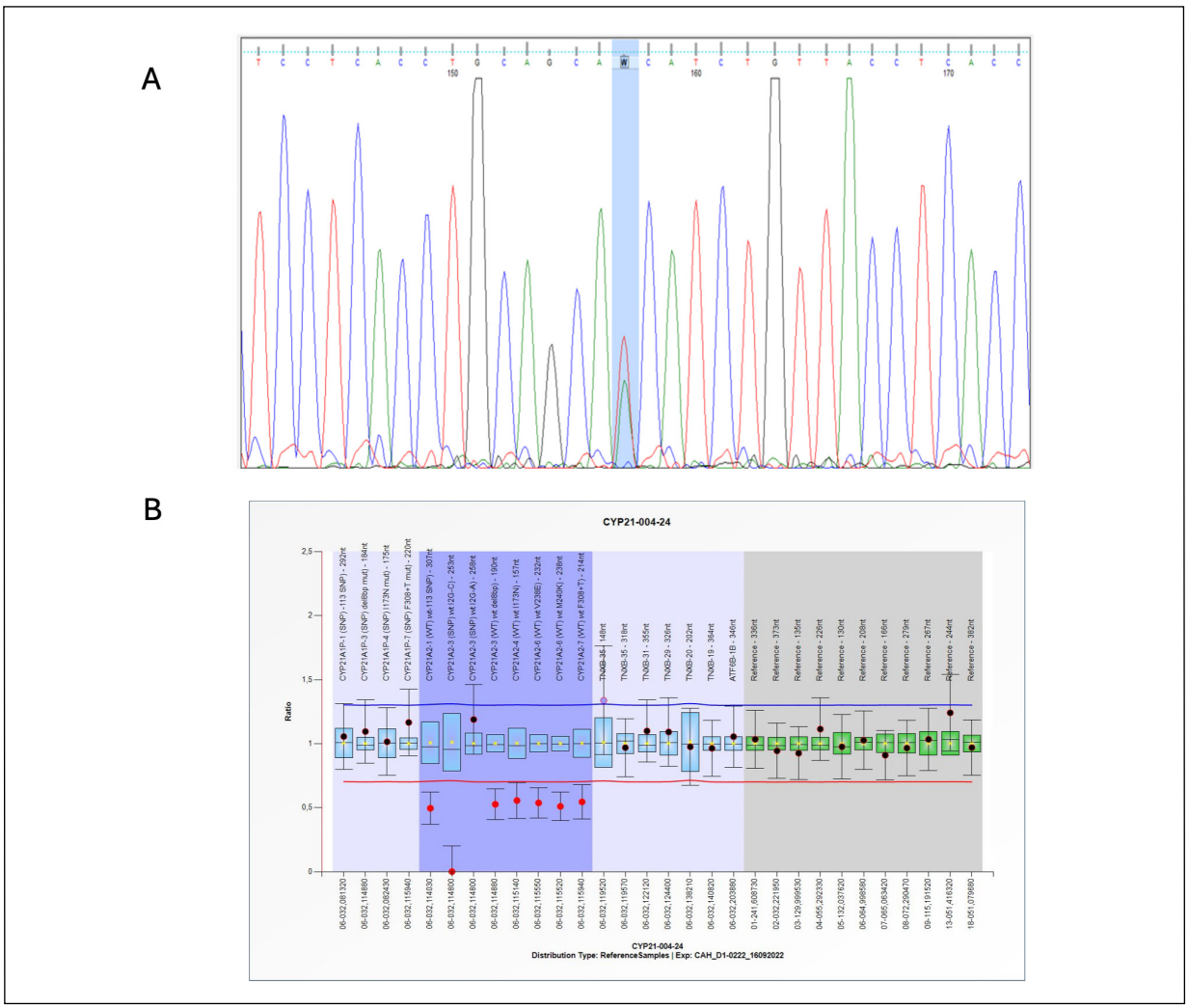

Figure 2. *A*, Sanger Sequencing identified the variant c.515T>A in exon 4 of the CYP21A2 gene in heterozygous state in proband's father; *B*, MLPA analysis detected a heterozygous deletion of exons 1, 3, 4, 6, and 7 in CYP21A2 gene in proband's mother. *Source*: Original.

