MOLECULAR-GENETIC CAUSES UNDERLYING PRIMARY ADRENAL INSUFFICIENCY: CURRENT INSIGHTS INTO DIAGNOSIS AND TREATMENTS

EDITED BY: Liliana Dain, Tania Bachega and
Maria Candida Barisson Villares Fragoso

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Topic Editors:
Liliana Dain, Centro Nacional de Genética Médica, Argentina
Tania Bachega, University of São Paulo, Brazil
Maria Candida Barisson Villares Fragoso, University of Sao Paulo, Chief of Adrenal Unit, Hospital das Clinicas da Universidade de Sao Paulo, Institute of Cancer of Sao Paulo, Brazil

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EDITED AND REVIEWED BY
Ralf Jockers,
Universitéde Paris, France

*CORRESPONDENCE
Maria Candida B.V. Fragoso
maria.villares@hc.fm.usp.br
Tânia A. S. S. Bachega
tbachega@usp.br
Liliana Dain
ldain@fbmc.fcen.uba.ar

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Editorial: Molecular -genetic causes underlying primary adrenal insufficiency: Current insights into diagnosis and treatment

Maria Candida B.V. Fragoso[1]*, Tânia A. S. S. Bachega[1]* and Liliana Dain[2,3]*

[1]Unidade de Adrenal, Disciplina de Endocrinologia, Laboratório de Hormônios e Genética Molecular (LIM 42), Hospital das Clínicas, São Paulo University, São Paulo, Brazil, [2]Buenos Aires University, Departamento de Diagnóstico Genético, Centro Nacional de Genética Médica- ANLIS, Buenos Aires, Argentina, [3]Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

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Editorial on the Research Topic

Molecular -genetic causes underlying primary adrenal insufficiency: Current insights into diagnosis and treatments

The loss of adrenal cortex function leads to glucocorticoid and/or mineralocorticoid deficiency, ranging from mild nonspecific symptoms to life-threatening shock conditions. Adrenal insufficiency (AI) is classified as primary, secondary or tertiary when the disease results from disorders affecting the adrenal cortex, anterior pituitary or hypothalamus, respectively. In newborns and children, genetic factors are the most frequent causes of AI, with emphasis on congenital adrenal hyperplasia, while acquired etiologies are more frequent in adults.

The diagnosis of all forms of AI is usually delayed because the initial presentation is often non-specific; despite significant advances in knowledge over the last decade, the diagnosis and management of adrenal insufficiency still represent a challenge for physicians, researchers, and also for patients. Moreover, the presence of genetic conditions resulting in AI is often underestimated in clinical practice and, consequently, leads to significant impairment of patients' quality of life. A relevant point is the need of special attention to patients with latent AI in order to prevent an adrenal crisis during stress conditions. Several studies have showed an increased morbidity and mortality rate in AI patients; therefore, prevention is of fundamental importance. The continuous education of both medical teams and patients/relatives on AI and the management of adrenal crisis is necessary to improve clinical outcomes. Recent studies have focused on developing new types of steroid

replacements to mimic the rhythm of cortisol secretion and function, as well as to decrease the metabolic adverse outcomes related to long-term therapy. Further advances in steroid replacements, oral and parenteral, will probably emerge in the coming years.

In this regard, in this issue Younes et al., presented a comprehensive review of the etiologies, diagnosis, and treatments of chronic and acute Primary AI. Management of AI in times of COVID-19 outbreak was also addressed in a promising article of Sabaddin et al., as patients are facing their primary disease and the risk/fear of COVID-19 infection. An especial attention was focused in this issue to Congenital Adrenal Hyperplasia (CAH) a group of autosomal recessive enzymatic disorders, caused by a deficiency of one of the enzymes required for cortisol biosynthesis in the adrenal cortex. CAH secondary to 21-hydroxylase deficiency is the most common form of CAH. Carrozza et al., Arriba et al., Kocova et al., and Marino et al., outstanding reviewed the current state of the art of this deficiency and its related condition CAH-X (CAH and Ehlers-Danlos Syndrome), ranging from the genetic and the molecular complexity of the locus, to its molecular diagnosis and management. Kim et al., drew attention to the increased prevalence of cardiometabolic risk factors in patients with CAH, claiming that a better understanding of the traditional and non-traditional risk factors in youth with CAH could help guide treatment options and prevent the onset of metabolic syndrome in adulthood, reducing overall patient morbidity. In addition, Ladjouze et al., added their experience in Algeria with the 3β-hydroxysteroid dehydrogenase type 2 deficiency, a rare cause of CAH with an estimated birth prevalence less than 1/1.000.000 and with fewer than 200 families reported worldwide.

Congenital disorders affecting adrenal function may also be associated with diseases of sex development as Finkielstain et al., addressed it in an excellent review article in this topic. Finally, Teoli et al., also described the impact of *NR0B1* (*DAX1*) genetic variants on clinical, hormonal, histological, spermiological aspects and gonadotropin treatment response in male patients with X-linked adrenal hypoplasia congenita (X-AHC).

To conclude, the purpose of this Research Topic was to compile in a single issue the most recent state of the art and new insights on AI, bringing together a comprehensive compendium of etiologies, diagnosis and treatments of the different AI disorders, based on the excellent contributions of the expert authors in the area.

Author contributions

MF, TB, and LD contributed to the conceptualization and writing of the Editorial. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Genes and Pseudogenes: Complexity of the RCCX Locus and Disease

Cinzia Carrozza[1,2], *Laura Foca*[1], *Elisa De Paolis*[1] *and Paola Concolino*[1]*

[1] Dipartimento di Scienze di Laboratorio e Infettivologiche, UOC Chimica, Biochimica e Biologia Molecolare Clinica, Fondazione Policlinico Universitario "Agostino Gemelli" IRCCS, Roma, Italy, [2] Dipartimento di Scienze Biotecnologiche di base, Cliniche Intensivologiche e Perioperatorie, Università Cattolica del Sacro Cuore, Roma, Italy

Copy Number Variations (CNVs) account for a large proportion of human genome and are a primary contributor to human phenotypic variation, in addition to being the molecular basis of a wide spectrum of disease. Multiallelic CNVs represent a considerable fraction of large CNVs and are strictly related to segmental duplications according to their prevalent duplicate alleles. RCCX CNV is a complex, multiallelic and tandem CNV located in the major histocompatibility complex (MHC) class III region. RCCX structure is typically defined by the copy number of a DNA segment containing a series of genes – the serine/threonine kinase 19 (*STK19*), the complement 4 (*C4*), the steroid 21-hydroxylase (*CYP21*), and the tenascin-X (*TNX*) – lie close to each other. In the Caucasian population, the most common RCCX haplotype (69%) consists of two segments containing the genes *STK19-C4A-CYP21A1P-TNXA-STK19B-C4B-CYP21A2-TNXB*, with a telomere-to-centromere orientation. Nonallelic homologous recombination (NAHR) plays a key role into the RCCX genetic diversity: unequal crossover facilitates large structural rearrangements and copy number changes, whereas gene conversion mediates relatively short sequence transfers. The results of these events increased the RCCX genetic diversity and are responsible of specific human diseases. This review provides an overview on RCCX complexity pointing out the molecular bases of Congenital Adrenal Hyperplasia (CAH) due to CYP21A2 deficiency, CAH-X Syndrome and disorders related to CNV of complement component C4.

Keywords: RCCX, haplotypes, Congenital Adrenal Hyperplasia (CAH), CAH-X, Copy Number Variation (CNV), Complement Component C4

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Liliana Dain,
Centro Nacional de Genética Médica, Argentina

Reviewed by:
Mirjana Kocova,
Saints Cyril and Methodius University of Skopje, North Macedonia
Shin Matsubara,
Suntory Foundation for Life Sciences, Japan

***Correspondence:**
Paola Concolino
paola.concolino@policlinicogemelli.it

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INTRODUCTION

Germline Copy Number Variation (CNV) is regarded as a particular DNA fragment with variable copies compared to a reference genome and primarily includes genome duplications and deletions (1). CNVs account for a large proportion of human genome (2), greatly influence cellular phenotypes such as gene expression (3), and are accountable for a plethora of diseases, in addition to representing relevant disease risk factors (4, 5). These observations raise the possibility that CNVs could be a primary contributor to human phenotypic variation and consequently evolve under selective pressures (5). Four major mechanisms have been proposed

as contributors to the generation of most CNVs, including nonallelic homologous recombination (NAHR), nonhomologous end-joining, fork stalling and template switching, and L1-mediated retrotransposition (4). Multiallelic CNVs constitute a considerable fraction of large CNVs and are strictly related to segmental duplications according to their prevalent duplicate alleles (6, 7). CNVs alleles with large, homologous, and tandem repeats are susceptible to rearrangements *via* NAHR mechanism (8) such as unequal crossover (9) and gene conversion (10). In this Review, we focus on the genetic complexity of the RCCX CNV discussing the molecular bases of related human diseases as Congenital Adrenal Hyperplasia (CAH).

RCCX CNV

RCCX CNV is a complex, multiallelic and tandem CNV located in the major histocompatibility complex (MHC) class III region (11, 12). It is an haplotypic structure typically defined by the copy number of a DNA segment containing a series of genes that lie close to each other: the serine/threonine kinase 19 (*STK19*), the complement 4 (*C4*), the steroid 21-hydroxylase (*CYP21*), and the tenascin-X (*TNX*) genes (13). *RCCX* CNV alleles commonly consist of one, two or three segments with the prevalence of approximately 17%, 69% and 14% in the Caucasian population (14). The **Figure 1A** shows the structure of the *RCCX* haplotype with two segments with the genes oriented as: *STK19-C4A-CYP21A1P-TNXA-STK19B-C4B-CYP21A2-TNXB* (15). *STK19* gene (originally called *G11* or *RP*), just upstream from *C4A*, encodes a nuclear Serine/Threonine Kinase protein recently identified as a regulator of NRAS activity (16–20). *STK19B*, immediately upstream from the *C4B* gene, consists only of 914 bases of the 3' end of the original gene because the *C4*/*CYP21*/*TNX* locus duplication caused the lost of a large part of the coding DNA in this region (14, 15). *C4A* and *C4B* genes encode the two isoforms of the fourth component of serum complement (C4), an essential element for the effector arm of the humoral immune response (21). Each human *C4* gene contains 41 exons, and the gene size shows a dichotomous size variation between ~22 kb and 16 kb. The longer gene is the result of the integration of the endogenous retrovirus *HERV-K*(*C4*) into intron 9 (22). Both the *C4A* and *C4B* 3' ends lie only 2466 bp upstream the *CYP21A1P* and *CYP21A2* transcriptional start sites, respectively. In addition, the promoter regions of *CYP21* genes are located in the *C4* intron 35 (23). *CYP21A2* gene encodes the steroid 21-hydroxylase enzyme (cytochrome P450c21), uniquely expressed in adrenal cortex, responsible for the biosynthesis of the two principal steroid hormones, aldosterone and cortisol. Both the *CYP21A2* functional gene and the *CYP21A1P* pseudogene consist in a total of ten exons spanning 3.4 kb. Sequence identity of 98% and approximately 96% characterizes their exons and intronic regions, respectively (24, 25).

With respect to the *C4* and *CYP21*, both the *TNXA* and *TNXB* genes are located in the opposite DNA strand with, consequently, an opposite transcriptional orientation. These genes partially

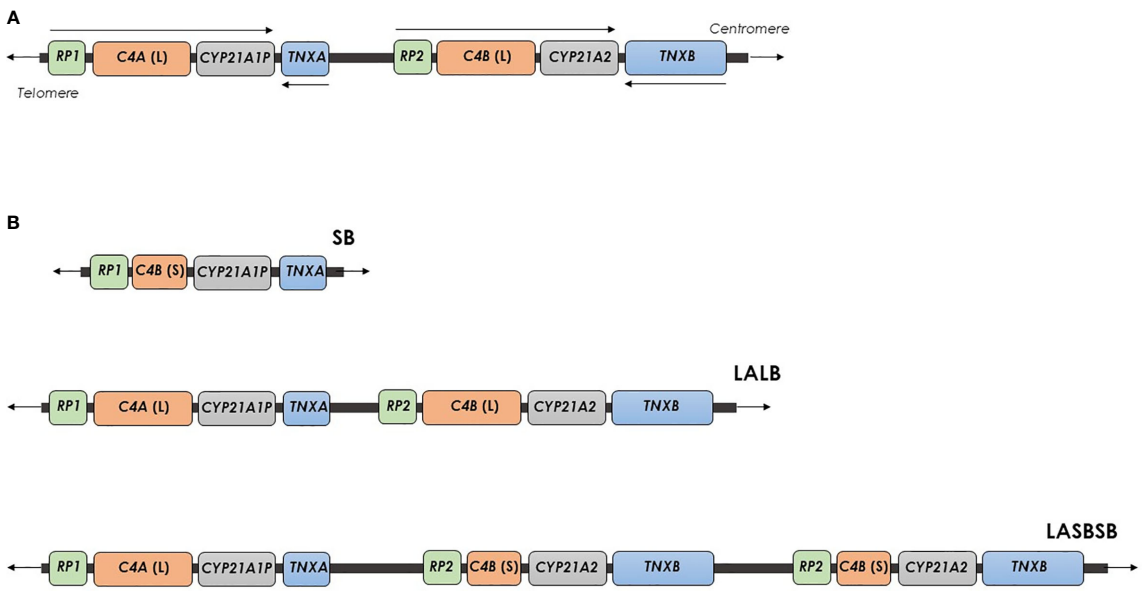

FIGURE 1 | Organization of the human *RCCX* CNV on chromosome 6. **(A)** *RCCX* structure with two segments containing the genes *STK19-C4A-CYP21A1P-TNXA-STK19B-C4B-CYP21A2-TNXB*, with a telomere-to-centromere orientation. Arrows indicates the transcriptional orientation of genes. **(B)** *RCCX* structures with one, two and three segments. Each segment is indicated with two letters, the first represents the allele of the *HERV-K*(*C4*) CNV [L, Long allele (insertion) or S, Short allele (deletion)], and the second indicates the types of *C4* gene (A or B). SB, A *RCCX* structure with one segment including a *C4B* gene without *HERV-K*(*C4*) insertion. LALB, A *RCCX* structure with two segments including two *C4* genes, A and B, both with *HERV-K*(*C4*) insertion. LA SB SB: A *RCCX* structure with three segments including a *C4A* gene with *HERV-K*(*C4*) insertion and two *C4B* genes without *HERV-K(C4*) insertion.

overlap the 3' ends of the *CYP21* genes: the last exon of *TNXA* and *TNXB* lies within the 3' untranslated region of exon 10 in *CYP21A1P* and *CYP21A2*, respectively, and contain fibronectin type III repeats (26, 27). *TNXB* gene, encoding the extracellular matrix protein TNX, consists of 68.2 kb of DNA and includes 44 exons (28). The *TNXB* gene appears to be unique in having both its 5' and 3' ends buried in other genes. In fact, several start sites located into or near the *CREB-RP* gene are responsible for the *TNXB* transcription initiation. The *CREB-RP* gene lie immediately upstream of *TNXB* and encoding a protein related to the CREB transcription factor (29, 30). *TNXA* is a duplicated section of *TNXB* and consists in a truncated pseudogene containing a 120 bp deletion that causes a frameshift and a premature stop codon that render the gene non-functional (31).

An haplotypic *RCCX* CNV structure is traditionally described by the copy number of the repeated segment of *RCCX* CNV (CNV allele), and, per segment, by the alleles of HERV-K(*C4*) CNV and the type of *C4* gene (13). Usually, a *RCCX* segment is indicated with two letters, the first representing the alleles of the *HERV-K*(*C4*) CNV [L: long allele (insertion allele) or S: short allele (deletion allele)] and the second indicating the type of *C4* gene (A or B). The multiplication of these two letters indicates the presence of two and three segments (**Figure 1B**) (11, 13). Very rare *RCCX* CNV alleles with four segments have been also reported (32, 33). In addition, in order to define the exact structure (presence or absence of *HERV-K*(*C4*) insertion and type of *C4* gene) of a *RCCX* CNV, specific molecular approaches have been proposed (11, 34).

RCCX-ASSOCIATED DISEASES

The genetic diversity of the *RCCX* is highly attributable to NAHR: unequal crossover facilitates large structural rearrangements and copy number changes, whereas gene conversion mediates relatively short sequence transfers (9, 10). The results of these events increase the *RCCX* genetic diversity and are responsible of specific human diseases.

CAH Due to 21-Hydroxylase Deficiency

CAH is a group of genetic autosomal recessive disorders that affects adrenal steroidogenesis in the adrenal cortex. The vast majority of the CAH cases, approximately 95%, are related to 21-hydroxylase deficiency due to pathogenic variants accounted in *CYP21A2* gene. 21-hydroxylase enzyme is responsible for the conversion of 17-hydroxyprogesterone to 11-deoxycortisol and progesterone to deoxycorticosterone (35, 36). The impairment of cortisol and aldosterone production is directly related to the clinical form of the disease that ranges from classic (CL) or severe to non-classic (NC) or mild late onset (37, 38). As above-mentioned, both the *CYP21A2* gene and its *CYP21A1P* pseudogene are composed by a total of 10 exons, sharing a high rate of homology (25, 39). The *CYP21A1P* pseudogene is inactivated by multiple deleterious variants (small insertions/deletions and point pathogenic variants) responsible for the synthesis of a non-functional protein. Intergenic recombination events represent more than 95% of deleterious variants leading to 21-hydroxylase deficiency. Approximately 75% of the deleterious variants are transferred by small conversions from the pseudogene during meiosis. These conversions can involve one (microconversions) or more pseudogene variants (40–42). Differently, 5-10% of CAH alleles observed in most populations are characterized by *CYP21A2* pathogenic variants that do not result in gene conversions (43–45).

The 20–25% of the cases of 21-hydroxylase deficiency is related to large misalignment due to unequal crossing over during meiosis process. This kind of event may cause gene deletion or amplification, and also broader deletions involving *CYP21A2* gene and the other contiguous genes (40–42). *CYP21A1P*/*CYP21A2* chimeric gene is the result of a recombination between *CYP21A1P* and *CYP21A2* genes, as an unequal crossing over occurs during meiosis. Based on the *C4B* form of the gene, i.e. long or short, the rearrangement results into a 26 or 32 Kb deletion, encompassing the 3′ end of *CYP21A1P*, all of the *C4B* gene, and the 5′ end of the *CYP21A2* gene. This event leads to a single non-functional chimeric gene containing the *CYP21A1P* at the 5' end and the *CYP21A2* at the 3' end (**Figure 2A**). To date 9 different chimeric *CYP21A1P*/*CYP21A2* genes have been found and characterized (46–55). In particular, two groups of chimeras, classic and attenuated, have been identified: chimeric genes where the junction site is located downstream of the c.293-13C/A>G mutation in the intron 2 (CH-1, CH-2, CH-3, CH-5, CH-6, CH-7, CH-8) are associated with the severe Salt Wasting form of CAH. In contrast, CH-4 and CH-9 chimeras, carrying the weaker *CYP21A1P* promoter and the sole p.(Pro30Leu) variant, are commonly related to a milder phenotype (47).

Unequal crossover is also the cause of copy number changes of *RCCX* segment. The most well-known case is an haplotypic *RCCX* CNV structure containing three distinct segments with two *CYP21A2* gene copies and one *CYP21A1P* pseudogene copy (56–62). Generally, the *CYP21A2* gene located downstream the *TNXA* gene shows a wild-type nucleotide sequence, or carries one or more deleterious variants. Conversely, the presence of the *CYP21A2* p.(Gln319Ter) mutation characterized the gene copy located next to *TNXB* gene (13, 57–64). To date, 8 different haplotypes with two active *CYP21A2* genes on a chromosome 6 have been detected (63). The absence of a clear correlation between genotype and phenotype observed in many individuals is solved by the existence of these rare haplotypes, underlying the need of the *RCCX* CNV assessment in the molecular diagnosis of 21-hydroxylase deficiency (56, 65, 66).

Finally, the complete deletion of *CYP21A2* gene can occur as the result of an unequal crossing over between *TNXA* and *TNXB* genes. This event produces a chromosome with two copies of *CYP21A2* gene and a chromosome where the arrangement of the *RCCX* segment shows the *C4-CYP21A1P-TNXA*/*TNXB* sequence, lacking *CYP21A2* gene copy. This condition is associated to the CAH-X Syndrome (67).

CAH-X Syndrome

Ehlers-Danlos syndromes (EDS) are a clinically and genetically heterogeneous group of heritable connective tissue disorders

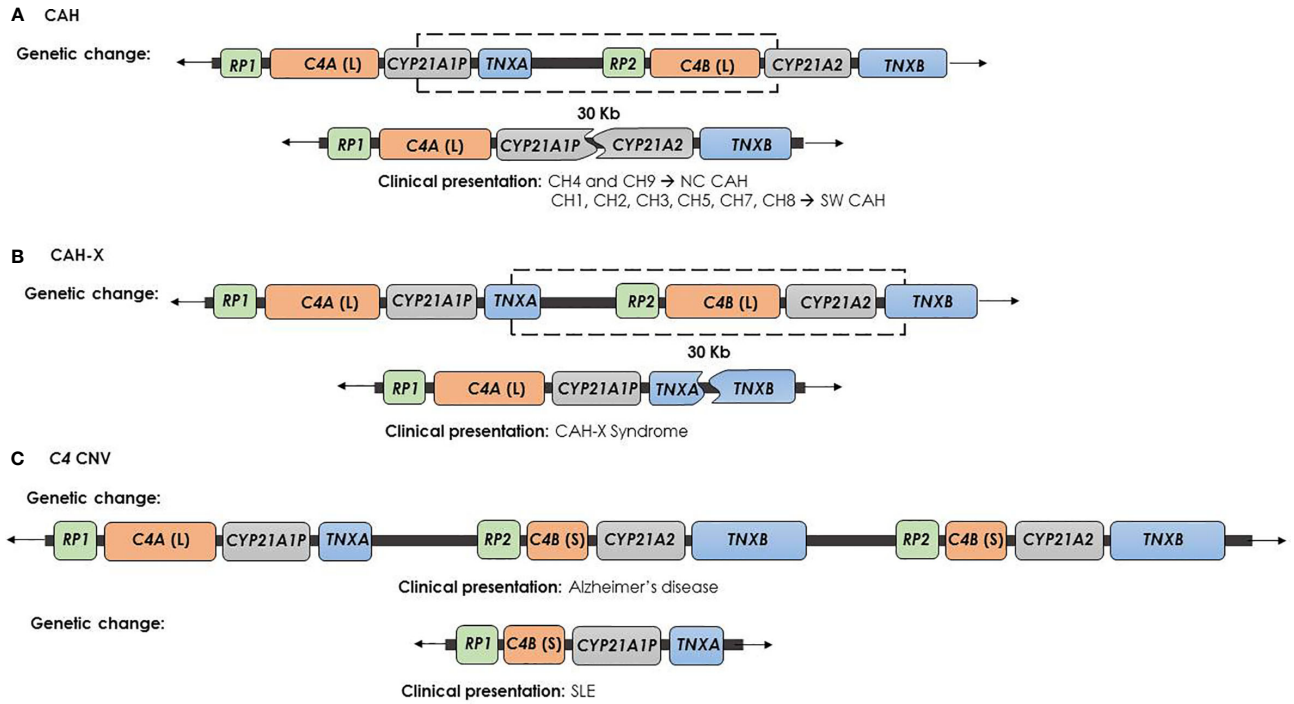

FIGURE 2 | Genetic changes and clinical presentation. **(A)** CAH chimera (*CYP21A1P*/*CYP21A2*) is caused by recombination between *CYP21A1P* and *CYP21A2* and causes the impairment of *CYP21A2* and the deletion of *C4B*, but leaves safe the *TNXB* gene. CH-1, CH-2, CH-3, CH-5, CH-6, CH-7 and CH-8 chimeras are involved in the severe SW form of CAH. In contrast, CH-4 and CH-9 chimeras are generally related to a milder phenotype. **(B)** CAH-X chimera (*TNXA*/*TNXB*) is caused by recombination between *TNXA* and *TNXB* and produces the complete deletion of *CYP21A2* gene, the impairment of *TNXB* gene and the deletion of *C4B*. This contiguous deletion is termed CAH-X and causes CAH-X syndrome. **(C)** Psychiatric and autoimmune diseases due to *C4* CNV: the high *C4B* copy number has been described as Alzheimer's disease risk factor, while the *C4A* deficiency was related to SLE.

characterized by joint hypermobility (JH), skin hyperextensibility, and tissue fragility. EDS is typically caused by autosomal dominant mutations in collagen-encoding genes or in genes encoding collagen-modifying enzymes (68). Tenascin-X deficiency causes a clinically distinct form of EDS due to homozygous or compound heterozygous pathogenic variants in the *TNXB* gene. Pathogenic variants account in the coding region of the EGF-like repeats or the fibronectin type III domain of the tenascin protein. The clinical phenotype resembles the classical EDS type with a pattern of autosomal recessive inheritance (69, 70). Heterozygosity for severe *TNXB* mutations causes *TNXB* haploinsufficiency and it is related to hypermobility type EDS (hEDS), characterized by JH, recurring joint dislocations, joint pain and structural cardiac valve abnormality (71). The CAH-X term was first used for the description of a specific subgroup of CAH affected subjects showing an EDS phenotype caused by *CYP21A2* monoallelic deletion extending into the *TNXB* gene (72). The result of this 30 Kb deletion, caused by a recombination event between *TNXA* and *TNXB* genes, is a chimeric *TNXA*/*TNXB* gene (**Figure 2B**) (73). To date, three *TNXA*/*TNXB* chimeras that differ in the junction site and result in a contiguous *CYP21A2* and *TNXB* gene deletion (CH-1 to CH-3) have been reported (72, 74, 75). CAH-X CH-1 is characterized by a *TNXA* pseudogene derived 120-bp deletion in exon 35 that causes the non-functionality of the gene and also results in decreased *TNX* expression in both dermal and serum, claiming an haploinsufficiency mechanism (69, 72). CAH-X CH-2 is characterized by the variant c.12174C>G (p.Cys4058Trp) (exon 40) derived from *TNXA* pseudogene. This substitution deletes a cysteine residue and leads to the loss of a critical disulfide bond in the tertiary structure of the *TNX* C-terminal fibrinogen-like domain (74). The third chimera, termed CAH-X CH-3, has *TNXB* exons 41-44 substituted by *TNXA* and it is characterized by a cluster of 3 closely linked variants also derived from *TNXA* pseudogene: the c.12218G>A (p.Arg4073His) in exon 41 and the c.12514G>A (p.Asp4172Asn) and the c.12524G>A (p.Ser4175Asn) in exon 43 (75). Computational studies showed that the p.(Arg4073His) variant interferes with TNX fibrinogen-like domain stability. In particular, the arginine 4073 is predicted to form a cation-pi interaction with the p.Phe4080 residue, which is lost in the p.(Arg4073His) change, penalizing the folding energy with a loss of 35 kcal/mol. The remaining variants in the cluster did not significantly affect the folding energies in the models (75). Differently to CAH-X CH-1 chimera, CH-2 and CH-3 not reduce the TNX expression but produce altered proteins and are associated with a dominant-negative effect.

All the *TNXA*/*TNXB* chimeras cause EDS in monoallelic or biallelic form regardless of CAH status, although patients with CAH usually show more severe EDS manifestations with respect to carriers without CAH (69, 72, 74–76). Approximately 10% of patients with CAH due to 21-hydroxylase deficiency are affected by CAH-X (74). Recently, Marino et al. reported that the overall prevalence of CAH-X in a large cohort of Argentine CAH patients was 14%, which was similar to that previously found in a large cohort from the National Institutes of Health and in the Chinese population (15% and 14% respectively) (77–79). In addition, Lao et al. reported a particularly high prevalence (29.2%) of CAH-X in 21-hydroxylase deficient patients carrying the 30 kb deletion (78).

Regarding clinical manifestations, CAH-X affected subjects show generalized JH, subluxation and chronic arthralgia, while cardiac abnormalities have been observed in about 25% (80). More severe clinical manifestations were found in patients with a biallelic than in those with a monoallelic form (8, 10). In addition, compared to haploinsufficiency, a dominant-negative effect causes a more severe phenotype displayed by greater skin and joint involvement (74). The diagnosis of EDS due to CAH-X relies mainly on clinical evaluations including physical examination for JH, skin characteristics and imaging. A serum tenascin-X test, based on enzyme-linked immunosorbent assay, has been developed to identify complete deficiency, but it is not accurate in identifying heterozygous forms (69, 81). Molecular diagnosis represents a valid support to the clinical evaluation of CAH-X and, in this context, Sanger sequencing results to be the most reliable an informative method for all *TNXB* variations, even if it is laborious and expensive (82).

Complement Component *C4* CNV

Complement component *C4* is a central protein in the classical and lectin pathways within the complement system (83). The two isotypes of C4, which differ by only four amino acids, demonstrate differential chemical reactivities: C4A displays higher affinity for amino group-containing antigens or immune complexes, and C4B for hydroxyl group-containing antigens (84, 85). In the general population, the most common *RCCX* haplotype consists of two segments with two C4 in tandem genes coding for *C4A* and *C4B*. So, approximately 60% of healthy individuals have two *C4A* and two *C4B* genes (14, 86, 87). However, deletions and duplications of *C4* genes are well documented and the human *C4* locus has been identified as a functional CNV hotspot within the *RCCX* region. *C4* isotypes involvement is described in several pathological conditions (88). For instance, an high *C4A* gene dosage represents a relevant schizophrenia risk factor, while both *C4A* or *C4B* high copy number is related to Alzheimer's disease (89, 90) (**Figure 2C**). The presence of one *C4A* or *C4B* gene is called heterozygous *C4A* or *C4B* deficiency, while the presence of no functional *C4A* or *C4B* genes causes complete *C4A* or *C4B* deficiency and is called homozygous *C4* deficiency (14). Homozygous deficiencies of complement *C4A* or *C4B* are detected in 1-10% of populations. Homozygous deficiency of *C4A* has been reported to associate with increased frequency of autoimmune diseases, whereas homozygous *C4B* deficiency has been associated with increased susceptibility of bacterial and enveloped viral infections (91, 92). Many studies support the association between homozygous *C4A* deficiency and systemic lupus erythematosus (SLE) (93–97) (**Figure 2C**).

C4 structural variations frequently arise in CAH affected subjects with relevant clinical implications, particularly in relation to psychiatric morbidity and autoimmunity (98, 99). Moreover, Lao et al. reported in a cohort of 145 CAH subjects with 21-hydroxylase deficiency, the correlation between *C4A* copy number and the externalization of psychiatric comorbidity (98). Interestingly, authors specified that *C4B* copy number was the determinant of *C4* serum levels in CAH patients because *C4B* copy number varied in CAH patients carrying the 30-Kb deletion and in NC patients carrying the p.(Val282Leu) variant. In fact, as a consequence of 30 Kb deletion, both *C4B* and *CYP21A2* genes are frequently lost concurrently, producing a *CYP21A1P*/*CYP21A2* or *CYP21A1P-TNXA*/*TNXB* chimera (**Figures 2A, B**). Conversely, the known association of the NC p.(Val282Leu) variant with high total *C4* copy number was found to be due to a duplication of *C4B* gene, not *C4A* (98, 100).

Recently, Falhammar et al. reported an increased prevalence of autoimmune disorders in a large cohort of Swedish patients with 21-hydroxylase deficiency (99). However, some limitations of the study were point out. In particular, the relatively young age of the patients and the possible protective effects of glucocorticoid treatment may have led to underestimates in the lifetime risks for autoimmune disorders (99).

The complex genetics of human histocompatibility complex provides evidences that *RCCX* genotype being related to *C4* could represent a further risk factor for additional illnesses in CAH affected subjects with 21-hydroxylase deficiency. However, the role of the *C4* gene dosage related to *CYP21A2* genotype in CAH patients needs to further investigations.

DISCUSSION

RCCX CNV represents a complex, multiallelic and tandem CNV in the MHC class III region. Genetic recombination events typically affect this genomic region due to the peculiar co-presence of genes and pseudogenes with high sequence homology, causing frequent misalignment during meiosis. The challenging related to the molecular diagnosis of 21-hydroxylase deficiency, owed to the complexity of the *RCCX* CNV structure, are well documented. For this reason, it is essential to refer to effective guidelines for the standardization of molecular genetic testing of CAH due to *CYP21A2* defects (101). In addition, as recently suggested, including CAH-X chimeras determination in 21-hydroxylase deficiency molecular testing would be particularly beneficial for individuals carrying an allele with the "30Kb deletion". In fact, a very early CAH-X diagnosis could be offered to young children before hypermobility evaluation is applicable, and to enable early screening for cardiac defects (102). However, a reflection is currently in progress on the need to carry out further studies in order to broader the

knowledge and the expertise on CAH-X before including respective methods in routine diagnostic procedures (103, 104).

Finally, novel and larger studies are required in order to elucidate the role of *C4* dosage in several disorders, especially in CAH patients with 21-hydroxylase deficiency.

AUTHOR CONTRIBUTIONS

LF and EP researched and wrote a first draft of the review. PC and CC revised the final version of the manuscript. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Zarrei M, MacDonald JR, Merico D, Scherer SW. A Copy Number Variation Map of the Human Genome. *Nat Rev Genet* (2015) 16:172–83. doi: 10.1038/nrg3871
2. Handsaker RE, Van Doren V, Berman JR, Genovese G, Kashin S, Boettger LM, et al. Large Multiallelic Copy Number Variations in Humans. *Nat Genet* (2015) 47:296–303. doi: 10.1038/ng.3200
3. Stranger BE, Forrest MS, Dunning M, Ingle CE, Beazley C, Thorne N, et al. Relative Impact of Nucleotide and Copy Number Variation on Gene Expression Phenotypes. *Science* (2007) 9:848–53. doi: 10.1126/science.1136678
4. Hu L, Yao X, Huang H, Guo Z, Cheng X, Xu Y, et al. Clinical Significance of Germline Copy Number Variation in Susceptibility of Human Diseases. *J Genet Genomics* (2018) 45:3–12. doi: 10.1016/j.jgg.2018.01.001
5. Saitou M, Gokcumen O. An Evolutionary Perspective on the Impact of Genomic Copy Number Variation on Human Health. *J Mol Evol* (2020) 88:104–19. doi: 10.1007/s00239-019-09911-6
6. Conrad DF, Pinto D, Redon R, Feuk L, Gokcumen O, Zhang Y, et al. Origins and Functional Impact of Copy Number Variation in the Human Genome. *Nature* (2010) 464:704–12. doi: 10.1038/nature08516
7. Redon R, Ishikawa S, Fitch KR, Feuk L, Perry GH, Andrews TD, et al. Global Variation in Copy Number in the Human Genome. *Nature* (2006) 444:444–54. doi: 10.1038/nature05329
8. Dittwald P, Gambin T, Szafranski P, Li J, Amato S, Divon MY, et al. NAHR-Mediated Copy-Number Variants in a Clinical Population: Mechanistic Insights Into Both Genomic Disorders and Mendelizing Traits. *Genome Res* (2013) 23:1395–409. doi: 10.1101/gr.152454.112
9. Lupski JR, Stankiewicz P. Genomic Disorders: Molecular Mechanisms for Rearrangements and Conveyed Phenotypes. *PloS Genet* (2005) 1:e49. doi: 10.1371/journal.pgen.0010049
10. Chen JM, Férec C, Cooper DN. Gene Conversion in Human Genetic Disease. *Genes (Basel)* (2010) 1:550–63. doi: 10.3390/genes1030550
11. Bánlaki Z, Szabó JA, Szilágyi Á, Patócs A, Prohászka Z, Füst G, et al. Intraspecific Evolution of Human RCCX Copy Number Variation Traced by Haplotypes of the CYP21A2 Gene. *Genome Biol Evol* (2013) 5:98–112. doi: 10.1093/gbe/evs121
12. Horton R, Wilming L, Rand V, Lovering RC, Bruford EA, Khodiyar VK, et al. Gene Map of the Extended Human MHC. *Nat Rev Genet* (2004) 5:889–99. doi: 10.1038/nrg1489
13. Doleschall M, Luczay A, Koncz K, Hadzsiev K, Erhardt É, Szilágyi Á, et al. A Unique Haplotype of RCCX Copy Number Variation: From the Clinics of Congenital Adrenal Hyperplasia to Evolutionary Genetics. *Eur J Hum Genet* (2017) 25:702–10. doi: 10.1038/ejhg.2017.38
14. Blanchong CA, Zhou B, Rupert KL, Chung EK, Jones KN, Sotos JF, et al. Deficiencies of Human Complement Component C4A and C4B and Heterozygosity in Length Variants of RP-C4-CYP21-TNX (RCCX) Modules in Caucasians. The Load of RCCX Genetic Diversity on Major Histocompatibility Complex-Associated Disease. *J Exp Med* (2000) 191:2183–96. doi: 10.1084/jem.191.12.2183
15. Bánlaki Z, Doleschall M, Rajczy K, Fust G, Szilágyi A. Fine-Tuned Characterization of RCCX Copy Number Variants and Their Relationship With Extended MHC Haplotypes. *Genes Immun* (2012) 13:530–5. doi: 10.1038/gene.2012.29
16. Sargent CA, Anderson MJ, Hsieh SL, Kendall E, Gomez-Escobar N, Campbell RD. Characterisation of the Novel Gene G11 Lying Adjacent to the Complement C4A Gene in the Human Major Histocompatibility Complex. *Hum Mol Genet* (1994) 3:481–88. doi: 10.1093/hmg/3.3.481
17. Shen L, Wu LC, Sanlioglu S, Chen R, Mendoza AR, Dangel AW, et al. Structure and Genetics of the Partially Duplicated Gene RP Located Immediately Upstream of the Complement C4A and the C4B Genes in the HLA Class III Region. Molecular Cloning, Exon-Intron Structure, Composite Retroposon, and Breakpoint of Gene Duplication. *J Biol Chem* (1994) 269:8466–76. doi: 10.1016/S0021-9258(17)37217-4
18. Gomez-Escobar N, Chou CF, Lin WW, Hsieh SL, Campbell RD. The G11 Gene Located in the Major Histocompatibility Complex Encodes a Novel Nuclear Serine/Threonine Protein Kinase. *J Biol Chem* (1998) 273:30954–60. doi: 10.1074/jbc.273.47.30954
19. Yin C, Zhu B, Zhang T, Liu T, Chen S, Liu Y, et al. Pharmacological Targeting of STK19 Inhibits Oncogenic NRAS-Driven Melanomagenesis. *Cell* (2019) 176:1113–27. doi: 10.1016/j.cell.2019.01.002
20. Gimple RC, Wang X. RAS: Striking at the Core of the Oncogenic Circuitry. *Front Oncol* (2019) 9:965. doi: 10.3389/fonc.2019.00965
21. Carroll MC. Complement and Humoral Immunity. *Vaccine* (2008) 26:I28–33. doi: 10.1016/j.vaccine.2008.11.022
22. Yu CY, Belt KT, Giles CM, Campbell RD, Porter RR. Structural Basis of the Polymorphism of Human Complement Components C4A and C4B: Gene Size, Reactivity and Antigenicity. *EMBO J* (1986) 5:2873–81. doi: 10.1002/j.1460-2075.1986.tb04582.x
23. Wijesuriya SD, Zhang G, Dardis A, Miller WL. Transcriptional Regulatory Elements of the Human Gene for Cytochrome P450c21 (Steroid 21-Hydroxylase) Lie Within Intron 35 of the Linked C4B Gene. *J Biol Chem* (1999) 274:38097–106. doi: 10.1074/jbc.274.53.38097
24. White PC, Grossberger D, Onufer BJ, Chaplin DD, New MI, Dupont B, et al. Two Genes Encoding Steroid 21-Hydroxylase are Located Near the Genes Encoding the Fourth Component of Complement in Man. *Proc Natl Acad Sci U.S.A.* (1985) 82:1089–93. doi: 10.1073/pnas.82.4.1089
25. White PC, New MI, Dupont B. Structure of Human Steroid 21-Hydroxylase Genes. *Proc Natl Acad Sci U.S.A.* (1986) 83:5111–5. doi: 10.1073/pnas.83.14.5111
26. Morel Y, Bristow J, Gitelman SE, Miller WL. Transcript Encoded on the Opposite Strand of the Human Steroid 21-Hydroxylase/Complement Component C4 Gene Locus. *Proc Natl Acad Sci USA* (1989) 86:6582–6. doi: 10.1073/pnas.86.17.6582
27. Matsumoto K, Arai M, Ishihara N, Ando A, Inoko H, Ikemura T. Cluster of Fibronectin Type III Repeats Found in the Human Major Histocompatibility Complex Class III Region Shows the Highest Homology With the Repeats in an Extracellular Matrix Protein, Tenascin. *Genomics* (1992) 12:485–91. doi: 10.1016/0888-7543(92)90438-x
28. Bristow J, Tee MK, Gitelman SE, Mellon SH, Miller WL. Tenascin-X: A Novel Extracellular Matrix Protein Encoded by the Human XB Gene Overlapping P450c21B. *J Cell Biol* (1993) 122:265–78. doi: 10.1083/jcb.122.1.265
29. Speek M, Barry F, Miller WL. Alternate Promoters and Alternate Splicing of Human Tenascin-X, a Gene With 5' and 3' Ends Buried in Other Genes. *Hum Mol Genet* (1996) 5:1749–58. doi: 10.1093/hmg/5.11.1749
30. Min J, Shukla H, Kozono H, Bronson SK, Weissman SM, Chaplin DD. A Novel Creb Family Gene Telomeric of HLA-DRA in the HLA Complex. *Genomics* (1995) 30:149–56. doi: 10.1006/geno.1995.9891
31. Gitelman SE, Bristow J and Miller WL. Mechanism and Consequences of the Duplication of the Human C4/P450c21/gene X Locus. *Mol Cell Biol* (1992) 12:2124–34. doi: 10.1128/mcb.12.7.3313-b
32. Koppens PF, Hoogenboezem T, Degenhart HJ. Duplication of the CYP21A2 Gene Complicates Mutation Analysis of Steroid 21-Hydroxylase Deficiency: Characteristics of Three Unusual Haplotypes. *Hum Genet* (2002) 111:405–10. doi: 10.1007/s00439-002-0810-7
33. Chung EK, Yang Y, Rennebohm RM, Lokki ML, Higgins GC, Jones KN, et al. Genetic Sophistication of Human Complement Components C4A and C4B and RP-C4-CYP21-TNX (RCCX) Modules in the Major Histocompatibility Complex. *Am J Hum Genet* (2002) 71:823–37. doi: 10.1086/342777
34. Sekar A, Bialas AR, de Rivera H, Davis A, Hammond TR, Kamitaki N, et al. Schizophrenia Risk From Complex Variation of Complement Component 4. *Nature* (2016) 530:177–83. doi: 10.1038/nature16549