ly, genetic analysis erroneously detected a homozygous c.515T>A genotype in the proband; however, parental segregation analysis clarified the correct heterozygous genotype. Indeed, due to the presence of heterozygous deletion, Sanger sequencing amplified only one allele, resulting in a false homozygous. The c.515T>A variant results in the substitution of isoleucine with asparagine at position 172 of the encoded protein (p.Ile172Asn). The large *CYP21A2* deletion (exons 1, 3, 4, 6, and 7) has been previously reported in the literature[10] but has not been documented in compound heterozygosity with the c.515T>A missense variant.

According to the EMQN best practice guidelines[11], this variant is classified as pathogenic and is responsible for the SV form of CAH, with approximately 1–2% residual 21-hydroxylase activity when present in homozygosity or compound heterozygosity. This finding contrasts with the initial clinical suspicion of non-classic CAH. Based on the confirmed diagnosis, the patient was started on hydrocortisone therapy (10 mg/m²/day) in three divided doses to centrally suppress adrenal androgen excess. The primary therapeutic goal was to stabilize bone age while ensuring normal growth. After 1 year of treatment, bone age advanced only slightly, and pubertal progression remained unchanged. This case underscores the importance of timely trio analysis and a multidisciplinary approach involving clinical, biochemical, and genetic evaluation for the accurate diagnosis of CAH and the implementation of targeted therapy.

Based on our review of the literature, the integration of biochemical and genetic analyses has been consistently demonstrated as essential for accurately identifying the specific form of CAH[12,13].

For example, Anastasovska et al[13] reported a case of a 14-day-old infant presenting with electrolyte imbalance and suspected SW CAH. Biochemical analysis revealed elevated 17-OHP, ACTH and testosterone levels. Genetic testing subsequently confirmed the diagnosis by identifying a genotype associated with the SW form. Similarly, Nasir et al[12] described a case of a 4-year-old boy born to consanguineous parents who initially presented with hyponatremia and hyperkalemia, raising suspicion of SW CAH. Further biochemical investigations showed increased 17-OHP and ACTH levels, while molecular testing identified compound heterozygosity consistent with the non-classic form, differing from the initial clinical suspicion. These cases underscore the critical role of combining biochemical and genetic analyses to accurately classify CAH subtypes and guide appropriate management. In this paper, we highlighted the importance of CAH screening for the early diagnosis of presymptomatic diseases. If the multidisciplinary approach is correctly applied, as described in the work, it could play a fundamental role. The synergistic combination of biochemical and genetic analysis allows for a timely diagnosis, favoring a rapid initiation of therapy. This process not only improves the patient's quality of life, but also reduces stress and anxiety for the family during the period of diagnostic uncertainty.

CONCLUSIONS

A multidisciplinary approach incorporating clinical, biochemical, and genetic analyses, supported by parental segregation, facilitates the early and accurate determination of CAH subtypes in cases of diagnostic uncertainty. This enables the implementation of appropriate therapies and helps prevent serious or potentially fatal complications associated with the classic form of CAH.

Therefore, our study contributes to expanding the existing literature and enhances the understanding of the biochemical and genetic aspects of congenital adrenal hyperplasia.

Artificial intelligence-assisted technologies:
No artificial intelligence-assisted technologies were used in the production of this article.

Authors' contributions:
Study conception and design: Marianna Ranaudo, Anastasia Dell'Elice; collection and interpretation of data: Marianna Ranaudo, Mirco Zucchelli, Maria Lucia Tommolini, Angelika Mohn; manuscript drafting: Marianna Ranaudo, Anastasia Dell'Elice, Aurora Navicella, Mirco Zucchelli, Maria Lucia Tommolini; manuscript editing: Aurora Navicella, Maria Minelli, Rossella Ferrante; approval to submit: Valentina Gatta.