35. Parsa AA, New MI. Steroid 21-Hydroxylase Deficiency in Congenital Adrenal Hyperplasia. *J Steroid Biochem Mol Biol* (2017) 165:2–11. doi: 10.1016/j.jsbmb.2016.06.015
36. El-Maouche D, Arlt W, Merke DP. Congenital Adrenal Hyperplasia. *Lancet* (2017) 17:31431–9. doi: 10.1016/S0140-6736(17)31431-9
37. Speiser PW. Nonclassic Adrenal Hyperplasia. *Rev Endocr Metab Disord* (2009) 10:77–82. doi: 10.1007/s11154-008-9097-x
38. Bachelot A, Chakthoura Z, Rouxel A, Dulon J, Touraine P. Classical Forms of Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency in Adults. *Horm Res* (2008) 69:203–11. doi: 10.1159/000113020
39. Higashi Y, Yoshioka H, Yamane M, Gotoh O, Fujii-Kuriyama Y. Complete Nucleotide Sequence of Two Steroid 21-Hydroxylase Genes Tandemly Arranged in Human Chromosome: A Pseudogene and a Genuine Gene. *Proc Natl Acad Sci USA* (1986) 83:2841–5. doi: 10.1073/pnas.83.9.2841
40. Higashi Y, Tanae A, Inoue H, Fujii-Kuriyama Y. Evidence for Frequent Gene Conversion in the Steroid 21-Hydroxylase P-450(C21) Gene: Implications for Steroid 21-Hydroxylase Deficiency. *Am J Hum Genet* (1988) 42:17–25.
41. Tusié-Luna MT, White PC. Gene Conversions and Unequal Crossovers Between CYP21 (Steroid 21-Hydroxylase Gene) and CYP21P Involve Different Mechanisms. *Proc Natl Acad Sci USA* (1995) 92:10796–800. doi: 10.1073/pnas.92.23.10796
42. Pignatelli D, Carvalho BL, Palmeiro A, Barros A, Guerreiro SG, Macut D. The Complexities in Genotyping of Congenital Adrenal Hyperplasia: 21-Hydroxylase Deficiency. *Front Endocrinol (Lausanne)* (2019) 10:432. doi: 10.3389/fendo.2019.00432
43. Concolino P, Costella A. Congenital Adrenal Hyperplasia (CAH) Due to 21-Hydroxylase Deficiency: A Comprehensive Focus on 233 Pathogenic Variants of CYP21A2 Gene. *Mol Diagn Ther* (2018) 22:261–80. doi: 10.1007/s40291-018-0319-y
44. Concolino P, Paragliola RM. Molecular Analysis of 21-Hydroxylase Deficiency Reveals Two Novel Severe Genotypes in Affected Newborns. *Mol Diagn Ther* (2021) 25:327–37. doi: 10.1007/s40291-021-00520-y
45. Simonetti L, Bruque CD, Fernández CS, Benavides-Mori B, Delea M, Kolomenski JE, et al. CYP21A2 Mutation Update: Comprehensive Analysis of Databases and Published Genetic Variants. *Hum Mutat* (2018) 39:5–22. doi: 10.1002/humu.23351
46. Lee HH. The Chimeric CYP21P/CYP21 Gene and 21-Hydroxylase Deficiency. *J Hum Genet* (2004) 49:65–72. doi: 10.1007/s10038-003-0115-2
47. Chen W, Xu Z, Sullivan A, Finkielstain GP, Van Ryzin C, Merke DP, et al. Junction Site Analysis of Chimeric CYP21A1P/CYP21A2 Genes in 21-Hydroxylase Deficiency. *Clin Chem* (2012) 58:421–30. doi: 10.1373/clinchem.2011.174037
48. Concolino P, Mello E, Minucci A, Giardina E, Zuppi C, Toscano V, et al. A New CYP21A1P/CYP21A2 Chimeric Gene Identified in an Italian Woman Suffering From Classical Congenital Adrenal Hyperplasia Form. *BMC Med Genet* (2009) 10:72. doi: 10.1186/1471-2350-10-72
49. Vrzalová Z, Hrubá Z, Hrabincová ES, Vrábelová S, Votava F, Koloušková S, et al. Chimeric CYP21A1P/CYP21A2 Genes Identified in Czech Patients With Congenital Adrenal Hyperplasia. *Eur J Med Genet* (2011) 54:112–7. doi: 10.1016/j.ejmg.2010.10.005
50. Chu X, Braun-Heimer L, Rittner C, Schneider PM. Identification of the Recombination Site Within the Steroid 21-Hydroxylase Gene (CYP21) of the HLA-B47, DR7 Haplotype. *Exp Clin Immunogenet* (1992) 9:80–5.
51. Helmberg A, Tabarelli M, Fuchs MA, Keller E, Dobler G, Schnegg I, et al. Identification of Molecular Defects Causing Congenital Adrenal Hyperplasia by Cloning and Differential Hybridization of Polymerase Chain Reaction-Amplified 21-Hydroxylase (CYP21) Genes. *DNA Cell Biol* (1992) 11:359–68. doi: 10.1089/dna.1992.11.359
52. Lee HH, Lee YJ, Chan P, Lin CY. Use of PCR-Based Amplification Analysis as a Substitute for the Southern Blot Method for CYP21 Deletion Detection in Congenital Adrenal Hyperplasia. *Clin Chem* (2004) 50:1074–6. doi: 10.1373/clinchem.2003.028597
53. Lee HH, Chang SF, Lee YJ, Raskin S, Lin SJ, Chao MC, et al. Deletion of the C4-CYP21 Repeat Module Leading to the Formation of a Chimeric CYP21P/CYP21 Gene in a 9.3-Kb Fragment as a Cause of Steroid 21-Hydroxylase Deficiency. *Clin Chem* (2003) 49:319–22. doi: 10.1373/49.2.319
54. White PC, New MI, Dupont B. HLA-Linked Congenital Adrenal Hyperplasia Results From a Defective Gene Encoding a Cytochrome P-450 Specific for Steroid 21-Hydroxylation. *Proc Natl Acad Sci USA* (1984) 81:7505–9. doi: 10.1073/pnas.81.23.7505
55. L'Allemand D, Tardy V, Grüters A, Schnabel D, Krude H, Morel Y. How a Patient Homozygous for a 30-Kb Deletion of the C4-CYP 21 Genomic Region can Have a Nonclassic Form of 21-Hydroxylase Deficiency. *J Clin Endocrinol Metab* (2000) 85:4562–7. doi: 10.1210/jcem.85.12.7018
56. Ezquieta B, Beneyto M, Mun˜oz-Pacheco R, Barrio R, Oyarzabal M, Lechuga JL, et al. Gene Duplications in 21-Hydroxylase Deficiency: The Importance of Accurate Molecular Diagnosis in Carrier Detection and Prenatal Diagnosis. *Prenat Diagn* (2006) 26:1172–8. doi: 10.1002/pd.1584
57. Kharrat M, Riahi A, Maazoul F, M'rad R, Chaabouni H. Detection of a Frequent Duplicated CYP21A2 Gene Carrying a Q318X Mutation in a General Population With Quantitative PCR Methods. *Diagn Mol Pathol* (2011) 20:123–7. doi: 10.1097/PDM.0b013e3181f24807
58. Parajes S, Quinteiro C, Domı´nguez F, Loidi L. High Frequency of Copy Number Variations and Sequence Variants at CYP21A2 Locus: Implication for the Genetic Diagnosis of 21-Hydroxylase Deficiency. *PloS One* (2008) 3: e2138. doi: 10.1371/journal.pone.0002138
59. Wedell A, Stengler B, Luthman H. Characterization of Mutations on the Rare Duplicated C4/CYP21 Haplotype in Steroid 21-Hydroxylase Deficiency. *Hum Genet* (1994) 94:50–4. doi: 10.1007/BF02272841
60. Kleinle S, Lang R, Fischer GF, Vierhapper H, Waldhauser F, Fo¨dinger M, et al. Duplications of the Functional CYP21A2 Gene Are Primarily Restricted to Q318X Alleles: Evidence for a Founder Effect. *J Clin Endocrinol Metab* (2009) 94:3954–8. doi: 10.1210/jc.2009-0487
61. Koppens PF, Hoogenboezem T, Degenhart HJ. CYP21 and CYP21P Variability in Steroid 21-Hydroxylase Deficiency Patients and in the General Population in the Netherlands. *Eur J Hum Genet* (2000) 8:827–36. doi: 10.1038/sj.ejhg.5200543
62. Concolino P, Mello E, Minucci A, Giardina B, Capoluongo E. Genes, Pseudogenes and Like Genes: The Case of 21-Hydroxylase in Italian Population. *Clin Chim Acta* (2013) 424:85–9. doi: 10.1016/j.cca.2013.05.019
63. Concolino P. A Rare CYP21A2 Haplotype Clarifies the Phenotype-Genotype Discrepancy in an Italian Patient With Non Classical Congenital Adrenal Hyperplasia (NC-CAH). *Mol Biol Rep* (2020) 47:3049–52. doi: 10.1007/s11033-020-05379-6
64. Tsai LP, Cheng CF, Chuang SH, Lee HH. Analysis of the CYP21A1P Pseudogene: Indication of Mutational Diversity and CYP21A2-Like and Duplicated CYP21A2 Genes. *Anal Biochem* (2011) 413:133–41. doi: 10.1016/j.ab.2011.02.016
65. Lekarev O, Tafuri K, Lane AH, Zhu G, Nakamoto JM, BullerBurckle AM, et al. Erroneous Prenatal Diagnosis of Congenital Adrenal Hyperplasia Owing to a Duplication of the CYP21A2 Gene. *J Perinatol* (2013) 33:76–8. doi: 10.1038/jp.2012.5
66. Sani I, Rossodivita AN, Mariani M, Costella A, Molinario R, Concolino P, et al. CYP21A2 Genetics: When Genotype Does Not Fit Phenotype. *Clin Biochem* (2016) 49:524–5. doi: 10.1016/j.clinbiochem.2015.07.022
67. Lee HH, Lee YJ, Lin CY. PCR-Based Detection of the CYP21 Deletion and TNXA/TNXB Hybrid in the RCCX Module. *Genomics* (2004) 83:944–50. doi: 10.1016/j.ygeno.2003.11.006
68. Malfait F, Francomano C, Byers P, Belmont J, Berglund B, Black J, et al. The 2017 International Classification of the Ehlers-Danlos Syndromes. *Am J Med Genet C Semin Med Genet* (2017) 175:8–26. doi: 10.1002/ajmg.c.31552
69. Schalkwijk J, Zweers MC, Steijlen PM, Dean WB, Taylor G, van Vlijmen IM, et al. A Recessive Form of the Ehlers-Danlos Syndrome Caused by Tenascin-X Deficiency. *N Engl J Med* (2001) 345:1167–75. doi: 10.1056/NEJMoa002939
70. Lindor NM, Bristow J. Tenascin-X Deficiency in Autosomal Recessive Ehlers-Danlos Syndrome. *Am J Med Genet A* (2005) 135:75–80. doi: 10.1002/ajmg.a.30671
71. Lao Q, Mallappa A, Rueda Faucz F, Joyal E, Veeraraghavan P, Chen W, et al. A TNXB Splice Donor Site Variant as a Cause of Hypermobility Type Ehlers-Danlos syndrome in Patients With Congenital Adrenal Hyperplasia. *Mol Genet Genomic Med* (2021) 9:e1556. doi: 10.1002/mgg3.1556
72. Merke DP, Chen W, Morissette R, Xu Z, Van Ryzin C, Sachdev V, et al. Tenascin-X Haploinsufficiency Associated With Ehlers-Danlos Syndrome in Patients With Congenital Adrenal Hyperplasia. *J Clin Endocrinol Metab* (2013) 98:E379–87. doi: 10.1210/jc.2012-3148
73. Lee HH. Chimeric CYP21P/CYP21 and TNXA/TNXB Genes in the RCCX Module. *Mol Genet Metab* (2005) 84:4–8. doi: 10.1016/j.ymgme.2004.09.009

74. Morissette R, Chen W, Perritt AF, Dreiling JL, Arai AE, Sachdev V, et al. Broadening the Spectrum of Ehlers Danlos Syndrome in Patients With Congenital Adrenal Hyperplasia. *J Clin Endocrinol Metab* (2015) 100: E1143–52. doi: 10.1210/jc.2015-2232
75. Chen W, Perritt AF, Morissette R, Dreiling JL, Bohn MF, Mallappa A, et al. Ehlers-Danlos Syndrome Caused by Biallelic TNXB Variants in Patients With Congenital Adrenal Hyperplasia. *Hum Mutat* (2016) 37:893–7. doi: 10.1002/humu.23028
76. Burch GH, Gong Y, Liu W, Dettman RW, Curry CJ, Smith L, et al. Tenascin-X Deficiency Is Associated With Ehlers-Danlos Syndrome. *Nat Genet* (1997) 17:104–8. doi: 10.1038/ng0997-104
77. Marino R, Garrido NP, Ramirez P, Notaristéfano G, Moresco A, Touzon MS, et al. Ehlers-Danlos Syndrome: Molecular and Clinical Characterization of TNXA/TNXB Chimeras in Congenital Adrenal Hyperplasia. *J Clin Endocrinol Metab* (2021) 22:dgab033. doi: 10.1210/clinem/dgab033
78. Lao Q, Brookner B, Merke DP. High-Throughput Screening for CYP21A1P-TNXA/TNXB Chimeric Genes Responsible for Ehlers-Danlos Syndrome in Patients With Congenital Adrenal Hyperplasia. *J Mol Diagn* (2019) 21:924–31. doi: 10.1016/j.jmoldx.2019.06.001
79. Gao Y, Lu L, Yu B, Mao J, Wang X, Nie M, et al. The Prevalence of the Chimeric TNXA/TNXB Gene and Clinical Symptoms of Ehlers-Danlos Syndrome With 21-Hydroxylase Deficiency. *J Clin Endocrinol Metab* (2020) 105:dgaa199. doi: 10.1210/clinem/dgaa199
80. Miller WL, Merke DP. Tenascin-X, Congenital Adrenal Hyperplasia, and the CAH-X Syndrome. *Horm Res Paediatr* (2018) 89:352–61. doi: 10.1159/000481911
81. Zweers MC, Bristow J, Steijlen PM, Dean WB, Hamel BC, Otero M, et al. Haploinsufficiency of TNXB Is Associated With Hypermobility Type of Ehlers-Danlos Syndrome. *Am J Hum Genet* (2003) 73:214–7. doi: 10.1086/376564
82. Lao Q, Merke DP. Letter to the Editor From Lao and Merke: "Ehlers-Danlos Syndrome: Molecular and Clinical Characterization of TNXA/TNXB Chimeras in Congenital Adrenal Hyperplasia". *J Clin Endocrinol Metab* (2021) 106:e2835–e2836. doi: 10.1210/clinem/dgab280
83. Mortensen S, Kidmose RT, Petersen SV, Szilágyi Á, Prohászka Z, Andersen GR. Structural Basis for the Function of Complement Component C4 Within the Classical and Lectin Pathways of Complement. *J Immunol* (2015) 194:5488–96. doi: 10.4049/jimmunol.1500087
84. Blanchong CA, Chung EK, Rupert KL, Yang Y, Yang Z, Zhou B, et al. Genetic, Structural and Functional Diversities of Human Complement Components C4A and C4B and Their Mouse Homologues, Slp and C4. *Int Immunopharmacol* (2001) 1:365–92. doi: 10.1016/s1567-5769(01)00019-4
85. Law SK, Dodds AW, Porter RR. A Comparison of the Properties of Two Classes, C4A and C4B, of the Human Complement Component C4. *EMBO J* (1984) 3:1819–23. doi: 10.1002/j.1460-2075.1984.tb02052.x
86. Chung EK, Yang Y, Rupert KL, Jones KN, Rennebohm RM, Blanchong CA, et al. Determining the One, Two, Three, or Four Long and Short Loci of Human Complement C4 in a Major Histocompatibility Complex Haplotype Encoding C4A or C4B Proteins. *Am J Hum Genet* (2002) 71:810–22. doi: 10.1086/342778
87. Chung EK, Wu YL, Yang Y, Zhou B, Yu CY. Human Complement Components C4A and C4B Genetic Diversities: Complex Genotypes and Phenotypes. *Curr Protoc Immunol* (2005). doi: 10.1002/0471142735.im1308s68
88. Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: A Key System for Immune Surveillance and Homeostasis. *Nat Immunol* (2010) 11:785–97. doi: 10.1038/ni.1923
89. Yilmaz M, Yalcin E, Presumey J, Aw E, Ma M, Whelan CW, et al. Overexpression of Schizophrenia Susceptibility Factor Human Complement C4A Promotes Excessive Synaptic Loss and Behavioral Changes in Mice. *Nat Neurosci* (2021) 24:214–24. doi: 10.1038/s41593-020-00763-8
90. Zorzetto M, Datturi F, Divizia L, Pistono C, Campo I, De Silvestri A, et al. Complement C4A and C4B Gene Copy Number Study in Alzheimer's Disease Patients. *Curr Alzheimer Res* (2017) 14:303–8. doi: 10.2174/1567205013666161013091934
91. Li N, Zhang J, Liao D, Yang L, Wang Y, Hou S. Association Between C4, C4A, and C4B Copy Number Variations and Susceptibility to Autoimmune Diseases: A Meta-Analysis. *Sci Rep* (2017) 7:42628. doi: 10.1038/srep42628
92. Samano ES, Ribeiro Lde M, Gorescu RG, Rocha KC, Grumach AS. Involvement of C4 Allotypes in the Pathogenesis of Human Diseases. *Rev do Hosp das Clin* (2004) 59:138–44. doi: 10.1590/s0041-87812004000300009
93. Wu YL, Yang Y, Chung EK, Zhou B, Kitzmiller KJ, Savelli SL, et al. Phenotypes, Genotypes and Disease Susceptibility Associated With Gene Copy Number Variations: Complement C4 CNVs in European American Healthy Subjects and Those With Systemic Lupus Erythematosus. *Cytogenet Genome Res* (2008) 123:131–41. doi: 10.1159/000184700
94. Yang Y, Chung EK, Wu YL, Savelli SL, Nagaraja HN, Zhou B, et al. Gene Copy-Number Variation and Associated Polymorphisms of Complement Component C4 in Human Systemic Lupus Erythematosus (SLE): Low Copy Number Is a Risk Factor for and High Copy Number is a Protective Factor Against SLE Susceptibility in European Americans. *Am J Hum Genet* (2007) 80:1037–54. doi: 10.1086/518257
95. Jüptner M, Flachsbart F, Caliebe A, Lieb W, Schreiber S, Zeuner R, et al. Low Copy Numbers of Complement C4 and Homozygous Deficiency of C4A May Predispose to Severe Disease and Earlier Disease Onset in Patients With Systemic Lupus Erythematosus. *Lupus* (2018) 27:600–9. doi: 10.1177/0961203317735187
96. Yang Y, Chung EK, Zhou B, Lhotta K, Hebert LA, Birmingham DJ, et al. The Intricate Role of Complement Component C4 in Human Systemic Lupus Erythematosus. *Curr Dir Autoimmun* (2004) 7:98–132. doi: 10.1159/000075689
97. Boteva L, Morris DL, Cortés-Hernández J, Martin J, Vyse TJ, Fernando MM. Genetically Determined Partial Complement C4 Deficiency States Are Not Independent Risk Factors for SLE in UK and Spanish Populations. *Am J Hum Genet* (2012) 90:445–56. doi: 10.1016/j.ajhg.2012.01.012
98. Lao Q, Jardin MD, Jayakrishnan R, Ernst M, Merke DP. Complement Component 4 Variations may Influence Psychopathology Risk in Patients With Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency. *Hum Genet* (2018) 137:955–60. doi: 10.1007/s00439-018-1959-z
99. Falhammar H, Frisén L, Hirschberg AL, Nordenskjöld A, Almqvist C, Nordenström A. Increased Risk of Autoimmune Disorders in 21-Hydroxylase Deficiency: A Swedish Population-Based National Cohort Study. *J Endocr Soc* (2019) 3:1039–52. doi: 10.1210/js.2019-00122
100. Chen W, Xu Z, Nishitani M, Van Ryzin C, McDonnell NB, Merke DP. Complement Component 4 Copy Number Variation and CYP21A2 Genotype Associations in Patients With Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency. *Hum Genet* (2012) 131:1889–94. doi: 10.1007/s00439-012-1217-8
101. 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) 28:1341–67. doi: 10.1038/s41431-020-0653-5
102. Lao Q, Merke DP. Molecular Genetic Testing of Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency Should Include CAH-X Chimeras. *Eur J Hum Genet* (2021) 29:1047–8. doi: 10.1038/s41431-021-00870-5
103. Baumgartner-Parzer S, Witsch-Baumgartner M, Hoeppner W. Reply to Lao Q and Merke Dp. *Eur J Hum Genet* (2021) 29:1045–6. doi: 10.1038/s41431-021-00869-y
104. Szilagyi A, Fust G. Diseases Associated With the Low Copy Number of the C4B Gene Encoding C4, the Fourth Component of Complement. *Cytogenet Genome Res* (2008) 123:118–30. doi: 10.1159/000184699

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Latent Adrenal Insufficiency: From Concept to Diagnosis

*Nada Younes, Isabelle Bourdeau and Andre Lacroix**

Division of Endocrinology, Department of Medicine and Research Center, Centre Hospitalier de l'Université de Montréal (CHUM), Montréal, QC, Canada

Primary adrenal insufficiency (PAI) is a rare disease and potentially fatal if unrecognized. It is characterized by destruction of the adrenal cortex, most frequently of autoimmune origin, resulting in glucocorticoid, mineralocorticoid, and adrenal androgen deficiencies. Initial signs and symptoms can be nonspecific, contributing to late diagnosis. Loss of zona glomerulosa function may precede zona fasciculata and reticularis deficiencies. Patients present with hallmark manifestations including fatigue, weight loss, abdominal pain, melanoderma, hypotension, salt craving, hyponatremia, hyperkalemia, or acute adrenal crisis. Diagnosis is established by unequivocally low morning serum cortisol/aldosterone and elevated ACTH and renin concentrations. A standard dose (250 µg) Cosyntropin stimulation test may be needed to confirm adrenal insufficiency (AI) in partial deficiencies. Glucocorticoid and mineralocorticoid substitution is the hallmark of treatment, alongside patient education regarding dose adjustments in periods of stress and prevention of acute adrenal crisis. Recent studies identified partial residual adrenocortical function in patients with AI and rare cases have recuperated normal hormonal function. Modulating therapies using rituximab or ACTH injections are in early stages of investigation hoping it could maintain glucocorticoid residual function and delay complete destruction of adrenal cortex.

Keywords: cortisol, aldosterone, ACTH, renin, autoimmunity, hypocortisolism, adrenal insufficiency

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***Correspondence:**
Andre Lacroix
andre.lacroix@umontreal.ca

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DEFINITION AND EPIDEMIOLOGY

The adrenal cortex produces glucocorticoids, mineralocorticoids and androgens, under the influence of adrenocorticotropic hormone (ACTH) and the renin-angiotensin system (1, 2). In the event of adrenal cortex destruction, primary adrenal insufficiency (PAI) develops and is characterized by reduced serum concentrations of all three hormones: cortisol, aldosterone and adrenal androgens (3). However, since intra-adrenal cortisol is required for epinephrine production by the adrenal medulla, PAI is often associated with decreased phenylethanolamine N-methyltransferase (PNMT) activity, resulting in adrenomedullary dysfunction (1, 4). Central adrenal insufficiency (AI), encompasses both secondary and tertiary AI caused by low ACTH and low corticotropin releasing hormone (CRH), respectively (5). However, since aldosterone production is mainly controlled by renin, angiotensin II and potassium (1, 6), it is not affected in central AI.

PAI was first described by Thomas Addison in 1855 in a case series of 11 patients and therefore it is often called Addison's disease (7). Since then, prevalence has been on the rise especially in Europe, reaching 117/million in central Italy in the late 1990s (8) and 144/million in Norway in 2007 (9). More recently, an even higher prevalence was documented in a 2016 Icelandic nationwide study of patients over 18 years of age: 221/million population (10). Furthermore, an annual average increase in the prevalence of PAI of 1.8% per year was reported in Germany from 2008 to 2012 (11). The annual incidence of PAI is estimated to be around 0.44-0.62 per 100,000 (9, 12). PAI is more frequently found in women than men (11, 13) with a M:F ratio of 1: 3.5 (14). Age of onset is typically around 30-50 years old (1, 9, 10, 14). Autoimmune destruction of adrenal cortex has surpassed tuberculosis as the most common cause of PAI, in particular in high income countries (1, 15). Comorbid autoimmune disorders can often be found in patients with autoimmune AI (AAI), reported to be 46.5 and 66%, respectively in the German (11) and Norwegian studies (9). Most common comorbid conditions were thyroid disease, type 1 diabetes, vitiligo, vitamin B12 deficiency and primary ovarian insufficiency (POI) (9–11). When occurring with other autoimmune disorders, AAI may be part of an autoimmune polyglandular syndrome (APS). APS type 1 or autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED) is a rare monogenic disease, in which the autoimmune regulator gene (*AIRE*) is mutated and at least 2 of the following three occur: chronic mucocutaneous candidiasis, hypoparathyroidism and PAI (16). Generally, its transmission is autosomal recessive but may be dominant (16). Other conditions may include POI, enteropathy and rarely lymphomas (6, 16). More commonly found is APS type 2, a polygenic condition in which genes encoding cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), protein tyrosine phosphatase, nonreceptor type 22 (PTPN22), the transcriptional regulator protein BACH2, and the CD25–interleukin-2 receptor have been implicated (16), along with associations to certain haplotypes including DR3-DQ2, DR4-DQ8, DRB1-0301, and DRB1-0404 (15, 17). It commonly associates with type 1 diabetes and autoimmune thyroid disease (16). When AAI is confirmed alongside other autoimmune diseases, excluding the cardinal ones needed for diagnosis of APS types 1 and 2, APS type 4 is diagnosed (5).

SIGNS AND SYMPTOMS OF AI-NATURAL HISTORY OF AUTOIMMUNE PAI-MORTALITY

Onset of AI can be insidious, with many of the symptoms and signs being nonspecific and often leading to a delay in diagnosis (1). In a retrospective study including 216 patients with AI (18), more than half of patients consulted at least 3 physicians and were falsely diagnosed with either gastrointestinal or psychiatric disorders most frequently. A 5 year delay in diagnosis was reported in as much as 20% of patients (18). Symptoms of cortisol deficiency are common to both primary and central AI. These include loss of appetite, weight loss, lethargy, gastrointestinal symptoms such as nausea, abdominal pain and vomiting (1, 6).

Because aldosterone deficiency exists in PAI but not in central AI, symptoms and signs of mineralocorticoid deficiency including, dizziness and orthostatic hypotension, salt craving, hyponatremia, hyperkalemia and hyperchloremic acidosis, can be found (1, 19). Orthostatic hypotension may also be secondary to cortisol deficiency *via* reduced expression of catecholamine receptors on blood vessels and therefore may be found in central AI, albeit less pronounced than in PAI (1, 20). Although hyponatremia can be present in both primary and central AI, the underlying mechanisms differ. In PAI, hyponatremia is secondary to aldosterone deficiency resulting in renal salt wasting and hypovolemia with an increased risk of dehydration and acute kidney injury (19, 21). In central AI, it is a result of increased vasopressin secondary to cortisol deficiency, which in turn results in water retention and euvolemic hyponatremia (22). Other possible laboratory abnormalities are hypercalcemia, mild normocytic anemia, lymphocytosis, eosinophilia and hypoglycemia which occurred more frequently in children (1, 6). There is also a significant decrease in natural killer cells cytotoxicity in patients with PAI, compromising innate immunity, hence contributing to increased viral infections (23). Androgen deficiency may result in low libido or reduced energy as well as thinning of axillary and pubic hair in post-menopausal women (1, 3). In pregnant women with unrecognized AI, establishing the diagnosis based on signs and symptoms is challenging, because cortisol deficiency symptoms may also be seen as part of a normal pregnancy. While only a few cases were diagnosed during pregnancy, the high maternal and fetal risks associated with unrecognized AI should prompt physicians to suspect the diagnosis in women with symptoms persisting into the second trimester or occurring secondary to illness or labor (24).

A particularly distinctive feature of PAI is melanoderma which can be explained by elevated ACTH due to loss of negative feedback control usually exerted by cortisol on corticotropic cells in anterior pituitary gland (1). Elevated plasma ACTH activates melanocortin 1 receptors (M1R), resulting in excessive pigmentation, especially on areas exposed to sun and friction such as face, neck, knuckles, creases in the hand, elbows, areola of the nipple, scrotum, labia and newly acquired scars (6, 19). Mucosal pigmentation can also be noted and clinicians should look for brown patchy discoloration on lips, palate and gingiva (19).

PAI may be underrecognized because of the nonspecific symptoms of cortisol deficiency, especially in its early stages. This is often called latent AI and should be suspected in the presence of unexplained health complaints related to stress, such as gastrointestinal symptoms, fatigue and weight loss, in particular in patients with a history of autoimmune disease (25, 26). Betterle et al. suggested that autoimmune adrenocortical destruction goes through 4 stages (27) (**Figure 1**). The very first stage of the disease is marked by aldosterone deficiency

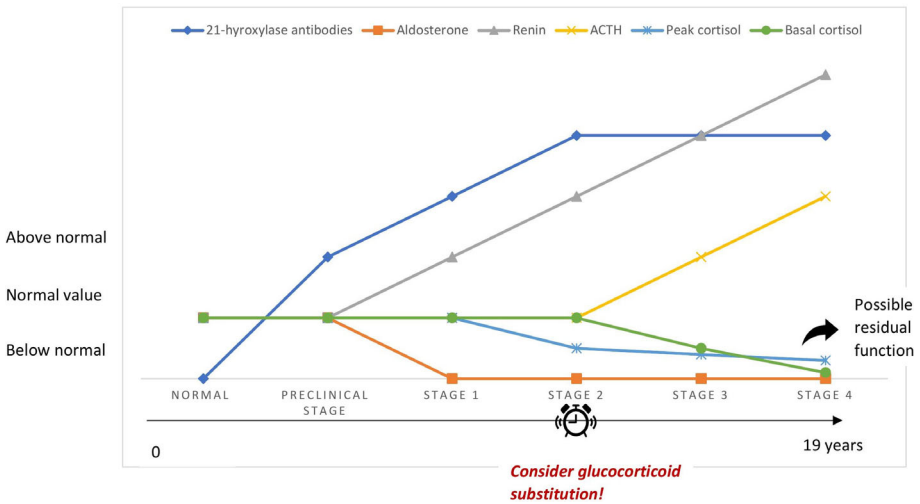

FIGURE 1 | Simplified schematic depiction of the 4 stages of autoimmune adrenocortical destruction.

and elevated renin concentrations suggesting that zona glomerulosa may be more prone to autoimmune destruction than zona fasciculata (27–29). The latter may be initially spared owing to the high local concentrations of cortisol responsible for suppressing antigen presentation to immune cells and procuring local anti-inflammatory properties (30). The second stage is characterized by an impaired cortisol response to synthetic ACTH administered intravenously, while in third and fourth stages, ACTH increases and serum cortisol drops, respectively (27). Early stages of AAI are difficult to diagnose because of the asymptomatic or pauci-symptomatic state since adrenal function is maintained by high plasma ACTH/renin (31). Stages 1 and 2 may be reversible in sporadic AAI. This was not true in APS types 1 and 2/4, where respectively stage 1 and stage 2 were defined as the point of no return with a cumulative risk of 100% (28). Progression of AAI occurred in most patients within 11 years, while no progression was seen after 19 years of follow up in a large cohort of 143 antibody positive patients. Progression was higher in males, patients who had APS type 1 and impaired adrenal function (28). However, antibody titers did not affect risk of progression, probably because AAI is mediated by cellular immunity and antibodies are in fact markers of the disease (27, 28). In 100 asymptomatic antibody positive patients followed up for 20 years, impairment of adrenal function occurred at an annual rate of 4.9% (29). Treatment with glucocorticoids in stressful conditions such as a major surgery or illness may be considered for patients at stages 1 and 2 and close monitoring of patients with higher risk of progression to clinical disease is warranted (children, APS type 1, high antibody titers and already at stage 1) (29, 32).

Because of the insidious course of the disease, patients may not be diagnosed until they present with adrenal crisis. Adrenal crisis is an acute, life-threatening condition more frequently seen in PAI than central AI (33). The frequency of adrenal crisis varied from 4.4 to 17/100 patient-years (11, 34, 35), depending on the definition used for adrenal crises and the characteristics of patients included in each of the studies. A prospective study by Hahner et al., evaluated the incidence of adrenal crisis in 423 patients followed up for 2 years. It was found to be persistently high even in adequately educated patients, reaching 8.3 crises/100 patient-years with a high mortality, estimated at 0.5 adrenal crisis related deaths/100 patient-years (36). A much lower incidence was reported in Switzerland despite insufficient patient education regarding their disease (37). Recently, an analysis of the biggest cohort described to date, including 2694 patients from the European Adrenal Insufficiency Registry, revealed an adrenal crisis incidence of 6.53/100 patient-years, with more than one episode per year in 16% of patients with PAI (38). In the absence of a universal definition of adrenal crisis, its recognition can prove to be difficult. The most used definition is an unstable hemodynamic state (absolute or relative hypovolemia/shock) recovering within 1 to 2 hours following parenteral glucocorticoid administration (4, 33). Although hyponatremia and hyperkalemia are very common features in adrenal crisis, clinicians should beware of patients with multiple episodes of vomiting and severe dehydration because they can present with normal levels of serum potassium and sodium (6). Adrenal crisis can be triggered by numerous precipitating events, namely gastrointestinal infections, fever, emotional stress, major surgery, pregnancy, strenuous physical activity and noncompliance (34–37, 39). In 7-14% of adrenal crises, no precipitating event could be identified (34, 36). Patients at higher risk of adrenal crisis were especially those who have a history of a previous adrenal crisis (threefold increase) (36), but also patients that are female (35), older (40) and have diabetes insipidus (35), type 1 diabetes mellitus (41) and cardiac, neurological or pulmonary comorbidities (35, 39). Conversely, the latter comorbidities were not shown to be associated with a higher risk of adrenal crises according to the new study by Quinkler et al. (38). However, they did have more infections associated with adrenal crisis occurrence, confirming

their immunocompromised state and inability to defend against viral infections owing to a natural killer cell dysfunction (23, 38). Eight % of annual cases of adrenal crises will need in hospital treatment and admission rates were highest in patients aged more than 60 years old (41). In children, adrenal crisis frequency was reported to be 3.4/100 patient-years and children diagnosed with salt wasting congenital adrenal hyperplasia (CAH), adrenal hypoplasia congenital (AHC) and AAI were at higher risk (42). Compared to the general population, patients with AI often complain of reduced quality of life and work capacity with more sick day leaves potentially explained by the inability of present treatment modalities to replicate circadian rhythm of cortisol production (6, 13). Another significant issue is the impact of PAI on fertility in women of reproductive age. Few cohort studies have shown that pregnancy rate decreases after diagnosis of PAI even in the absence of associated POI, and a higher risk of cesarean delivery, impaired fetal growth, preterm birth and low birth weight is found (43). Particularly, a German study including 39 women with CAH secondary to 21-hydroxylase deficiency and 54 with AAI, reported reduced fertility only in classic CAH and APS type 2, based on answers from a self-reporting questionnaire (44). This suggests that a more severe course of disease and/or the presence of other comorbid autoimmune disorders largely impact fertility outcomes. There are no clear explanations for the underlying mechanisms of reduced fertility in women with AAI, in the absence of POI. Perhaps their reduced quality of life and overall dissatisfaction with current treatment modalities may play a significant role in hindering successful pregnancies.

ETIOLOGIES

The most common cause of PAI in the adult population is autoimmune destruction of the adrenal cortex (1). Other potential causes include infections, infiltrative diseases, adrenal hemorrhage, surgery, and drugs (1, 6). They are summarized in **Table 1**. AAI remains a diagnosis of exclusion and in the absence of positive antibodies directed against 21-hydroxylase, other diagnoses should be explored; an abdominal computed tomography should be performed. In the event of enlarged adrenal glands, differential diagnoses include an active tuberculous infection, systemic fungal infections in immunocompromised patients, metastases and lymphoma (1). In young men with negative antibodies to 21-hydroxylase, adrenoleukodystrophy should be suspected, even in the absence of neurological symptoms (1, 6). Adrenoleukodystrophy is an X-linked recessive disorder, hence affecting only boys, characterized by defects in *ABCD1* gene causing elevated serum very long chain fatty acids. The clinical spectrum of adrenoleukodystrophy includes cerebral manifestations that may manifest in childhood such as behavior changes, school difficulties, cognitive deficits up to dementia, psychoses and loss of vision and speech. It may be associated with adrenomyeloneuropathy presenting later in middle age with progressive lower body stiffness and weakness (45). The diagnosis of adrenoleukodystrophy should be confirmed by molecular genetic testing (46). In children, however, CAH represents 83% of PAI diagnoses compared to 9.7, 6.1 and 1.2% for AHC, autoimmune AI and adrenoleukodystrophy, respectively (42). 97.2% of CAH were secondary to 21-hydroxylase deficiency in a Chinese pediatric cohort followed up for 29 years (47). A detailed discussion of these diagnoses is outside the scope of this review and will be discussed elsewhere.

PATHOGENESIS OF AUTOIMMUNE AI

AAI is an autoimmune destructive process affecting all three zones of the adrenal cortex in which lymphocytes infiltrate the adrenal parenchyma leading to adrenal fibrosis and atrophy. It is a slow process and AI may not be clinically relevant until most of adrenocortical cells are destroyed (48). Antibodies directed against 21-hydroxylase are specific to AAI and are rarely seen in the general population; hence their presence alongside positive adrenal cortex antibodies allow to accurately diagnose AAI in 99% of cases (19, 49, 50). The presence of antibodies targeting interferon-α2 and interferon-ω should prompt genetic testing for APS type 1 (19, 49). While the detection of anti 21-hydroxylase antibodies is diagnostic of AAI, they have no direct role in pathogenesis and are only biologic markers of autoimmunity (48, 49). In fact, cytotoxic T-cells auto-reactive to steroidogenic enzymes, in particular 21-hydroxylase, infiltrate the adrenal cortex in response to proinflammatory chemokines, CXCL9 and CXCL10, released intrinsically by adrenocortical cells. Hence, adrenocortical cells contribute to their own destruction in the presence of both, a genetic predisposition and environmental triggers (48). Possible triggers are thought to be caused by local viral infections with increased tropism to adrenocortical cells, such as herpes simplex virus 1, cytomegalovirus and adenovirus, as well as interferon alfa treatments and the relatively new checkpoint inhibitors targeting CTLA-4, programmed cell death protein 1 (PD-1), and PD-1 ligand (PD-L1), used in melanoma and lung cancer treatment (48). Genetic variants implicated in AAI development include the autosomal recessive mutation in AIRE gene commonly responsible for APS type 1 (50) but was also implicated in AAI independently of APS type 1 in a recent genome wide association study (51), which reported 2 new alterations in *AIRE*, the strongest one being p.R471C. It also described nine independent risk loci implicated in central immunological tolerance (51), in particular PTPN22, CTLA4, and BACH2 loci whose role in pathogenesis is already established (50, 51) and SH2B3 and SIGLEC5 (51). HLA class II genes also play a central role in predisposition to isolated AAI or APS type 2/4. While HLA DRB1-0301 and DRB1-040 are associated with AAI, it seems that DRB1-0403 is protective against development of Addison's disease (50). Other polymorphisms worth mentioning include the MHC class I chain-related gene A (MICA) allele 5.1, the CIITA (MHC class II transactivator), the master regulator of MHC class II expression, STAT4, PD-L1, and the vitamin D receptor (50). All susceptibility loci described to date are in genes involved in adaptive or innate immunity (52), particularly affecting

TABLE 1 | Etiologies of primary adrenal insufficiency in adults (1, 5, 6, 45).

Etiology	*Associated clinical/biological and radiological features*
AUTOIMMUNE ADRENALITIS	Bilateral adrenal atrophy on computed tomography.
ISOLATED	Polygenic- positive 21-hydroxylase antibodies ± adrenal cortex antibodies/17-hydroxylase antibodies/steroid side chain cleavage enzyme autoantibodies- other AI diseases associated.
APS TYPE 1	Autosomal recessive AIRE mutation- positive interferon antibodies- chronic mucocutaneous candidiasis, hypoparathyroidism, ectodermal dystrophy.
APS TYPE 2	Polygenic- Type 1 diabetes and autoimmune thyroid disease
INFECTIONS	
ACTIVE TUBERCULOUS INFECTION	Enlarged adrenal glands on computed tomography, calcifications may be seen.
SYSTEMIC FUNGAL INFECTIONS	
WATERHOUSE-FRIDERICHSEN SYNDROME	Altered mental state, hypotension, fever, acute adrenal crisis.
SEPTIC CHOC	
OPPORTUNISTIC INFECTIONS IN IMMUNOCOMPROMISED	
INFILTRATIVE DISEASES	Enlarged adrenal glands on computed tomography.
METASTASES FROM LUNG, BREAST, OR KIDNEY CARCINOMAS	Known primary cancer.
SARCOIDOSIS	Other signs specific to infiltrative disease (mediastinal lymph nodes, chronic kidney disease, hypoparathyroidism, diabetes mellitus…)
AMYLOIDOSIS	
HEMOCHROMATOSIS	Hypophysitis may be associated with infiltrative diseases.
ADRENAL HEMORRHAGE	Sudden pain accompanied with acute adrenal crisis.
COAGULATION DISORDERS	Adrenal hemorrhage on computed tomography.
ANTICOAGULANT TREATMENT	Warfarin.
ANTIPHOSPHOLIPID SYNDROME	Positive cardiolipin antibodies, lupus anticoagulant and anti-beta-2 glycoprotein 1.
BILATERAL ADRENALECTOMY	
PRIMARY ADRENAL LYMPHOMA	Enlarged adrenal glands on computed tomography.
DRUGS	
INCREASE CORTISOL METABOLISM	Induction of P450-cytochrome enzymes, CYP3A4, CYP2B1, CYP2B2: phenytoin, rifampicin, phenobarbital
IMPAIRED STEROIDOGENESIS	Ketoconazole, fluconazole, mitotane, metyrapone, etomidate, aminoglutethimide, trilostane. Abiraterone acetate.
ANTAGONIZE GLUCOCORTICOID ACTION ON PERIPHERAL TISSUES	Mifepristone
ADRENOLYTIC	Mitotane
TRIGGER AUTOIMMUNE REACTION	Nivolumab and pembrolizumab
ADRENOLEUKODYSTROPHY	Young men X-linked recessive Defects in *ABCD1* gene Negative antibodies to 21-hydroxylase Elevated serum very long chain fatty acids Progressive neurological deficit, hypogonadism

regulatory T-cell function leading to local intra-adrenal self-reactive cytotoxic T-cells (53). However, novel therapies targeting the cellular immune response or specific genes implicated in AAI pathogenesis have yet to emerge.

ESTABLISHING THE DIAGNOSIS

The Endocrine Society Clinical Practice Guideline published in 2016 recommend confirming the diagnosis of PAI with a corticotropin stimulation test to assess adrenocortical function (3). An algorithm depicting the proposed diagnostic approach in PAI in adults is shown in **Figure 2**. In PAI, adrenal glands are unresponsive to corticotropin stimulation because zona fasciculata is already maximally stimulated by elevated endogenous ACTH (54) and because adrenal cortex is replaced by fibrous tissue (48). In cases when confirmatory test is not possible, a morning serum cortisol of less than 140 nmol/L paired with a morning plasma ACTH above 2-fold the upper limit of normal is consistent with PAI. Measuring plasma renin and aldosterone is also recommended to document mineralocorticoid deficiency (3). There are two types of synthetic corticotropin analogs (ACTH 1-24) that can be used in the corticotropin stimulation test: cosyntropin (Cortrosyn, Amphastar Pharmaceuticals. Inc) and tetracosactrin (Synacthen, Novartis Pharma, Switzerland) and since they both exist in 250 µg formulations, the standard dose test is more practical and is recommended by the Endocrine Society Clinical Practice guideline (3). Diagnostic cutoff values of serum cortisol depend on the assay used: in most immunoassays, 500 nmol/L is often used as the cutoff to establish diagnosis (6). However, newer monoclonal immunoassays such as the Elecsys®Cortisol II from

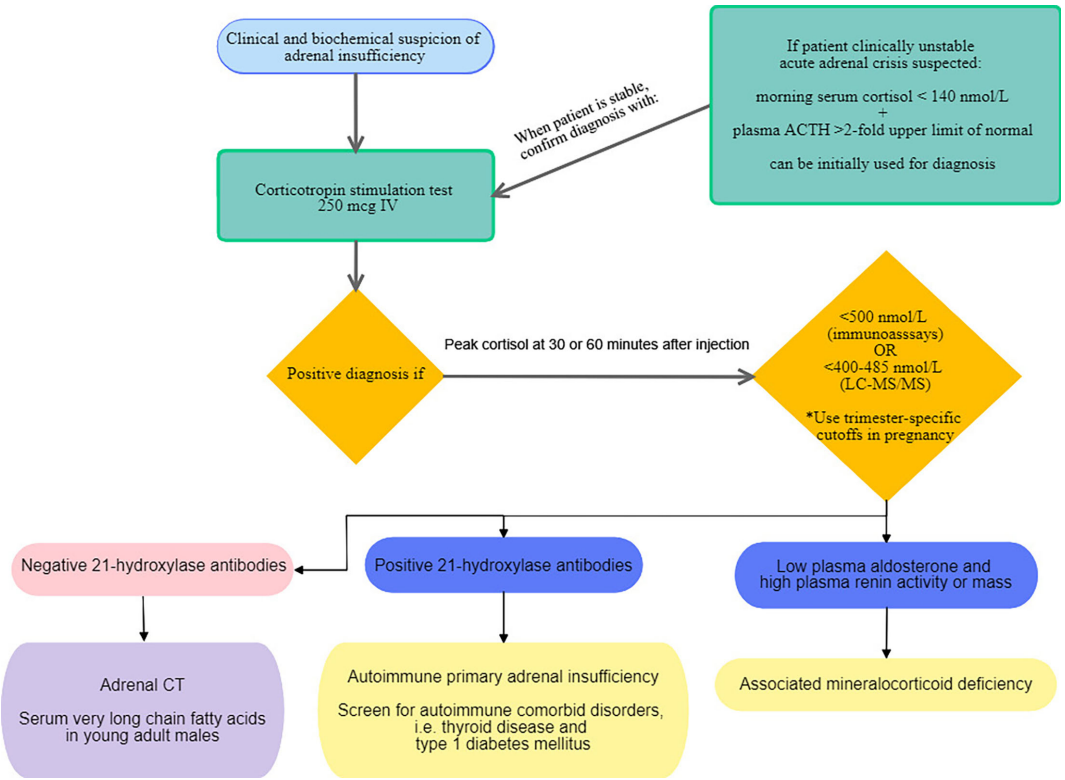

FIGURE 2 | Proposed diagnostic approach for primary adrenal insufficiency in adults.

Roche Diagnostics, have lower cross reactivity with other steroids, thus giving values that are 20-30% lower than those with older assays (55, 56). Two different studies suggested new cutoff thresholds when using the Elecsys®Cortisol II: 374 nmol/L (55) and 403 nmol/L at 30 minutes (56). Furthermore, because liquid chromatography tandem mass spectrometry (LC-MS/MS) measures cortisol more accurately than immunoassays, cutoff values were also redefined to be lower, respectively 400-412 and 485 nmol/L, at 30 and 60 minutes following corticotropin analog injection (56, 57). This would potentially reduce overdiagnosis of AI. Additionally, a baseline cortisol less than 55 nmol/L successfully predicted abnormal response to stimulation test (56). A recent retrospective study conducted on 370 patients in Spain (58), confirmed need for sex-specific and assay-specific cutoff values when interpreting corticotropin test to reduce false positives and increase specificity. It also allowed for a better diagnostic agreement between sampling times at 30 and 60 minutes compared to using general cutoff values. Sampling at 30 minutes following injection of corticotropin analog accurately diagnosed 95% of PAI. However, this was not true for central AI, where sampling at 60 minutes showed better diagnostic accuracy (58). Corticotropin stimulation test can be done anytime of the day because in PAI response to ACTH is independent of circadian rhythm, but morning testing might be more accurate to avoid overdiagnosis in healthy individuals (59, 60). A metanalysis including 13 studies showed that the low dose corticotropin test (1 μg) had better accuracy for diagnosing central AI compared to the standard dose (250 μg) (61). Even so, the low dose is less practical because it necessitates dilution from supplied ampules of 250 μg and is subject to human and technical errors (3, 61). Also, samples are best withdrawn 20 to 30 minutes following injection in order to avoid false positives (59, 61). Neither corticotropin stimulation test nor metyrapone test often used to diagnose central AI, can replace insulin tolerance test considered to be the gold standard for evaluation of hypothalamic pituitary axis. A study by Giordano et al. including 31 patients with central AI, failed to demonstrate superiority of either low dose or standard dose, when both achieved same diagnostic accuracy (62). Another potential role for low dose corticotropin test would be in establishing diagnosis of latent AAI. In fact, it was found abnormal in 88.4% of 33 patients with abnormal cortisol response compared to 66.6% for the standard dose, suggesting that the standard dose might miss some cases of latent PAI (63). More studies are needed to elucidate the diagnostic role of low dose corticotropin testing. Moreover, several factors may affect interpretation of cortisol response to stimulation tests. In particular, cortisol binding globulin (CBG) which binds the majority of circulating cortisol, leaving only 5-10% of plasma cortisol free (1, 6), can be responsible for pitfalls in diagnosis. Because assays measure total serum cortisol and not free cortisol, conditions increasing CBG such as pregnancy, oral contraceptive pills and mitotane,

lead to normal cortisol values, falsely reassuring clinicians (6, 64). Hence, trimester-specific cutoff values were suggested to eliminate false negatives: 700, 800 and 900 nmol/L, respectively in first, second and third trimester (65). In situations where CBG is reduced (sepsis, cirrhosis, nephrotic syndrome, hyperthyroidism and SERPINA6 gene polymorphisms), low cortisol values must be interpreted with caution (6, 66). In such patients, measurement of salivary cortisol has the benefit of being a direct, noninvasive measurement of free cortisol and correlates well with circadian variations of serum cortisol (67). While it is more often used in the diagnosis of hypercortisolism, a recent study demonstrated an added benefit of measuring salivary cortisol in response to corticotropin stimulation in particular in patients taking oral estrogens and in cases of indeterminate serum cortisol at 60 minutes, defined as values between 500 and 599 nmol/L (68). The diagnostic cutoff used in this study was 26 nmol/L (68). Another emerging noninvasive diagnostic test is the measurement of salivary cortisol or cortisone at 60 minutes following administration of 500 μg nasal tetracosactide with mucoadhesive chitosan. This generated the same 60-minute plasma cortisol response as seen with 250 μg intravenous tetracosactide and slightly lower levels of salivary cortisol and cortisone with the nasal formulation (69). Granted, this noninvasive diagnostic method is safe and convenient for patients, it requires additional studies before wide application can be recommended. Finally, measurement of anti 21-hydroxylase antibodies is necessary to establish autoimmune etiology. Commercially available assays include immunofluorescence and autoantibody assays. However, clinicians should keep in mind that they are not standardized and variations in between assays exist (3, 6). When AAI is diagnosed, screening for other comorbid autoimmune diseases should be undertaken; in particular autoimmune thyroid disease (70), type 1 diabetes (3) and POI, especially when steroid side chain cleavage enzyme autoantibodies are detected (29).