Availability of data and material:
All data generated or analysed during this study are included in this published article or its supplementary material.

Conflicts of interest:
The authors declare that they have no conflict of interest to disclose.

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This case report does not require an Ethics Committee approval, as per national law.

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REFERENCES

1. Baranowski ES, Arlt W, Idkowiak J. Monogenic disorders of adrenal steroidogenesis. Horm Res Paediatr 2018; 89: 292-310.
2. Forest MG. Recent advances in the diagnosis and management of congenital adrenal hyperplasia due to 21-hydroxylase deficiency. Hum Reprod Update 2004; 10: 469-85.
3. Speiser PW, Azziz R, Baskin LS, Ghizzoni L, Hensle TW, Merke DP, Meyer-Bahlburg HF, Miller WL, Montori VM, Oberfield SE, Ritzen M, White PC; Endocrine Society. Congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab 2010; 95: 4133-4160.
4. Wedell A. An update on the molecular genetics of congenital adrenal hyperplasia: diagnostic and therapeutic aspects. J Pediatr Endocrinol Metab 1998; 11: 581-590.
5. Nimkarn S, Gangishetti PK, Yau M, New MI. 21-Hydroxylase-Deficient Congenital Adrenal Hyperplasia. 2002 Feb 26 [updated 2016 Feb 4]. In: Adam MP, Feldman J, Mirzaa GM, Pagon RA, Wallace SE, Amemiya A, editors. GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993–2025.
6. Uslar T, Olmos R, Martínez-Aguayo A, Baudrand R. Clinical update on congenital adrenal hyperplasia: recommendations from a multidisciplinary adrenal program. J Clin Med 2023; 12: 3128.
7. Lee HH, Lee YJ, Lin CY. PCR-based detection of the CYP21 deletion and TNXA/TNXB hybrid in the RCCX module. Genomics 2004; 83: 944-950.
8. Sørensen KM, Andersen PS, Larsen LA, Schwartz M, Schouten JP, Nygren AO. Multiplex ligation-dependent probe amplification technique for copy number analysis on small amounts of DNA material. Anal Chem 2008; 80: 9363-9368.
9. Ferrante R, Tumini S, Saltarelli MA, Di Rado S, Scorrano V, Tommolini ML, Zucchelli M, Lauriola F, Lisi G, Lauriti G, Marino N, Stuppia L, Rossi C, Bucci I. A very early diagnosis of complete androgeninsensitivitysyndrome due to a novelvariant in the AR gene: a neonatal case study. Biomedicines 2024; 12: 1742.
10. Xi N, Song X, Wang XY, Qin SF, He GN, Sun LL, Chen XM. 2+0 CYP21A2 deletion carrier - a limitation of the genetic testing and counseling: A case report. World J Clin Cases 2021; 9: 6789-6797.
11. Baumgartner-Parzer S, Witsch-Baumgartner M, Hoeppner W. EMQN best practice guidelines for molecular genetic testing and reporting of 21-hydroxylase deficiency. Eur J Hum Genet 2020; 28: 1341-1367.
12. Nasir H, Ali SI, Haque N, Grebe SK, Kirmani S. Compound heterozygosity for a whole gene deletion and p.R124C mutation in CYP21A2 causing nonclassic congenital adrenal hyperplasia. Ann Pediatr Endocrinol Metab 2018; 23: 158-161.
13. Anastasovska V, Kocova M, Zdraveska N, Stojiljkovic M, Skakic A, Klaassen K, Pavlovic S. A novel 9 bp deletion (c.1271_1279del-GTGCCCGCG) in exon 10 of CYP21A2 gene causing severe congenital adrenal hyperplasia. Endocrine 2021; 73: 196-202.