MANAGEMENT OF CHRONIC AND ACUTE PAI

Cortisol release follows a circadian and ultradian rhythm. It peaks early in the morning then gradually declines to reach nadir around midnight (6). Pulsatile release of cortisol every 60-90 minutes seems to be intrinsically related to interactions between the pituitary and the adrenal glands and might be independent of supra-pituitary influences (71). The Endocrine Society Clinical Practice Guideline recommends treating all patients with PAI with glucocorticoids and mineralocorticoids when aldosterone deficiency is confirmed (**Figure 3**). The authors agreed that hydrocortisone or cortisone acetate, given in two or three divided oral doses, should be the preferred therapeutic choices (3). Total daily dose should be the equivalent of 15-25 mg of hydrocortisone for adults, with the highest dose given in the morning, in an attempt to replicate the physiologic circadian rhythm (3). However, Caetano et al. recently showed that the daily hydrocortisone dose sufficient to substitute for glucocorticoid deficiency, without signs of under replacement, in 25 adults with AI, was significantly less than that recommended by the Endocrine Society Clinical Practice Guideline (72). The mean replacement dose reported in their study was 7.6 ± 3.5 mg/m2, reflecting daily endogenous cortisol production (72). A recent systematic review of 47 studies reported that although prednisolone therapy increased risk of dyslipidemia and cardiovascular disease, it was as safe and efficacious as hydrocortisone (73). It also suggested that lower doses of hydrocortisone (less than 20 mg/day) had better clinical outcomes, and failed to conclusively demonstrate an added benefit of modified release hydrocortisone or continuous subcutaneous hydrocortisone infusion using insulin pumps (73). Current available regimens fail to replicate both circadian and ultradian rhythmicity of cortisol, potentially explaining the persistently low quality of life that patients with AI often complain about. In fact, non-pulsatile cortisol secretion was found to be associated with poor quality of sleep, poor working memory performance and mood disorders (74, 75). An in-depth discussion of novel forms of glucocorticoid substitution and their role in better mimicking physiologic cortisol secretion will be covered in other chapters of this special topic.

Overreplacement of glucocorticoids predisposes to elevated blood pressure, diabetes, osteoporosis and obesity, and thus should be avoided (6). Monitoring of glucocorticoid therapy is based solely on clinical assessment using signs and symptoms of over and under replacement such as body weight, blood pressure, energy levels, hyperpigmentation and bone mineral density (3). Use of ACTH measurement is especially unreliable and will most definitely lead to overreplacement owing to the disrupted negative feedback of cortisol on ACTH (3). Although hair cortisol concentrations were found to be useful in identifying children overtreated with hydrocortisone, it requires a 1 cm thick hair sample and the technique is not yet widely available (76). Also, gene expression emerged recently as a potential tool for monitoring hydrocortisone therapy. In particular, expression of DSIPI, DDIT4 and FKBP5 increased 2 hours after hydrocortisone infusion and correlated well with normal serum cortisol levels (77).

Mineralocorticoid deficiency is treated with fludrocortisone, approximately 50-200 mcg in adults, in one single morning dose, and ad libitum salt consumption (3). Higher doses are often needed in specific circumstances such as in children because of early mineralocorticoid resistance, in athletic people and in very hot climates because of salt wasting due to excessive perspiration (3, 6). Clinicians should look for signs of overreplacement (hypertension, peripheral edema, hypokalemia) and those of under-replacement (orthostatic hypotension, salt craving and hyperkalemia). Adequate mineralocorticoid replacement can also be determined according to plasma renin concentration, which should be kept in the upper reference range (3).

Until this day, no evidence exists to support dehydroepiandrosterone (DHEA) replacement therapy in all patients with PAI. A trial therapy of 25-50 mg of DHEA for

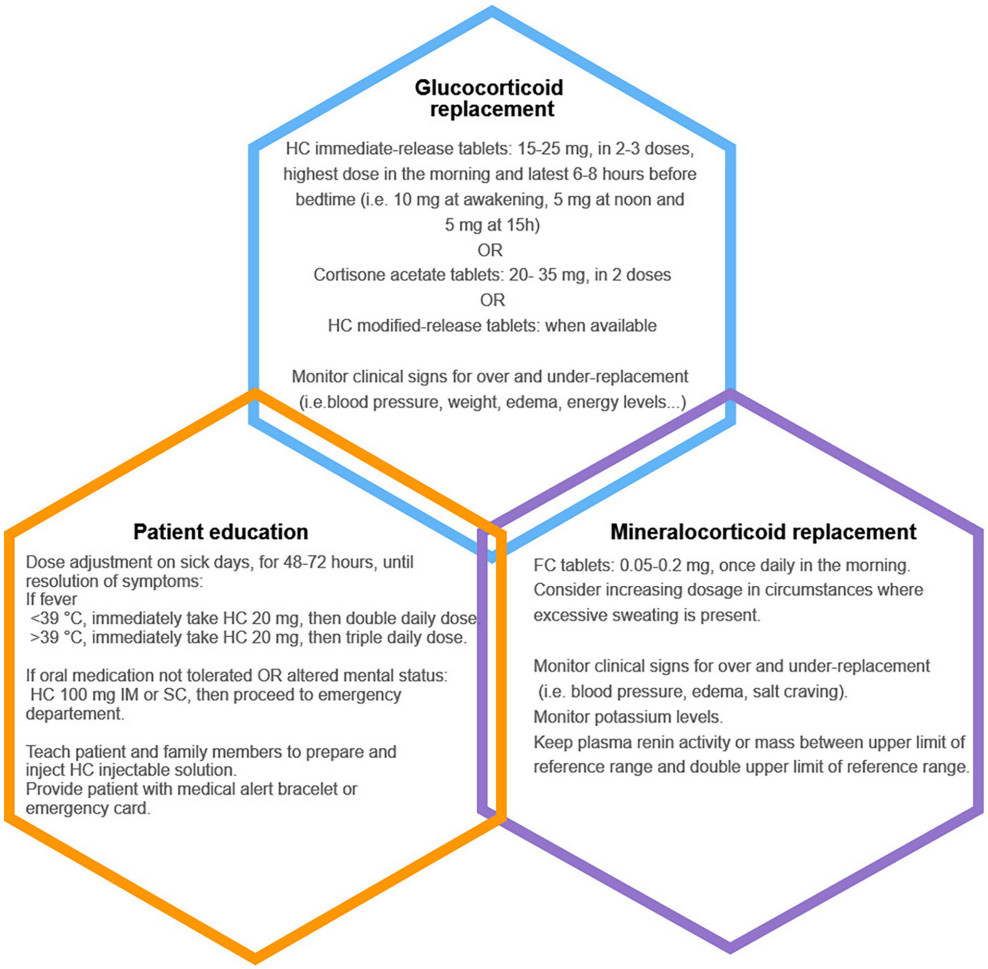

FIGURE 3 | Summary of the three pillars of management in adults with primary adrenal insufficiency. HC, hydrocortisone; FC, fludrocortisone; IM, intramuscularly; SC, subcutaneously.

6 months can be initiated in premenopausal women complaining of reduced libido and depressive moods despite adequate glucocorticoid and mineralocorticoid therapy (3, 6). Unfortunately, well standardized and reliable DHEA formulations are lacking in many countries.

Patient education on dose adaptation and sick day rules is an essential part of management (**Figure 3**). Because all patients with PAI are at risk of adrenal crisis, physicians should insist on increasing patient awareness to their disease by implementing treatment strategies, participating in group sessions, and prescribing at home hydrocortisone injection kits in case of emergencies (78). Hydrocortisone dose adjustments are required during sick days: patients should double or triple their daily oral dose for 48-72 hours until full recovery. If oral medication cannot be tolerated or absorbed, 100 mg of parenteral hydrocortisone should be promptly administered before seeking medical care (3). Subcutaneous hydrocortisone injection is an alternative in patients who cannot self-inject hydrocortisone intramuscularly. It was shown that cortisol increased rapidly following subcutaneous hydrocortisone with a delay of only 11 minutes when compared to intramuscular hydrocortisone (79). A medical alert bracelet or an emergency card can help health care providers identify patients with PAI requiring lifesaving hydrocortisone administration in emergent situations (78). During adrenal crises, it is recommended to administer 100 mg of intravenous hydrocortisone followed by either continuous infusion of 200 mg of hydrocortisone per 24 hours or 50 mg intravenously every 6 hours alongside adequate isotonic saline infusion. Tapering of hydrocortisone can begin 24-48 hours following adrenal crisis, when patients can tolerate oral medication. Fludrocortisone can be re-introduced when hydrocortisone dose is less than 50 mg/day (3, 6). Stress dosing is also needed before dental and minor surgeries (25-75 mg/24h of hydrocortisone, depending on type of procedure). Major surgeries, trauma and delivery require same dosing regimen as in adrenal crises management (3). Management of PAI in special populations including children, patients with CAH, pregnant women and endurance athletes is not discussed in this review.

RESIDUAL GLUCOCORTICOID FUNCTION AND FUTURE PERSPECTIVES

Adrenocortical plasticity is a well-established concept defined by the ability of subcapsular adrenocortical stem cells to proliferate, then migrate into the three zones of the adrenal cortex where zone-specific differentiation occurs, and cells acquire steroidogenic function; all under the influence of ACTH (54). Adrenal mass is also influenced by ACTH: when deficient, adrenal atrophy and hypofunction develop, whereas when increased, adrenal hyperplasia and hyperfunction occur (54). Adrenocortical stem cells may hold the key to understanding the mechanisms of a new emerging concept in autoimmune adrenalitis; that of residual glucocorticoid function. Indeed, residual glucocorticoid production was described in 30-50% of patients years after AAI was diagnosed (80, 81), more commonly in men and in those with a more recent diagnosis. In contrast, residual mineralocorticoid production was only found in 13.5% of patients (80). It was hypothesized that the lack of expression by adrenocortical stem cells of 21-hydroxylase and other enzymes implicated in steroidogenesis, may protect adrenal cortex from complete autoimmune destruction by retaining the possibility of repopulation by intact stem cells (54). Also, turnover of adrenocortical stem cells differs according to sex in rodents and could possibly explain why endogenous residual function is more commonly observed in men (54). Elevated ACTH in PAI may also contribute to the stimulation of proliferation and differentiation of adrenocortical stem cells. However, once hydrocortisone replacement therapy is introduced, endogenous cortisol production declines alongside the ACTH decline and it is not known whether this is responsible for a more rapid loss of residual function or it is but the natural history of autoimmune adrenalitis that is responsible for the loss of function over time (82). Glucocorticoid precursors such as 11-deoxycortisol, 11-deoxycorticosterone and corticosterone, are potential biomarkers of residual endogenous adrenal function because their concentrations correlated well with serum cortisol (81, 82). Although, some patients retained residual adrenal function, they were not protected from increased risk of adrenal crisis and quality of life was still significantly altered (80). Spontaneous recovery of endogenous adrenal function in AAI is rarely described in the literature, 7-16 years following diagnosis, with most cases being partial recovery (83–85). Recent studies suggested that treatment with immunomodulators could be a step forward to reverse autoimmune destruction and allow for regeneration of adrenal cortex. Two of 13 patients who were given subcutaneous tetracosactide for a period of 20 weeks had urine glucocorticoid metabolite in the median range of healthy individuals and peak serum cortisol concentrations above 400 nmol/L, allowing for cessation of glucocorticoid therapy (86). A more recent study evaluated the effect of dual therapy with rituximab and depot tetracosactide in 13 patients with AAI (87). Although dual therapy did not allow full recovery of adrenal function, as defined in this study by peak cortisol > 550 nmol/L at week 48 of treatment, it showed that endogenous cortisol production, quantified by urine metabolites, was increased in 62% of patients (87). Much remains to be explored in regenerative therapies and their role in recovering adrenal function. More studies are needed to allow for a better treatment approach that will mimic physiologic production of cortisol in order to ameliorate quality of life and reduce the morbidity related to PAI.

CONCLUSION

AAI remains a life-threatening condition if not recognized early. Despite medical advances, adrenal crises still occur and quality of life of patients is largely impacted. Attention should be especially given to patients with latent AI, at early stages of adrenal destruction, to prevent adrenal crises from developing and going unnoticed. Impending novel therapies are being explored to determine the best approach to utilize residual adrenal cortisol production and to regenerate adrenocortical cells destroyed by the autoimmune process. Until this is a validated practice, the only effective treatment remains adequate glucocorticoid and mineralocorticoid replacement and patient and physician education.

AUTHOR CONTRIBUTIONS

NY, IB, and AL contributed to conception and design of this review. NY wrote the first draft of the manuscript. IB and AL wrote sections of the manuscript and revised the final draft. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Oelkers W. Adrenal Insufficiency. *N Engl J Med* (1996) 335(16):1206–12. doi: 10.1056/NEJM199610173351607
2. Pazderska A, Pearce SH. Adrenal Insufficiency - Recognition and Management. *Clin Med Lond Engl* (2017) 17(3):258–62. doi: 10.7861/clinmedicine.17-3-258
3. Bornstein SR, Allolio B, Arlt W, Barthel A, Don-Wauchope A, Hammer GD, et al. Diagnosis and Treatment of Primary Adrenal Insufficiency: An Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab* (2016) 101(2):364–89. doi: 10.1210/jc.2015-1710
4. Dineen R, Thompson CJ, Sherlock M. Adrenal Crisis: Prevention and Management in Adult Patients. (2019). Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6566489/ (Accessed 2021 Apr 19).
5. Charmandari E, Nicolaides NC, Chrousos GP. Adrenal Insufficiency. *Lancet Lond Engl* (2014) 383(9935):2152–67. doi: 10.1016/S0140-6736(13)61684-0
6. Husebye ES, Pearce SH, Krone NP, Kämpe O. Adrenal Insufficiency. *Lancet Lond Engl* (2021) 397(10274):613–29. doi: 10.1016/S0140-6736(21)00136-7
7. On the Constitutional and Local Effects of Disease of the Supra-Renal Capsules/by Thomas Addison. [Internet]. Available at: https://wellcomecollection.org/works/xsmzqpdw (Accessed 2021 Apr 17).
8. Laureti S, Vecchi L, Santeusanio F, Falorni A. Is the Prevalence of Addison's Disease Underestimated? *J Clin Endocrinol Metab* (1999) 84(5):1762. doi: 10.1210/jcem.84.5.5677-7
9. Erichsen MM, Løvås K, Skinningsrud B, Wolff AB, Undlien DE, Svartberg J, et al. Clinical, Immunological, and Genetic Features of Autoimmune Primary

Adrenal Insufficiency: Observations From a Norwegian Registry. *J Clin Endocrinol Metab* (2009) 94(12):4882–90. doi: 10.1210/jc.2009-1368
10. Olafsson AS, Sigurjonsdottir HA. Increasing Prevalence of Addison Disease: Results From a Nationwide Study. *Endocr Pract Off J Am Coll Endocrinol Am Assoc Clin Endocrinol* (2016) 22(1):30–5. doi: 10.4158/EP15754.OR
11. Meyer G, Neumann K, Badenhoop K, Linder R. Increasing Prevalence of Addison's Disease in German Females: Health Insurance Data 2008-2012. *Eur J Endocrinol* (2014) 170(3):367–73. doi: 10.1530/EJE-13-0756
12. Løvås K, Husebye ES. High Prevalence and Increasing Incidence of Addison's Disease in Western Norway. *Clin Endocrinol (Oxf)* (2002) 56(6):787–91. doi: 10.1046/j.1365-2265.2002.t01-1-01552.x
13. Chabre O, Goichot B, Zenaty D, Bertherat JGroup 1. Epidemiology of Primary and Secondary Adrenal Insufficiency: Prevalence and Incidence, Acute Adrenal Insufficiency, Long-Term Morbidity and Mortality. *Ann Endocrinol* (2017) 78(6):490–4. doi: 10.1016/j.ando.2017.10.010
14. Kong MF, Jeffcoate W. Eighty-Six Cases of Addison's Disease. *Clin Endocrinol (Oxf)* (1994) 41(6):757–61. doi: 10.1111/j.1365-2265.1994.tb02790.x
15. Bancos I, Hahner S, Tomlinson J, Arlt W. Diagnosis and Management of Adrenal Insufficiency. *Lancet Diabetes Endocrinol* (2015) 3(3):216–26. doi: 10.1016/S2213-8587(14)70142-1
16. Husebye ES, Anderson MS, Kämpe O. Autoimmune Polyendocrine Syndromes. *N Engl J Med* (2018) 378(12):1132–41. doi: 10.1056/NEJMra1713301
17. Dittmar M, Kahaly GJ. Polyglandular Autoimmune Syndromes: Immunogenetics and Long-Term Follow-Up. *J Clin Endocrinol Metab* (2003) 88(7):2983–92. doi: 10.1210/jc.2002-021845
18. Bleicken B, Hahner S, Ventz M, Quinkler M. Delayed Diagnosis of Adrenal Insufficiency Is Common: A Cross-Sectional Study in 216 Patients. *Am J Med Sci* (2010) 339(6):525–31. doi: 10.1097/MAJ.0b013e3181db6b7a
19. Saverino S, Falorni A. Autoimmune Addison's Disease. *Best Pract Res Clin Endocrinol Metab* (2020) 34(1):101379. doi: 10.1016/j.beem.2020.101379
20. Walker BR, Connacher AA, Webb DJ, Edwards CR. Glucocorticoids and Blood Pressure: A Role for the Cortisol/Cortisone Shuttle in the Control of Vascular Tone in Man. *Clin Sci Lond Engl 1979* (1992) 83(2):171–8. doi: 10.1042/cs0830171
21. Arai K, Papadopoulou-Marketou N, Chrousos GP. Aldosterone Deficiency and Resistance. In: *Endotext [Internet]* (2000). South Dartmouth (MA: MDText.com, Inc. Available at: http://www.ncbi.nlm.nih.gov/books/NBK279079/ (Accessed 2021 Apr 19).
22. Oelkers W. Hyponatremia and Inappropriate Secretion of Vasopressin (Antidiuretic Hormone) in Patients With Hypopituitarism. *N Engl J Med* (1989) 321(8):492–6. doi: 10.1056/NEJM198908243210802
23. Bancos I, Hazeldine J, Chortis V, Hampson P, Taylor AE, Lord JM, et al. Primary Adrenal Insufficiency Is Associated With Impaired Natural Killer Cell Function: A Potential Link to Increased Mortality. *Eur J Endocrinol* (2017) 176(4):471–80. doi: 10.1530/EJE-16-0969
24. Yuen KCJ, Chong LE, Koch CA. Adrenal Insufficiency in Pregnancy: Challenging Issues in Diagnosis and Management. *Endocrine* (2013) 44 (2):283–92. doi: 10.1007/s12020-013-9893-2
25. Yamamoto T. Latent Adrenal Insufficiency: Concept, Clues to Detection, and Diagnosis. *Endocr Pract Off J Am Coll Endocrinol Am Assoc Clin Endocrinol* (2018) 24(8):746–55. doi: 10.4158/EP-2018-0114
26. Yamamoto T. History of Stress-Related Health Changes: A Cue to Pursue a Diagnosis of Latent Primary Adrenal Insufficiency. *Intern Med Tokyo Jpn* (2014) 53(3):183–8. doi: 10.2169/internalmedicine.53.1156
27. Betterle C, Scalici C, Presotto F, Pedini B, Moro L, Rigon F, et al. The Natural History of Adrenal Function in Autoimmune Patients With Adrenal Autoantibodies. *J Endocrinol* (1988) 117(3):467–75. doi: 10.1677/joe.0.1170467
28. Naletto L, Frigo AC, Ceccato F, Sabbadin C, Scarpa R, Presotto F, et al. The Natural History of Autoimmune Addison's Disease From the Detection of Autoantibodies to Development of the Disease: A Long-Term Follow-Up Study on 143 Patients. *Eur J Endocrinol* (2019) 180(3):223–34. doi: 10.1530/EJE-18-0313
29. Betterle C, Coco G, Zanchetta R. Adrenal Cortex Autoantibodies in Subjects With Normal Adrenal Function. *Best Pract Res Clin Endocrinol Metab* (2005) 19(1):85–99. doi: 10.1016/j.beem.2004.11.008
30. Gan EH, Pearce SH. Management of Endocrine Disease: Regenerative Therapies in Autoimmune Addison's Disease. *Eur J Endocrinol* (2017) 176 (3):R123–35. doi: 10.1530/EJE-16-0581
31. Ketchum CH, Riley WJ, Maclaren NK. Adrenal Dysfunction in Asymptomatic Patients With Adrenocortical Autoantibodies. *J Clin Endocrinol Metab* (1984) 58(6):1166–70. doi: 10.1210/jcem-58-6-1166
32. Coco G, Dal Pra C, Presotto F, Albergoni MP, Canova C, Pedini B, et al. Estimated Risk for Developing Autoimmune Addison's Disease in Patients With Adrenal Cortex Autoantibodies. *J Clin Endocrinol Metab* (2006) 91 (5):1637–45. doi: 10.1210/jc.2005-0860
33. Rushworth RL, Torpy DJ, Falhammar H. Adrenal Crisis(2019). Available at: https://www.nejm.org/doi/10.1056/NEJMra1807486 (Accessed 2021 Apr 19).
34. Reisch N, Willige M, Kohn D, Schwarz H-P, Allolio B, Reincke M, et al. Frequency and Causes of Adrenal Crises Over Lifetime in Patients With 21-Hydroxylase Deficiency. *Eur J Endocrinol* (2012) 167(1):35–42. doi: 10.1530/EJE-12-0161
35. Hahner S, Loeffler M, Bleicken B, Drechsler C, Milovanovic D, Fassnacht M, et al. Epidemiology of Adrenal Crisis in Chronic Adrenal Insufficiency: The Need for New Prevention Strategies. *Eur J Endocrinol* (2010) 162(3):597–602. doi: 10.1530/EJE-09-0884
36. Hahner S, Spinnler C, Fassnacht M, Burger-Stritt S, Lang K, Milovanovic D, et al. High Incidence of Adrenal Crisis in Educated Patients With Chronic Adrenal Insufficiency: A Prospective Study. *J Clin Endocrinol Metab* (2015) 100(2):407–16. doi: 10.1210/jc.2014-3191
37. Notter A, Jenni S, Christ E. Evaluation of the Frequency of Adrenal Crises and Preventive Measures in Patients With Primary and Secondary Adrenal Insufficiency in Switzerland. *Swiss Med Wkly* (2018) 148:w14586. doi: 10.4414/smw.2018.14586
38. Quinkler M, Murray RD, Zhang P, Marelli C, Petermann R, Isidori AM, et al. Characterization of Patients With Adrenal Insufficiency and Frequent Adrenal Crises. *Eur J Endocrinol* (2021) 184(6):761–71. doi: 10.1530/EJE-20-1324
39. Smans LCCJ, van der Valk ES, Hermus ARMM, Zelissen PMJ. Incidence of Adrenal Crisis in Patients With Adrenal Insufficiency. *Clin Endocrinol (Oxf)* (2016) 84(1):17–22. doi: 10.1111/cen.12865
40. Rushworth RL, Torpy DJ. A Descriptive Study of Adrenal Crises in Adults With Adrenal Insufficiency: Increased Risk With Age and in Those With Bacterial Infections. *BMC Endocr Disord* (2014) 14:79. doi: 10.1186/1472-6823-14-79
41. White K, Arlt W. Adrenal Crisis in Treated Addison's Disease: A Predictable But Under-Managed Event. *Eur J Endocrinol* (2010) 162(1):115–20. doi: 10.1530/EJE-09-0559
42. Eyal O, Levin Y, Oren A, Zung A, Rachmiel M, Landau Z, et al. Adrenal Crises in Children With Adrenal Insufficiency: Epidemiology and Risk Factors. *Eur J Pediatr* (2019) 178(5):731–8. doi: 10.1007/s00431-019-03348-1
43. Bensing S, Giordano R, Falorni A. Fertility and Pregnancy in Women With Primary Adrenal Insufficiency. *Endocrine* (2020) 70(2):211–7. doi: 10.1007/s12020-020-02343-z
44. Remde H, Zopf K, Schwander J, Quinkler M. Fertility and Pregnancy in Primary Adrenal Insufficiency in Germany. *Horm Metab Res Horm Stoffwechselforschung Horm Metab* (2016) 48(5):306–11. doi: 10.1055/s-0035-1565183
45. Raymond GV, Moser AB, Fatemi A. X-Linked Adrenoleukodystrophy, in: (1993). Seattle (WA): University of Washington, Seattle: GeneReviews® [Internet]. Available at: http://www.ncbi.nlm.nih.gov/books/NBK1315/ (Accessed 2021 Apr 26).
46. Engelen M, Kemp S, de Visser M, van Geel BM, Wanders RJA, Aubourg P, et al. X-Linked Adrenoleukodystrophy (X-ALD): Clinical Presentation and Guidelines for Diagnosis, Follow-Up and Management. *Orphanet J Rare Dis* (2012) 7:51. doi: 10.1186/1750-1172-7-51
47. Wijaya M, Huamei M, Jun Z, Du M, Li Y, Chen Q, et al. Etiology of Primary Adrenal Insufficiency in Children: A 29-Year Single-Center Experience. *J Pediatr Endocrinol Metab JPEM* (2019) 32(6):615–22. doi: 10.1515/jpem-2018-0445
48. Hellesen A, Bratland E, Husebye ES. Autoimmune Addison's Disease - An Update on Pathogenesis. *Ann Endocrinol* (2018) 79(3):157–63. doi: 10.1016/j.ando.2018.03.008

49. Husebye E, Løvås K. Pathogenesis of Primary Adrenal Insufficiency. *Best Pract Res Clin Endocrinol Metab* (2009) 23(2):147–57. doi: 10.1016/j.beem.2008.09.004
50. Falorni A, Brozzetti A, Perniola R. From Genetic Predisposition to Molecular Mechanisms of Autoimmune Primary Adrenal Insufficiency. *Front Horm Res* (2016) 46:115–32. doi: 10.1159/000443871
51. Eriksson D, Røyrvik EC, Aranda-Guillén M, Berger AH, Landegren N, Artaza H, et al. GWAS for Autoimmune Addison's Disease Identifies Multiple Risk Loci and Highlights AIRE in Disease Susceptibility. *Nat Commun* (2021) 12 (1):959. doi: 10.1038/s41467-021-21015-8
52. Mitchell AL, Pearce SHS. Autoimmune Addison Disease: Pathophysiology and Genetic Complexity. *Nat Rev Endocrinol* (2012) 8(5):306–16. doi: 10.1038/nrendo.2011.245
53. Betterle C, Presotto F, Furmaniak J. Epidemiology, Pathogenesis, and Diagnosis of Addison's Disease in Adults. *J Endocrinol Invest* (2019) 42 (12):1407–33. doi: 10.1007/s40618-019-01079-6
54. Pearce SHS, Gan EH, Napier C. Management of Endocrine Disease: Residual Adrenal Function in Addison's Disease. *Eur J Endocrinol* (2021) 184(2):R61–7. doi: 10.1530/EJE-20-0894
55. Raverot V, Richet C, Morel Y, Raverot G, Borson-Chazot F. Establishment of Revised Diagnostic Cut-Offs for Adrenal Laboratory Investigation Using the New Roche Diagnostics Elecsys® Cortisol II Assay. *Ann Endocrinol* (2016) 77 (5):620–2. doi: 10.1016/j.ando.2016.05.002
56. Javorsky BR, Raff H, Carroll TB, Algeciras-Schimnich A, Singh RJ, Colón-Franco JM, et al. New Cutoffs for the Biochemical Diagnosis of Adrenal Insufficiency After ACTH Stimulation Using Specific Cortisol Assays. *J Endocr Soc* (2021) 5(4):bvab022. doi: 10.1210/jendso/bvab022
57. Ueland GÅ, Methlie P, Øksnes M, Thordarson HB, Sagen J, Kellmann R, et al. The Short Cosyntropin Test Revisited: New Normal Reference Range Using Lc-Ms/Ms. *J Clin Endocrinol Metab* (2018) 103(4):1696–703. doi: 10.1210/jc.2017-02602
58. Ortiz-Flores AE, Santacruz E, Jiménez-Mendiguchia L, García-Cano A, Nattero-Chávez L, Escobar-Morreale HF, et al. Role of Sampling Times and Serum Cortisol Cut-Off Concentrations on the Routine Assessment of Adrenal Function Using the Standard Cosyntropin Test in an Academic Hospital From Spain: A Retrospective Chart Review. (2018). Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5942445/ (Accessed 2021 Apr 29).
59. Park YJ, Park KS, Kim JH, Shin CS, Kim SY, Lee HK. Reproducibility of the Cortisol Response to Stimulation With the Low Dose (1 Microg) of ACTH. *Clin Endocrinol (Oxf)* (1999) 51(2):153–8. doi: 10.1046/j.1365-2265.1999.00773.x
60. Dickstein G, Shechner C, Nicholson WE, Rosner I, Shen-Orr Z, Adawi F, et al. Adrenocorticotropin Stimulation Test: Effects of Basal Cortisol Level, Time of Day, and Suggested New Sensitive Low Dose Test. *J Clin Endocrinol Metab* (1991) 72(4):773–8. doi: 10.1210/jcem-72-4-773
61. Kazlauskaite R, Evans AT, Villabona CV, Abdu TAM, Ambrosi B, Atkinson AB, et al. Corticotropin Tests for Hypothalamic-Pituitary- Adrenal Insufficiency: A Metaanalysis. *J Clin Endocrinol Metab* (2008) 93(11):4245–53. doi: 10.1210/jc.2008-0710
62. Giordano R, Picu A, Bonelli L, Balbo M, Berardelli R, Marinazzo E, et al. Hypothalamus-Pituitary-Adrenal Axis Evaluation in Patients With Hypothalamo-Pituitary Disorders: Comparison of Different Provocative Tests. *Clin Endocrinol (Oxf)* (2008) 68(6):935–41. doi: 10.1111/j.1365-2265.2007.03141.x
63. Dekkers OM, Timmermans JM, Smit JWA, Romijn JA, Pereira AM. Comparison of the Cortisol Responses to Testing With Two Doses of ACTH in Patients With Suspected Adrenal Insufficiency. *Eur J Endocrinol* (2011) 164(1):83–7. doi: 10.1530/EJE-10-0621
64. van Seters AP, Moolenaar AJ. Mitotane Increases the Blood Levels of Hormone-Binding Proteins. *Acta Endocrinol (Copenh)* (1991) 124(5):526–33. doi: 10.1530/acta.0.1240526
65. Lebbe M, Arlt W. What Is the Best Diagnostic and Therapeutic Management Strategy for an Addison Patient During Pregnancy? *Clin Endocrinol (Oxf)* (2013) 78(4):497–502. doi: 10.1111/cen.12097
66. Wallace I, Cunningham S, Lindsay J. The Diagnosis and Investigation of Adrenal Insufficiency in Adults. *Ann Clin Biochem* (2009) 46(5):351–67. doi: 10.1258/acb.2009.009101
67. Inder WJ, Dimeski G, Russell A. Measurement of Salivary Cortisol in 2012 - Laboratory Techniques and Clinical Indications. *Clin Endocrinol (Oxf)* (2012) 77(5):645–51. doi: 10.1111/j.1365-2265.2012.04508.x
68. Nolan BJ, Sorbello J, Brown N, Dimeski G, Inder WJ. Characterization of the Serum and Salivary Cortisol Response to the Intravenous 250 µg ACTH1-24 Stimulation Test. *Endocrine* (2018) 59(3):520–8. doi: 10.1007/s12020-017-1505-0
69. Elder CJ, Vilela R, Johnson TN, Taylor RN, Kemp EH, Keevil BG, et al. Pharmacodynamic Studies of Nasal Tetracosactide With Salivary Glucocorticoids for a Noninvasive Short Synacthen Test. *J Clin Endocrinol Metab* (2020) 105(8):2692–703. doi: 10.1210/clinem/dgaa323
70. Yamamoto T. Comorbid Latent Adrenal Insufficiency With Autoimmune Thyroid Disease. *Eur Thyroid J* (2015) 4(3):201–6. doi: 10.1159/000433532
71. Lightman SL, Conway-Campbell BL. The Crucial Role of Pulsatile Activity of the HPA Axis for Continuous Dynamic Equilibration. *Nat Rev Neurosci* (2010) 11(10):710–8. doi: 10.1038/nrn2914
72. Caetano CM, Sliwinska A, Madhavan P, Grady J, Malchoff CD. Empiric Determination of the Daily Glucocorticoid Replacement Dose in Adrenal Insufficiency. *J Endocr Soc* (2020) 4(11):bvaa145. doi: 10.1210/jendso/bvaa145
73. Kiko N, Kalhan A. Comparison of Various Glucocorticoid Replacement Regimens Used in Chronic Adrenal Insufficiency: A Systematic Review. *Dubai Diabetes Endocrinol J* (2020) 26(2):50–68. doi: 10.1159/000508321
74. Kalafatakis K, Russell GM, Harmer CJ, Munafo MR, Marchant N, Wilson A, et al. Ultradian Rhythmicity of Plasma Cortisol Is Necessary for Normal Emotional and Cognitive Responses in Man. *Proc Natl Acad Sci* (2018) 115 (17):E4091–100. doi: 10.1073/pnas.1714239115
75. Kalafatakis K, Russell GM, Lightman SL. Mechanisms in Endocrinology: Does Circadian and Ultradian Glucocorticoid Exposure Affect the Brain? *Eur J Endocrinol* (2019) 180(2):R73–89. doi: 10.1530/EJE-18-0853
76. Noppe G, van Rossum EFC, Vliegenthart J, Koper JW, van den Akker ELT. Elevated Hair Cortisol Concentrations in Children With Adrenal Insufficiency on Hydrocortisone Replacement Therapy. *Clin Endocrinol (Oxf)* (2014) 81 (6):820–5. doi: 10.1111/cen.12551
77. Sævik ÅB, Wolff AB, Björnsdottir S, Simunkova K, Hynne MS, Dolan DWP, et al. Potential Transcriptional Biomarkers to Guide Glucocorticoid Replacement in Autoimmune Addison's Disease. *J Endocr Soc* (2021) 5(3): bvaa202. doi: 10.1210/jendso/bvaa202
78. Guignat L. Therapeutic Patient Education in Adrenal Insufficiency. *Ann Endocrinol* (2018) 79(3):167–73. doi: 10.1016/j.ando.2018.03.002
79. Hahner S, Burger-Stritt S, Allolio B. Subcutaneous Hydrocortisone Administration for Emergency Use in Adrenal Insufficiency. *Eur J Endocrinol* (2013) 169(2):147–54. doi: 10.1530/EJE-12-1057
80. Sævik ÅB, Åkerman A-K, Methlie P, Quinkler M, Jørgensen AP, Höybye C, et al. Residual Corticosteroid Production in Autoimmune Addison Disease. *J Clin Endocrinol Metab* (2020) 105(7):2430–41. doi: 10.1210/clinem/dgaa256.
81. Vulto A, Bergthorsdottir R, van Faassen M, Kema IP, Johannsson G, van Beek AP. Residual Endogenous Corticosteroid Production in Patients With Adrenal Insufficiency. *Clin Endocrinol (Oxf)* (2019) 91(3):383–90. doi: 10.1111/cen.14006
82. Napier C, Allinson K, Gan EH, Mitchell AL, Gilligan LC, Taylor AE, et al. Natural History of Adrenal Steroidogenesis in Autoimmune Addison's Disease Following Diagnosis and Treatment. *J Clin Endocrinol Metab* (2020) 105(7):2322–30. doi: 10.1210/clinem/dgaa187
83. Smans LCCJ, Zelissen PMJ. Partial Recovery of Adrenal Function in a Patient With Autoimmune Addison's Disease. *J Endocrinol Invest* (2008) 31(7):672–4. doi: 10.1007/BF03345623
84. Baxter M, Gorick S, Swords FM. Recovery of Adrenal Function in a Patient With Confirmed Addison's Disease. *Endocrinol Diabetes Metab Case Rep* (2013) 2013:130070. doi: 10.1530/EDM-13-0070
85. Nordin BE. Addison's Disease With Partial Recovery. *Proc R Soc Med* (1955) 48(12):1024–6.
86. Gan EH, MacArthur K, Mitchell AL, Hughes BA, Perros P, Ball SG, et al. Residual Adrenal Function in Autoimmune Addison's Disease: Improvement After Tetracosactide (ACTH1-24) Treatment. *J Clin Endocrinol Metab* (2014) 99(1):111–8. doi: 10.1210/jc.2013-2449

87. Napier C, Gan EH, Mitchell AL, Gilligan LC, Rees DA, Moran C, et al. Residual Adrenal Function in Autoimmune Addison's Disease—Effect of Dual Therapy With Rituximab and Depot Tetracosactide. *J Clin Endocrinol Metab* (2020) 105(4):e1250–9. doi: 10.1210/clinem/dgz287

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REVIEW
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Disorders of Sex Development of Adrenal Origin

Gabriela P. Finkielstain[1†], *Ana Vieites*[1], *Ignacio Bergadá*[1] *and Rodolfo A. Rey*[1,2*]

[1] Centro de Investigaciones Endocrinológicas "Dr. César Bergadá" (CEDIE), CONICET – FEI – División de Endocrinología, Hospital de Niños Ricardo Gutiérrez, Buenos Aires, Argentina, [2] Universidad de Buenos Aires, Facultad de Medicina, Departamento de Biología Celular, Histología, Embriología y Genética, Buenos Aires, Argentina

Disorders of Sex Development (DSD) are anomalies occurring in the process of fetal sexual differentiation that result in a discordance between the chromosomal sex and the sex of the gonads and/or the internal and/or external genitalia. Congenital disorders affecting adrenal function may be associated with DSD in both 46,XX and 46,XY individuals, but the pathogenic mechanisms differ. While in 46,XX cases, the adrenal steroidogenic disorder is responsible for the genital anomalies, in 46,XY patients DSD results from the associated testicular dysfunction. Primary adrenal insufficiency, characterized by a reduction in cortisol secretion and overproduction of ACTH, is the rule. In addition, patients may exhibit aldosterone deficiency leading to salt-wasting crises that may be life-threatening. The trophic effect of ACTH provokes congenital adrenal hyperplasia (CAH). Adrenal steroidogenic defects leading to 46,XX DSD are 21-hydroxylase deficiency, by far the most prevalent, and 11β-hydroxylase deficiency. Lipoid Congenital Adrenal Hyperplasia due to StAR defects, and cytochrome P450scc and P450c17 deficiencies cause DSD in 46,XY newborns. Mutations in SF1 may also result in combined adrenal and testicular failure leading to DSD in 46,XY individuals. Finally, impaired activities of 3βHSD2 or POR may lead to DSD in both 46,XX and 46,XY individuals. The pathophysiology, clinical presentation and management of the above-mentioned disorders are critically reviewed, with a special focus on the latest biomarkers and therapeutic development.

Keywords: adrenal insufficiency, aldosterone, congenital adrenal hyperplasia, cortisol, DSD, glucocorticoid, lipoid, mineralocorticoid

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Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Spain

****Correspondence:***
Rodolfo A. Rey
rodolforey@cedie.org.ar

†Present address:
Gabriela P. Finkielstain,
Department of Medical Affairs, Takeda Pharma S.A., Buenos Aires, Argentina

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1 INTRODUCTION

The term Disorders of Sex Development (DSD) refers to a wide range of anomalies occurring in the process of fetal sexual differentiation of the gonads and/or the genitalia, resulting in discordance between the chromosomal sex and the gonads and/or the internal and/or external genitalia.

1.1 The Physiology of Fetal Sex Differentiation

The chromosomal sex is determined at fertilization, depending on whether the spermatozoon carries an X or a Y chromosome. Nevertheless, during the first six weeks of embryogenesis in the human, there is no evidence of sex differences. This period is, therefore, called "undifferentiated" and is characterized by the existence of bipotential gonadal ridges, two sets of unipotential internal

ducts –the Wolffian and the Müllerian ducts–, and bipotential urogenital sinus and primordia of external genitalia, in both the XX and the XY embryo.

During the 7th week, the onset of the expression of SRY (Sex-determining region on the Y chromosome) in the XY embryo drives the indifferent gonad towards testicular differentiation by disrupting the existing balance between pro-testicular and pro-ovarian genes (1, 2). The testis secretes androgens and anti-Müllerian hormone (AMH), whose actions are critical in the process of genital differentiation (**Figure 1**). Androgens are responsible for Wolffian duct development into the epididymis, vas deferens and seminal vesicle, and the virilization of the urogenital sinus and the external genitalia. The urogenital sinus gives rise to the bladder, the proximal portion of the urethra and the prostate. The genital tubercle forms the penis, the labioscrotal folds differentiate into the scrotum and the urogenital folds fuse to form the penile urethra. The genital and the urinary systems flow into a single orifice. On the other hand, AMH induces the regression of the Müllerian ducts.

In the XX embryo, the ovaries do not produce androgens or AMH at this stage of development. Therefore, the Wolffian ducts regress, and the urogenital sinus and external genitalia follow the female pathway with no need for estrogen activity. The Müllerian ducts form the Fallopian tubes, the uterus and the upper part of the vagina. The urogenital sinus gives rise to the bladder, the urethra and the lower part of the vagina. The genital tubercle forms the clitoris, the labioscrotal folds differentiate into the labia majora and the urogenital folds into the labia minora. A detailed description of the physiology and the molecular and cellular biology of sex differentiation in mammals is available elsewhere (4).

1.2 Pathogenesis of DSD

It is simple to understand that physiologically abnormal gonads containing dysgenetic testicular and/or ovarian tissue may develop in fetuses with sex chromosome abnormalities, such as 46,XX/46,XY, 45,X/46,XY or other sex chromosome mosaicisms or chimerism. These are known as "sex-chromosome DSD". However, DSD can also occur in individuals with typical 46,XX or 46,XY karyotypes. The underlying pathogenic mechanisms involve either an androgen excess in the XX fetus or a deficient testicular hormone activity in the XY fetus (5, 6).

1.2.1 Virilization of the 46,XX Fetus

Excessive androgen action induces virilization of the XX fetus (**Figure 2A**). If there is exposure during the first trimester of intrauterine life, the final development of the external genitalia may be from completely male, when androgen levels are very high, to a milder virilization when androgen levels are lower. The different degrees of virilization have been classified by Prader in stages 1 to 5 (**Figures 2B–D**) (8). A later exposure to intrauterine androgens can no longer provoke a fusion of the labioscrotal folds but results in clitoris enlargement and labial swelling and rugosity.

Androgens may have different origins. Exaggerated production may arise from the adrenal cortex, that normally synthesizes androgens (**Figures 2A** and **3**), or from their lack of aromatization to estrogens by the placenta. Alternatively, androgen excess results from the existence of testicular tissue, in disorders such as ovotesticular or testicular DSD, or from maternal sources, such as adrenal or ovarian neoplasms, non-neoplastic disorders or androgenic drug use (**Figure 2A**).

1.2.1.1 Androgens of Fetal or Fetoplacental Origin

Excessive androgen production from adrenal origin results from pathogenic variants in genes involved in steroidogenesis and encoding 21-hydroxylase, 11β-hydroxylase, 3β-hydroxysteroid dehydrogenase or P450 oxidoreductase. Congenital adrenal hyperplasia (by 21-hydroxylase alteration) is the commonest cause of virilization of the XX fetus, and it will be discussed in detail in this review.

Androgens of fetal gonadal origin may also be the cause of virilization *in utero* of 46,XX patients: the existence of dysgenetic testicular tissue, alone (46,XX testicular DSD) or associated with ovarian tissue (46,XX ovotesticular DSD).

Androgens are converted to estrogens through the action of the enzyme P450 aromatase (**Figure 3**). During pregnancy, the fetal component of the placenta expresses aromatase and is the major site of estrogen synthesis. Inactivating mutations in *CYP19A1*, encoding aromatase, result in an accumulation of fetal androgens that provoke the virilization of the XX fetus (9). Maternal virilization occurs during pregnancy but disappears progressively after delivery.

Adrenal function is not altered in patients with testicular/ovotesticular DSD or with placental aromatase deficiency; therefore, these conditions will not be further discussed in this review.

1.2.1.2 Androgens of Maternal Origin

Virilization is notoriously milder when the excess of androgens is of maternal source because the placenta has a protective role by aromatizing them to estrogens (10). Nonetheless, some degree of virilization may occur in the 46,XX fetus if her mother suffered from androgen-secreting neoplasms, such as granulosa/theca cell tumors, thecomas and Sertoli-Leydig cell tumors of the ovary, or androgen secreting adrenal carcinomas and adenomas. In these cases, virilization of the mother persists until treatment, whereas virilization of the fetus partially regresses (10). Other non-neoplastic disorders characterized by androgen production are pregnancy luteomas and hyperreactio luteinalis (11). Since these are self-limited disorders of pregnancy, virilization wanes in both the mother and the newborn after birth.

1.2.2 Undervirilization of the 46,XY Fetus

Insufficient testicular hormone action on target organs results in undervirilization of the XY newborn. When the lack of androgen action is complete, the newborn has an entirely female aspect of the external genitalia, and the condition may go undiagnosed until pubertal age (5, 6). The underlying etiologies can be classified into three groups: testicular dysgenesis, steroid synthesis defects and target organ defects.

Dysgenetic DSD is due to abnormalities in the process of testis differentiation (2), leading to a fetal-onset hypogonadism characterized by low or undetectable testosterone and AMH levels (12). The newborn has female or ambiguous external

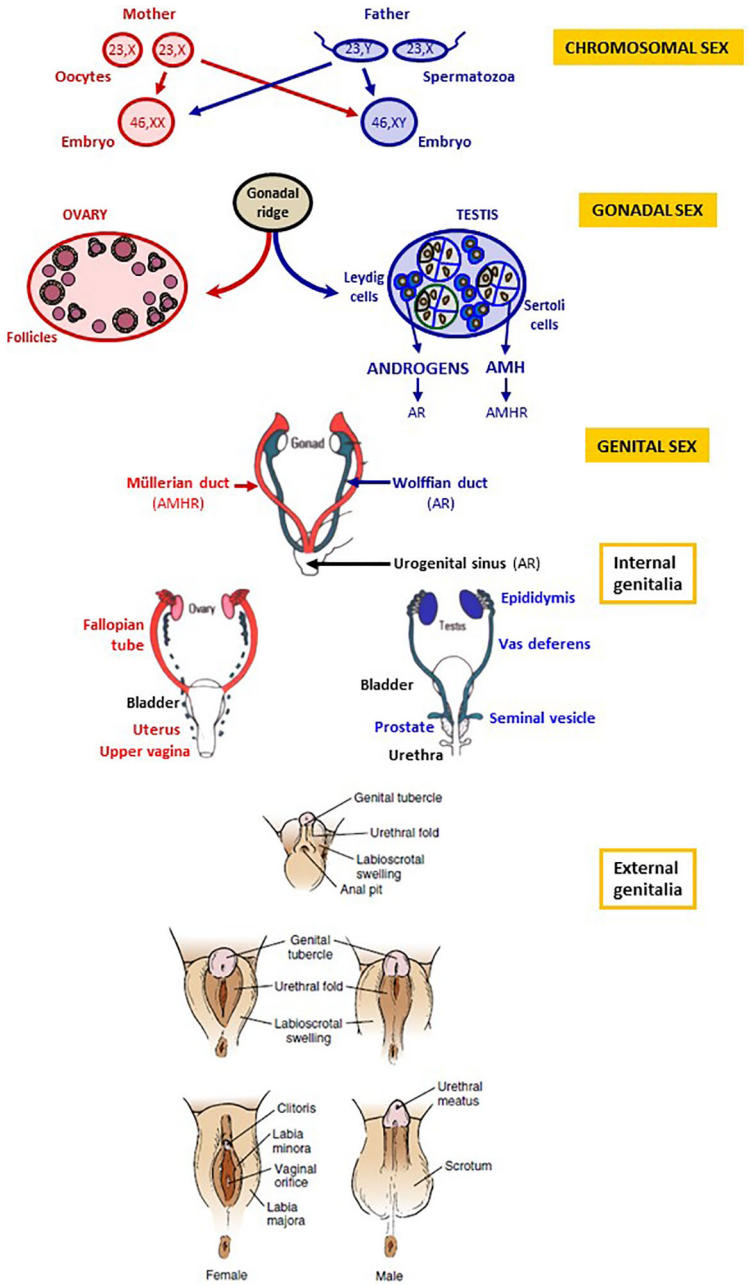

FIGURE 1 | Chromosomal, gonadal and genital sex. Chromosomal sex is determined at fertilization, according to the X or Y chromosome carried by the spermatozoon. Gonadal sex differentiation occurs during the 7th week of gestation: testes secrete androgens and anti-Müllerian hormone (AMH). The ovaries do not produce androgens and AMH in the first trimester of gestation. Genital differentiation is driven by testicular hormones: androgens produced by Leydig cells bind to the androgen receptor (AR) and induce the differentiation of the Wolffian ducts into the epididymides, the vasa deferentia and the seminal vesicles as well as the virilization of the urogenital sinus and of the external genitalia. In the absence of androgen action, the Wolffian ducts regress, and the urogenital sinus and the external genitalia undergo female differentiation. AMH, secreted by Sertoli cells, binds to the AMH receptor (AMHR) and provokes Müllerian duct regression; in the absence of AMH action, Müllerian ducts form the Fallopian tubes, the uterus and the upper vagina. Reproduced with permission from: Freire AV, Ropelato MG, Rey RA. Ovaries and Testes. In: Kovacs CS, Deal CL, editors. Maternal-Fetal and Neonatal Endocrinology: Physiology, Pathophysiology, and Clinical Management. Elsevier, 2020, pp 625-641. Copyright © 2000 Elsevier Inc (3).

genitalia and persistence of Müllerian derivatives, i.e. uterus and Fallopian tubes.

A dissociated testicular dysfunction occurs in patients with normal AMH production but impaired androgen secretion (12). These patients have female or ambiguous external genitalia but do not have uterus and Fallopian tubes. The defect in androgen production may be limited to the testis, e.g. in Leydig cell hypoplasia due to mutations in *LHCGR*, the gene encoding the

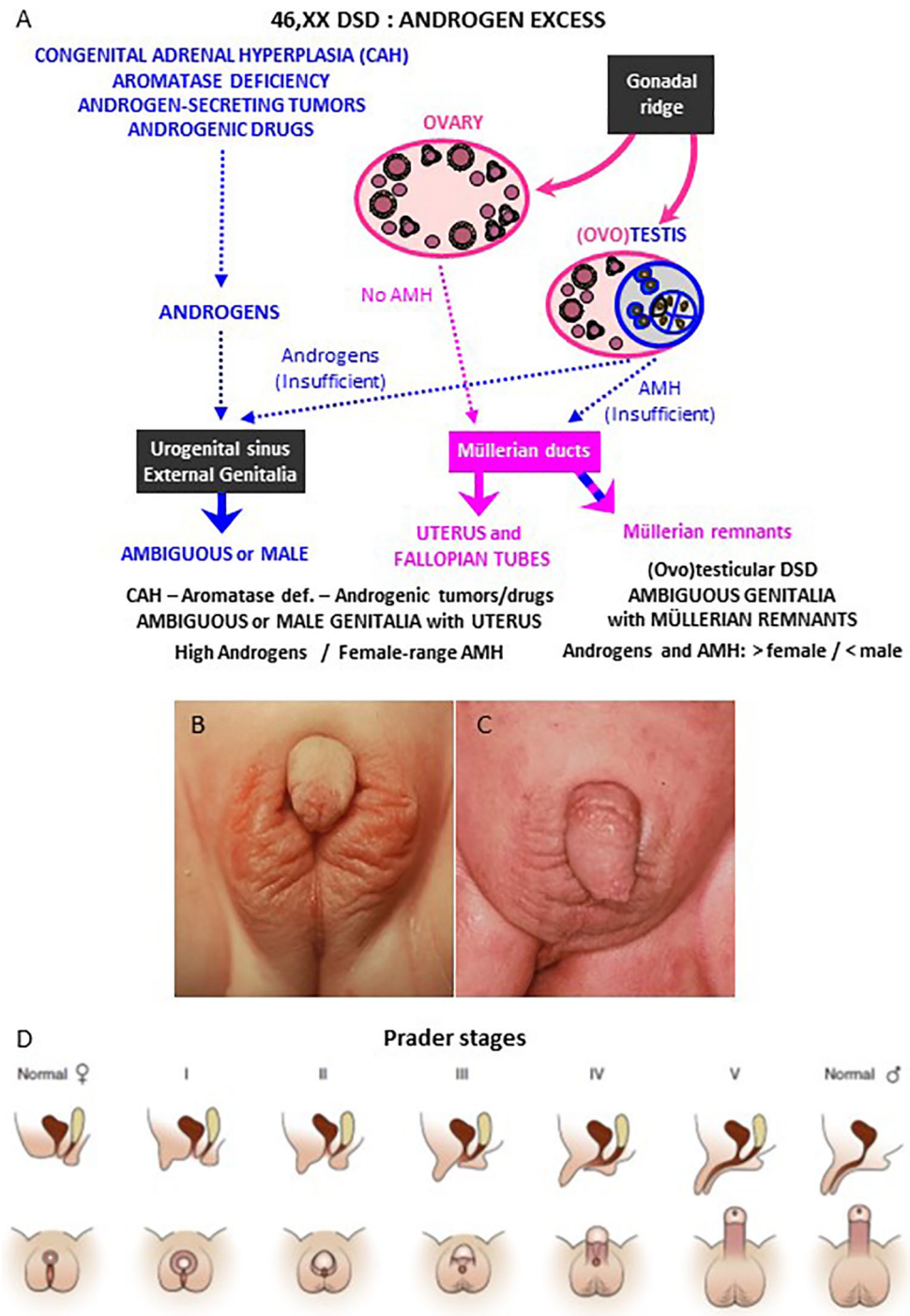

FIGURE 2 | Genital virilization in 46,XX individuals. **(A)** Pathophysiology of virilization: virilization of external genitalia may occur in 46,XX patients with ovaries and hyperandrogenism of adrenal (congenital adrenal hyperplasia) or extra-adrenal (aromatase deficiency, androgenic tumors or drugs) origin; alternatively, virilization of external genitalia with partial regression of Müllerian ducts may occur in 46,XX patients with testicular or ovotesticular DSD. **(B, C)** External genitalia of 46,XX patients with congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency: Prader stage III **(B)** and stage V **(C)**. **(D)** Schematic of Prader staging for patients with CAH. Reprinted with permission from Rey RA, Josso N. Diagnosis and treatment of Disorders of Sexual Development. In: Jameson JL, De Groot LC, de Kretser DM, Giudice LC, Grossman A, Melmed S, Potts JT, Weir GC, eds. Endocrinology: Adult and Pediatric, 7th edition. Philadelphia: Elsevier Saunders; 2016:2086-2118. Copyright © 2016 Elsevier Inc (7). **(B, C)** kindly provided by Dr. M. Podestá, Buenos Aires, Argentina.

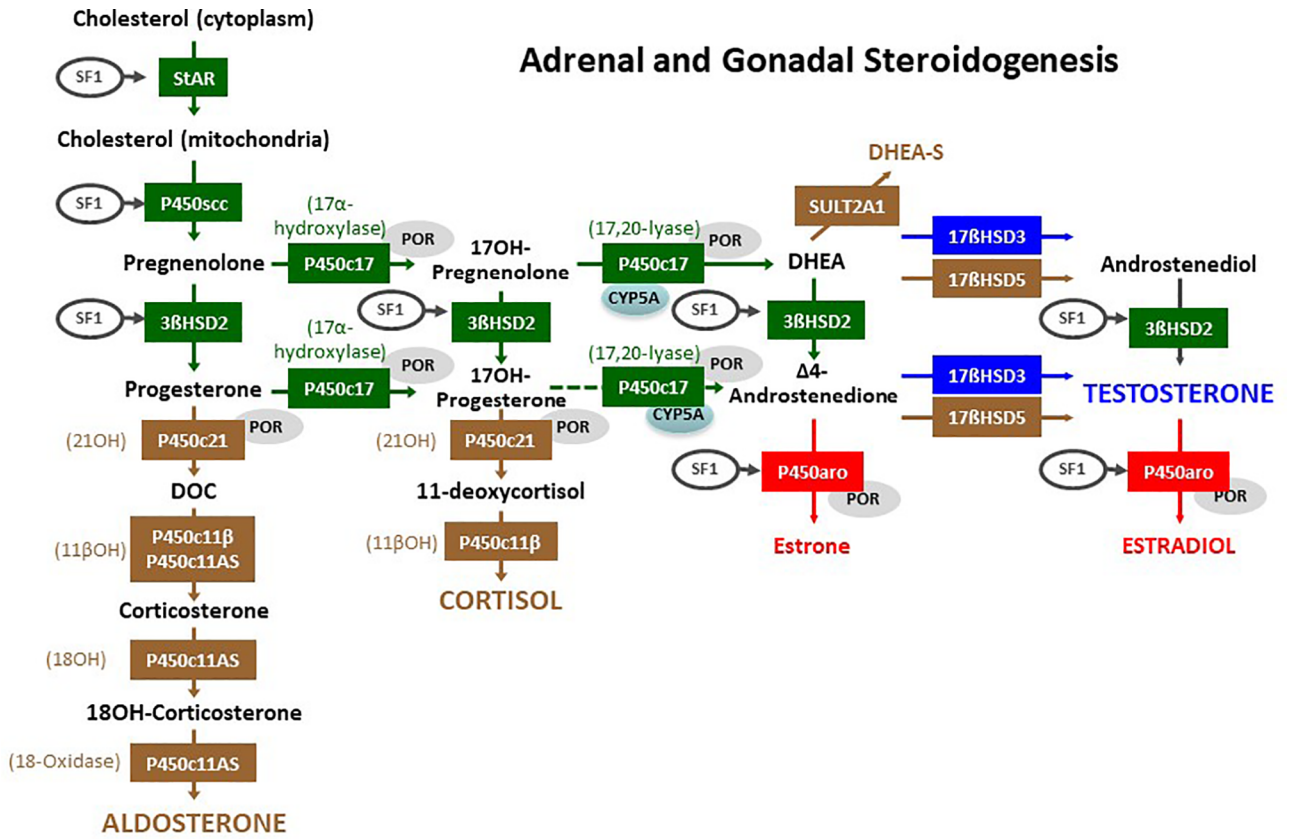

FIGURE 3 | Adrenal and gonadal steroidogenesis. The initial steroidogenic steps (in green) are identical in the adrenals and the gonads. The steps in brown are specific of the adrenal cortex, and the steps in blue or red are specific of the gonads. Steroidogenic Acute Regulatory (StAR) protein enables cholesterol influx into the mitochondria. Cytochrome P450 side chain cleavage (P450scc) enzyme removes the cholesterol side chain yielding the first C21, Δ5-steroid pregnenolone. All Δ5-steroids are converted to Δ4-steroids by 3β-hydroxysteroid dehydrogenase type 2 (3βHSD2). In the zona glomerulosa of the adrenal cortex, the first Δ4-steroid progesterone, is converted to deoxycorticosterone (DOC) by the 21-hydroxylase (21OH) activity of cytochrome P450c21; subsequently, the 11β-hydroxylase (11βOH) activity of P450c11β (encoded by *CYP11B1*) or of the aldosterone synthase (P450c11AS, encoded by *CYP11B2*) catalyzes DOC conversion to corticosterone, and finally P450c11AS, through its 18-hydroxylase (18OH) and 18-methyl oxidase (18-oxidase) activities respectively yields 18-hydroxycorticosterone (18OH-corticosterone) and aldosterone. In the zona fasciculata, cytochrome P450c17 converts pregnenolone and progesterone to 17-hydroxypregnenolone (17OH-Pregnenolone) and 17-hydroxyprogesterone (17OH-Progesterone), which is subsequently converted to 11-deoxycortisol by 21OH and to cortisol by 11βOH. In the zona reticularis of the adrenal cortex and in the gonads, the 17,20-lyase activity of P450c17 is facilitated by cytochrome b5 (CYP5A) yielding dehydroepiandrosterone (DHEA) and only secondarily androstenedione. DHEA may be sulphated to DHEA-S by sulfotransferase 2A1 (SULT2A1) in the adrenal. Gonadal 17β-hydroxysteroid dehydrogenase (17βHSD) type 3 converts DHEA to androstenediol and androstenedione to testosterone; in the adrenal these steps are minorly catalyzed by 17βHSD type 5 (encoded by *AKR1C3*). In the ovary, cytochrome P450 aromatase (P450aro) converts androstenedione to estrone and testosterone to estradiol. The activity of many of these enzymes is induced by steroidogenic factor 1 (SF1, also known as AD4BP, encoded by *NR5A1*) or by the cytochrome P450 oxidoreductase (POR). Reproduced with modifications from: Rey RA, Grinspon RP. Normal male sexual differentiation and aetiology of disorders of sex development. Best Practice & Research Clinical Endocrinology & Metabolism (2011) 25:221-238. doi: 10.1016/j.beem.2010.08.013. Copyright © 2010 Elsevier Ltd (6).

LH/hCG receptor, or in *HSD17B3*, which codes for 17β-hydroxysteroid dehydrogenase type 3, responsible for the conversion of androstenedione to testosterone (13). The other defects of androgen synthesis affect steroidogenic steps shared by the testis and the adrenal cortex (**Figure 3**) and will be described in detail in this review.

Finally, undervirilization of the XY fetus may result from defects in androgen target organs. Testicular androgen and AMH production is normal, but either dihydrotestosterone synthesis from testosterone is defective or the androgen receptor function is impaired (6).

2 DSD ASSOCIATED WITH ADRENAL DISORDERS

Congenital disorders affecting adrenal function may be associated with DSD in both 46,XX and 46,XY individuals, yet with a different underlying pathophysiology. While in 46,XX cases, the adrenal dysfunction is responsible for DSD, in 46,XY patients DSD results from the associated testicular dysfunction. In the vast majority of the cases, there is a primary adrenal insufficiency characterized by a reduction in cortisol secretion and overproduction of ACTH. In addition to cortisol deficiency,

patients may exhibit different degrees of aldosterone deficiency leading to salt-wasting adrenal crises that can be severe and sometimes fatal. The trophic effect of ACTH provokes adrenal cortex hyperplasia, which justifies the denomination of congenital adrenal hyperplasia (CAH).

CAH is a group of autosomal recessive disorders resulting in defects in one of the proteins or enzymes involved in cortisol biosynthesis: steroidogenic acute regulatory protein (StAR), P450 cholesterol side-chain cleavage enzyme (P450scc), P450 17α-hydroxylase/17,20-lyase (P450c17), P450 oxidoreductase (POR), 3β-hydroxysteroid dehydrogenase type 2 (3βHSD2), P450 21-hydroxylase (21OH or P450c21), or 11β-hydroxylase (11βOH) (**Figure 3**). The first report of CAH dates from 1865, describing a man with female internal genitalia and enlarged adrenals who experienced sudden death (14). The various forms of CAH lead to different hormonal imbalances. Production of glucocorticoids, mineralocorticoids and sex steroids might be either compromised or, in some cases, normal. Most forms of CAH can be subdivided into classic (or severe), presenting at birth, and non-classic, diagnosed later in life because of mild hyperandrogenism leading to growth and bone age acceleration, precocious pubarche and increase in penile or clitoris size in childhood (14, 15).

DSD is present as a consequence of androgen excess in 46,XX or deficiency in 46,XY patients, according to the specific enzymatic defect and the severity of impairment (14). Adrenal steroidogenic defects leading to 46,XX DSD are 21-hydroxylase deficiency (21OHD), by far the most prevalent cause, and 11β-hydroxylase deficiency (11βOHD). On the other hand, Lipoid Congenital Adrenal Hyperplasia due to StAR defects, and P450scc and P450c17 deficiencies cause DSD in 46,XY newborns. Steroidogenic Factor 1 (SF1, also known as AD4BP) defects may also result in combined adrenal and testicular failure leading to DSD in 46,XY individuals. Finally, impaired 3βHSD2 and POR functions result in both 46,XX and 46,XY DSD.

2.1 46,XX DSD of Adrenal Origin

The common pathogenesis of DSD in 46,XX patients is the excessive androgen production by the adrenal cortex resulting from cortisol synthesis blockage (**Table 1**). The resulting increase in pituitary ACTH secretion, due to failure of the negative feedback, leads to the accumulation of cortisol steroid precursors that are derived to the androgen synthesis pathway (**Figure 3**).

2.1.1 21Hydroxylase Deficiency (21OHD)

2.1.1.1 Pathophysiology and Clinical Presentation

The enzyme 21OH (P450c21) catalyzes the conversion of 17-hydroxyprogesterone into 11-deoxycortisol in the zona fasciculata and progesterone into 11-deoxycorticosterone (DOC) in the zona glomerulosa of the adrenal cortex. 21OHD (MIM 201910) due to mutations in *CYP21A2* (MIM 613815) represents the most common form, accounting for approximately 95% of CAH (16). *CYP21A2* and its highly homologous pseudogene *CYP21A1P* map to 6p21.3, about 30 kb apart. Due to the high homology in their sequences, mutations causing 21OHD typically occur from unequal recombination events between *CYP21A2* and *CYP21A1P*, abolishing enzymatic activity in different degrees (17). The estimated incidence, based on neonatal screening programs, ranges between 1/14,000 to 1/18,000 live births (14). Prevalence of heterozygous carries is around 1/60 (15).

21OHD shows a wide spectrum of phenotypes, no longer representing a clear cut between the classic and non-classic forms, as historically reported, but depicting a continuum between both forms which depends on the remaining enzyme activity (15, 18). Classic CAH is the most severe form, and it is currently the most common cause of DSD and of primary adrenal insufficiency during childhood (15, 18). Inadequate cortisol production leading to increased ACTH secretion results in accumulation of steroid precursors upstream of 21OH action, namely progesterone and 17-hydroxyprogesterone, which are derived to the adrenal androgen pathway *via* the "classic" (**Figure 3**) and "backdoor" (**Figure 4**) pathways. Consequently, affected 46,XX fetuses experience virilization of external genitalia in early stages of development. There is a failure of separate vaginal formation, with the urogenital sinus emptying into the urethra leading to a single opening of the urinary and reproductive tracts, like in the male. Genital tubercle trophism is stimulated by androgens resulting in clitoral enlargement, whereas labioscrotal folds become more or less fused. Different degrees of virilization are quantified by a scale ranging from I to V, developed by Prader (**Figure 2D**). Hyperpigmentation is one of the clinical features that 21OHD cases may present due to hypersecretion of ACTH in the fetal stage. At variance with external virilization, normal uterine development derived from Müllerian structures is observed internally, owing to normally absent AMH production by the ovaries in the first trimester of fetal life (**Figure 2A**).

In the classic salt wasting form of 21OHD, residual enzymatic activity is less than 1%, with both cortisol and aldosterone deficiencies resulting in life-threatening adrenal crises in the first 2 weeks of life, which can be anticipated if neonatal screening for CAH is performed. Simple virilizing forms retain about 1-2% of enzymatic activity; therefore, there is minimal but sufficient aldosterone production to prevent salt wasting crises. However, because all patients have some degree of salt-wasting, and clinical presentation overlap, this subclassification is no longer fully reliable.

In childhood, pseudoprecocious and central precocious puberty, advanced bone age and impaired final height -are common features of the disease as a consequence of periods of hyperandrogenism and long-term glucocorticoid treatment (15). Later in life, common long-term complications in female adolescents and young adults with CAH include pubertal dysfunction, menstrual abnormalities, and fertility issues (19, 20). Pathophysiology of these complications includes an excess of C19 steroids of classic adrenal steroidogenesis and of the more recently explored alternative or "backdoor" pathway involving 11-oxo-steroids (21, 22). High levels of 17-hydroxyprogesterone and progesterone play a role in menstrual abnormalities: serum progesterone >0.6 ng/mL in the follicular phase leads to reduced LH pulse frequency and amplitude, and oligo/amenorrhea (23, 24). Fertility is impaired, especially in salt-wasting forms, but pregnancy and delivery rates are not reduced (23, 25). Potential causes of reduced fertility include anatomical issues due to hyperandrogenism, unfavorable cervical mucus for sperm

TABLE 1 | Distinctive features of DSD associated with adrenal dysfunction in 46,XX patients.

Protein Activity	Gene Chromosome	Transmission/ Heterozygous carrier*	Mineralocorticoid pathway	Glucocorticoid pathway	Other steroids**	Signs of hyperandrogenism	Other features
P450c21 21-hydroxylase	*CYP21A2* 6p21.33	AR	DOC low Aldosterone low PRA high Sodium low Potassium high	Cortisol low 11-deoxycortisol low	Pregnenolone high 17OH-pregnenolone high Progesterone high 17OH-progesterone very high DHEA high Androstenedione very high Testosterone high 11-oxygenated androgens high	Prader I to V	Salt-wasting Hypoglycemia Hyperpigmentation Ovaries, uterus and Fallopian tubes present AMH in female range
P450c11β 11β-hydroxylase	*CYP11B1* 8q24.3	AR	DOC high Aldosterone low PRA low Sodium normal Potassium low/normal	Cortisol low 11-deoxycortisol high	Pregnenolone mildly high 17OH-pregnenolone mildly high Progesterone mildly high 17OH-progesterone mildly high DHEA mildly high Androstenedione mildly high Testosterone mildly high 11-oxygenated androgens mildly high	Prader I to V	Hypertension Hyperpigmentation Ovaries, uterus and Fallopian tubes present AMH in female range
3βHSD2 3β-hydroxysteroid dehydrogenase	*HSD3B2* 1p12	AR	DOC low Aldosterone low PRA high Sodium low Potassium high	Cortisol low 11-deoxycortisol low	Pregnenolone high 17OH-pregnenolone high Progesterone low 17OH-progesterone low DHEA high Androstenedione low Testosterone high	Prader I to III	Salt-wasting Hypoglycemia Hyperpigmentation Ovaries, uterus and Fallopian tubes present AMH in female range
POR P450 oxidoreductase, cofactor to P450scc, P450c17 and P450aro	*POR* 7q11.23	AR	DOC mildly high PRA mildly low Sodium normal/high Potassium normal/low	Cortisol mildly low 11-deoxycortisol mildly low	Pregnenolone mildly high 17OH-pregnenolone mildly high Progesterone high 17OH-progesterone high DHEA low Androstenedione low Testosterone mildly high	Prader I to IV	Hyperpigmentation Estradiol low Ovaries, uterus and Fallopian tubes present AMH in female range (rare) Hypertension Hypoglycemia

**Mode of transmission: AR, autosomal recessive. Heterozygous carrier: phenotype observed in heterozygous carriers of pathogenic gene variants.*

***Steroid levels are considered low, normal or high as compared to reference values for females (46,XX chromosomal sex).*

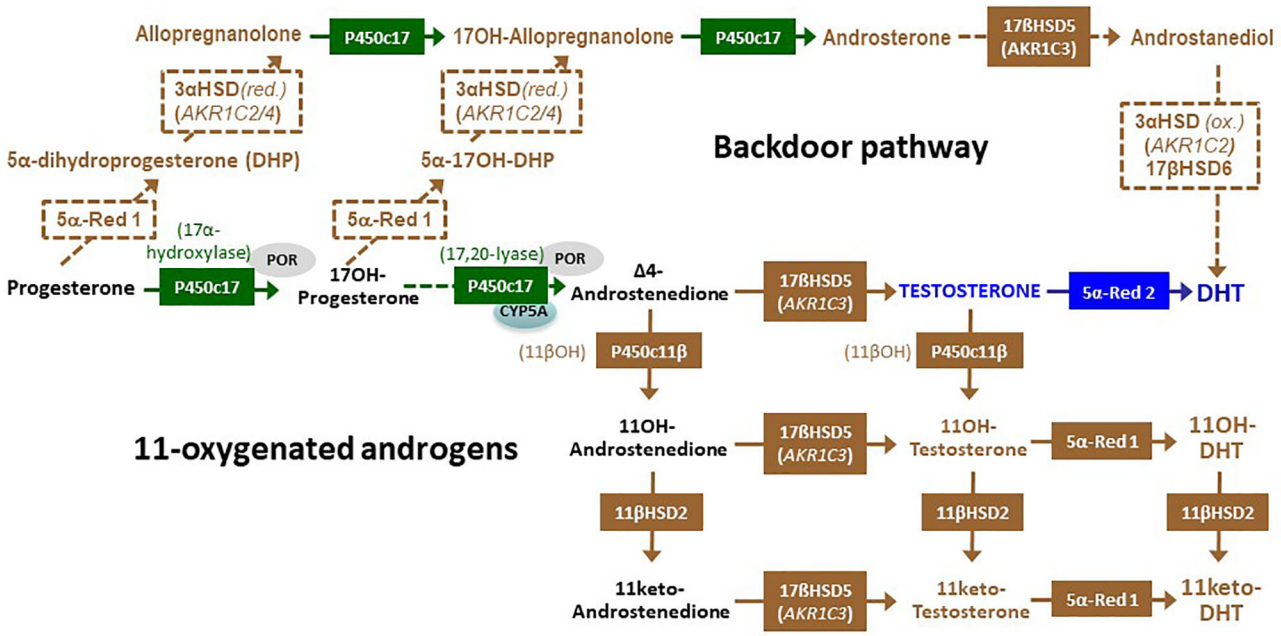

FIGURE 4 | The alternative or "backdoor" pathway of androgen synthesis and the 11-oxygentaed androgens. In the "backdoor" pathway, progesterone is converted to 5α-dihydroprogesterone (DHP) by 5α-reductase type 1 (5α-Red 1) and subsequently reduced to allopregnanolone by 3α-hydroxysteroid dehydrogenase (3α-HSD) encoded by either *AKR1C2* or *AKR1C4*. Similarly, 17-hysroxyprogetserone (17OH-progesterone) is converted to 5α-17-hydroxy DHP and to 17hydroxy-allopregnanolone (17OH Allopregnanolone). Cytochrome P450c17, though its 17,20 lyase activity, catalyzes 17OH Allopregnanolone conversion to androsterone. Subsequently, androsterone can be metabolized by adrenal 17β-hydroxysteroid dehydrogenase type 5 (17βHSD5) to yield androstanediol, which is oxidized by 3α-HSD encoded by *AKR1C2* and finally transformed to dihydrotestosterone (DHT) by 17βHSD type 6 (also known as retinol dehydrogenase or RoDH), without involving testosterone. Blockage of cortisol synthesis resulting in the accumulation of 17-hydroxyprogesterone and androstenedione leads to the synthesis of 11-oxygenated androgens: the 11β-hydroxylase activity of cytochrome P450c11β catalyzes the conversion of androstenedione and testosterone to 11-hydroxyandrostenedione (11OH-androstenedione) and 11-hydroxytestosterone (11OH-testosterone), respectively. Subsequently, 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) converts them to 11keto-androstenedione and 11keto-testosterone. Finally, 5α-Red 1 leads to the synthesis of 11-hydroxy-DHT and 11-keto-DHT.

migration, and endometrial thickening impairing embryo implantation and psychosocial factors (24). Ovarian adrenal rest tumors (OART) are rare in females with CAH, in contrast to the higher prevalence of testicular adrenal rest tumors (TART) present in males and are usually secondary to longstanding poor hormonal control (20).

2.1.1.2 Diagnosis

Diagnosis of 21OHD in 46,XX newborns is based on a positive neonatal screening in those countries where this procedure is established, or by elevated serum levels of 17-hydroxyprogesterone, typically above 1000 ng/dl (**Table 1**). Screening laboratories are expected to employ a second-tier screen by mass spectrometry, which is preferred to other methods such as genetic studies; immunoassays have a higher rate of false-positive results. If liquid chromatography–tandem mass spectrometry is not available, occasionally an ACTH stimulation test is recommended to distinguish 21OHD from other adrenal steroidogenic defects, especially in individuals with borderline 17-hydroxyprogesterone (26). The use of 11-deoxycortisol may show advantages and avoid false positive results sometimes observed with 17-hydroxyprogesterone (27). Other laboratory findings, such as elevated testosterone and androstenedione with normal female levels of gonadotropins and AMH, are not needed to certify the diagnosis. In childhood, the diagnostic criteria are the same.

Life-threatening salt-wasting forms, representing about 75% of classic CAH, are generally due to gene deletions or conversions, stop codons, frame shifts or variants severely affecting 21OH activity, thus impairing both glucocorticoid and mineralocorticoid synthesis. The clinical signs of salt loss, i.e. low sodium and elevated potassium, are usually seen between days 5 and 15 after birth. Simple virilizing forms are usually associated with missense gene variants, which retain enough enzyme activity to produce the small amounts of aldosterone required to maintain salt balance. As mentioned, even those cases classified as simple virilizing may show a subclinical degree of aldosterone deficiency. Although genetic testing searching for *CYP21A2* variants is not used as the first-line diagnostic test, genotyping is key for establishing affected carriers in the family (15).

2.1.2 11β-Hydroxylase Deficiency (11βOHD)

2.1.2.1 Pathophysiology and Clinical Presentation

The microsomal cytochrome P450c11β, with 11β-hydroxylase activity, is encoded *CYP11B1* (MIM 610613) and catalyzes one of the final steps in cortisol biosynthesis: the conversion of 11-deoxycortisol (S compound) and 11-deoxycorticosterone (DOC)

to cortisol and corticosterone, respectively (**Figure 3**). Mutations in *CYP11B1* gene cause 11βOHD (MIM 202010), the second most common form of CAH accounting for 0.2-8% of all cases. The estimated prevalence of this condition is 1 in 100,000 births, with higher prevalence in Muslim and Moroccan Jewish Middle Eastern populations (28). Impairment in both cortisol and corticosterone production causes increased ACTH secretion with accumulation of 11-deoxycortisol and DOC, respectively, which are shunted to the androgen pathway causing different degrees of virilization in affected females (29). Compared to females with 21OHD, those with 11βOHD are more virilized; intriguingly, the extent of masculinization, however, correlates poorly with the degree of hyperandrogenemia (30).

In childhood, persistent androgen excess may result in pseudoprecocious puberty, rapid somatic growth and accelerated bone maturation leading to premature epiphyseal closure and short stature (31). Later in life, hyperandrogenism results in delayed menarche. Lower fertility rates have been reported. So far, there is one report of pregnancy in a 26-year-old woman with severe 11βOHD deficiency (32).

Mild to moderate hypertension is present in two-thirds of patients with classic 11βOHD. Despite 11-deoxycorticosterone being a less potent mineralocorticoid than aldosterone, its accumulation causes salt retention and hyporeninemic hypokalemic hypertension, mainly in older children and adults. However, as newborns are relatively resistant to mineralocorticoids, salt loss might be present, but it is usually mild and transient (33).

Rare cases of non-classic 11βOHD have been described, presenting later in life with milder virilization, precocious pseudopuberty, hirsutism or menstrual irregularities.

2.1.2.2 Diagnosis

Diagnosis of 11βOHD is based on elevated basal concentrations of DOC and hyperresponsiveness of 11-deoxycortisol during ACTH test (>3 times the upper limit of normal) (**Table 1**). Low cortisol and normal or suppressed plasma renin activity is also present (14). Nevertheless, 11βOHD diagnosis may be challenging in neonates, due to several reasons. Newborns often do not present with hypertension and suppressed renin. Another potential source of error is the mild to moderate elevations of 17-hydroxyprogesterone often observed, leading to an erroneous diagnosis of 21OHD deficiency. Lastly, in case deoxycorticosterone and 11-deoxycortisol are not specifically measured, the diagnosis may be missed. Molecular genetic testing confirms the diagnosis of 11βOHD when mutations in *CYP11B1* gene are found.

2.2 46,XY DSD Associated With Adrenal Dysfunction

As already mentioned, DSD in 46,XY patients do not result from adrenal failure, but from hypoandrogenemia due to the associated testicular steroidogenic defect (**Figure 3** and **Table 2**).

2.2.1 StAR and P450scc Deficiencies

2.2.1.1 Pathophysiology and Clinical Presentation

StAR protein has a crucial role in facilitating the influx of cholesterol between the outer and the inner mitochondrial membranes; subsequently, P450scc enzyme, encoded by *CYP11A1* gene, catalyzes the conversion of cholesterol to pregnenolone, the first and rate-limiting step in the synthesis of all steroid hormones (34).

The pathophysiology of StAR and P450scc deficiencies is similar except that lipid droplet accumulation typical of Lipoid Congenital Adrenal Hyperplasia (LCAH) caused by StAR deficiency does not occur in P450scc deficiency (34). There is a severe impairment of steroidogenesis in adrenals and gonads, leading to minimal concentrations of all steroids. Adrenal insufficiency leads to failure to thrive, salt wasting due to aldosterone deficiency, hypoglycemia due to cortisol deficiency, and consequent elevation of ACTH and plasma renin activity (33). Testicular failure is limited to Leydig cell dysfunction, with hypoandrogenism leading to defective virilization of the Wolffian ducts, the urogenital sinus and the external genitalia. Conversely, because AMH is normally produced by Sertoli cells in early fetal life, there is no uterus or Fallopian tubes (6)..

A distinctive feature in the pathophysiology of StAR deficiency is explained by the "two-hit disease model" (35): the first hit is the absence of StAR, which reduces cholesterol import and, therefore, adrenal and testicular steroidogenesis. However, a small amount of steroidogenesis remains by StAR-independent mechanisms. The second hit occurs when the newly synthesized intracellular cholesterol, cholesterol esters and their autoxidation products progressively accumulate in lipid droplets, leading to grossly enlarged adrenals, and destroy residual StAR-independent steroidogenic mechanisms (33). Leydig cell destruction early in gestation causes deficient testosterone production. As expected, fetal sex development of 46,XX individuals is not altered; these patients are born with normal female genitalia, and most of them enter puberty normally due to StAR independent steroidogenesis. However, later in adolescence gonadotropic stimulus results in cellular damage affecting mainly the luteal phase, leading to irregular menses (33). Low levels of estradiol might be insufficient for embryo implantation, resulting in infertility (25). Thus far, pregnancy has been reported in three women with a StAR gene mutation who presented with spontaneous puberty and menarche. These pregnancies were achieved using reproductive technology: clomiphene citrate in one and IVF in the remaining two patients (36–38).

LCAH (MIM 201710) is the most severe form of CAH, caused by pathogenic variants in the *STAR* gene (MIM 600617) (35). Despite being a rare defect, LCAH is more frequently seen in certain populations, such as East Asian, Arab and Swiss due to the presence of mutations with founder effect. For example, mutation Q258X was found in more than 70% of affected alleles in Japan and Korea representing about half of all reported cases (39). So far, more than 40 mutations have been described in 190 patients (34, 40, 41). P450scc deficiency (MIM 613743), due to *CYP11A1* mutations (MIM 118485), is a rare disorder that can present at any time, from infancy to early childhood. To date, less than 40 cases have been reported (14).

Typically, 46,XY affected infants are born with female or ambiguous genitalia and present with neonatal adrenal crises. Hyperpigmentation is frequent, associated with elevation of

TABLE 2 | Distinctive features of DSD associated with adrenal dysfunction in 46,XY patients.

Protein Activity	Gene Chromosome	Transmission/ Heterozygous carrier*	Mineralocorticoid pathway	Glucocorticoid pathway	Other steroids**	Genitalia and gonads***	Other features
StAR Mitochondrial cholesterol transfer	*STAR* 8p11.23	AR	DOC low Aldosterone low PRA high Sodium low Potassium high	Cortisol low 11-deoxycortisol low	Pregnenolone low 17OH-pregnenolone low Progesterone low 17OH-progesterone low DHEA low Androstenedione low Testosterone low	EG: from female to male with hypospadias No uterus Testes with lipoid degeneration of Leydig cells	Salt-wasting Hypoglycemia Hyperpigmentation Lipoid adrenal hyperplasia Clinical androgen deficiency at puberty AMH in male range
P450scc Cholesterol side-chain cleavage	*CYP11A1* 15q24.1	AR	DOC low Aldosterone low PRA high Sodium low Potassium high	Cortisol low 11-deoxycortisol low	Pregnenolone low 17OH-pregnenolone low Progesterone low 17OH-progesterone low DHEA low Androstenedione low Testosterone low	EG: from female to male with hypospadias No uterus Testes with impaired Leydig cell androgen synthesis	Salt-wasting Hypoglycemia Hyperpigmentation Clinical androgen deficiency at puberty AMH in male range
P450c17 17α-hydroxylase, 17,20-lyase	*CYP17A1* 10q24.32	AR	DOC high Aldosterone low PRA low Sodium normal/high Potassium low	Cortisol low 11-deoxycortisol low	Pregnenolone high 17OH-pregnenolone high Progesterone mildly high 17OH-progesterone mildly high DHEA low Androstenedione low Testosterone low	EG: from female to male with hypospadias No uterus Testes with impaired Leydig cell androgen synthesis	Hypertension Hypoglycemia Hyperpigmentation Clinical androgen deficiency at puberty AMH in male range
P450c17 Isolated 17,20-lyase	*CYP17A1* 10q24.32	AR	Not affected	Not affected	Pregnenolone mildly high 17OH-pregnenolone high Progesterone mildly high 17OH-progesterone high DHEA low Androstenedione low Testosterone low	EG: from clitoromegaly with some labial fusion to male with hypospadias No uterus Testes with impaired Leydig cell androgen synthesis	Clinical androgen deficiency at puberty AMH in male range
Cytochrome b5, type A Cofactor to 17,20-lyase	*CYB5A* 18q22.3	AR	Not affected	Not affected	Pregnenolone mildly high 17OH-pregnenolone high Progesterone mildly high 17OH-progesterone	EG: from clitoromegaly with some labial fusion to male with hypospadias No uterus Testes with impaired Leydig cell androgen synthesis	Clinical androgen deficiency at puberty AMH in male range

(Continued)

TABLE 2 | Continued

Protein Activity	Gene Chromosome	Transmission/ Heterozygous carrier*	Mineralocorticoid pathway	Glucocorticoid pathway	Other steroids**	Genitalia and gonads***	Other features
					high DHEA low Androstenedione low Testosterone low		
POR P450 oxidoreductase, cofactor to P450scc, P450c17 and P450aro	*POR* 7q11.23	AR	DOC mildly high PRA mildly low Sodium normal/high Potassium normal/ low	Cortisol mildly low 11-deoxycortisol mildly low	Pregnenolone mildly high 17OH-pregnenolone mildly high Progesterone high 17OH-progesterone high DHEA low Androstenedione low Testosterone low	EG: from female to male with hypospadias No uterus Testes with impaired Leydig cell androgen synthesis	Hyperpigmentation Clinical androgen deficiency at puberty AMH in male range (rare) Hypertension Hypoglycemia
3βHSD2 3β-hydroxysteroid dehydrogenase	*HSD3B2* 1p12	AR	DOC low Aldosterone low PRA high Sodium low Potassium high	Cortisol low 11-deoxycortisol low	Pregnenolone high 17OH-pregnenolone high Progesterone low 17OH-progesterone low DHEA high Androstenedione low Testosterone low	EG: from female to male with hypospadias No uterus Testes with impaired Leydig cell androgen synthesis	Salt-wasting Hypoglycemia Hyperpigmentation Clinical androgen deficiency at puberty AMH in male range
SF1 (AD4BP) Regulator of StAR, P450scc, 3βHSD2	*NR5A1* 9q33.3	AD/AR	(rare) DOC low Aldosterone low PRA high Sodium low Potassium high	(rare) Cortisol low 11-deoxycortisol low	Pregnenolone low 17OH-pregnenolone low Progesterone low 17OH-progesterone low DHEA low Androstenedione low Testosterone low	EG: from female to male with hypospadias Uterus or Müllerian remnants may be present Testes: variable degree of dysgenesis	Clinical androgen deficiency at puberty AMH low (rare) Salt-wasting Hypoglycemia Hyperpigmentation

**Mode of transmission: AD, autosomal dominant; AR, autosomal recessive. Heterozygous carrier: phenotype observed in heterozygous carriers of pathogenic gene variants.*
***Steroid levels are considered low, normal or high as compared to reference values for males (46,XY chromosomal sex).*
****EG, external genitalia.*

ACTH. Affected patients are generally raised as girls (34).However, a number of cases with mild forms resulting in normal male genitalia and late-onset adrenal insufficiency have also been reported (42). In these cases, the presence of testicular adrenal rest tumors (TART) has been described, leading to primary testicular failure with oligospermia and elevated FSH (43).

2.2.1.2 Diagnosis

The diagnosis of DSD due to StAR or P450scc deficiency is suspected in a 46,XY newborn, phenotypically female or with ambiguous, hyperpigmented genitalia and failure to thrive in the first weeks of life. All gonadal and adrenal steroids are very low, ACTH, renin and LH are elevated, and AMH levels are within the normal range for chromosomal sex (**Table 2**). The differential diagnosis with other steroidogenic defects is based on the low levels of all steroids. In 46,XY patients with female or ambiguous genitalia, hyperpigmentation, absence of uterus in ultrasonography, male-range AMH levels and low levels of adrenal steroids with ACTH elevation distinguishes StAR and P450scc deficiencies from Leydig cell hypoplasia (6, 34). The differential diagnosis between StAR and P450scc deficiencies is limited to sequencing of *STAR* and *CYP11A1* genes. The enlarged adrenal size usually observed in LCAH is not seen in P450scc deficiency. However, adrenal size alone cannot distinguish both conditions (34).

2.2.2 17α-Hydroxylase, 17,20-Lyase Deficiency (P450c17D)

2.2.2.1 Pathophysiology and Clinical Presentation

P450c17 is a microsomal P450 enzyme expressed in both adrenals and gonads that catalyzes two major reactions in the steroidogenic pathway: the 17α-hydroxylation followed by the 17,20-lyase reactions resulting in the synthesis of 17α-hydroxylated glucocorticoids and sex steroids by the adrenal glands and gonads, respectively (33). Complete P450c17D (MIM 202110) is a rare form of CAH accounting for 1% of the cases, caused by mutations in *CYP17A1* gene (MIM 609300). To date, just over 100 mutations have been reported, some of them being more frequent in certain populations such as Dutch Friedlaenders, Southeast Asian and Brazilians, due to mutations with founder effect (44).

Steroidogenesis in adrenals and gonads is severely impaired, causing deficiency of cortisol and sex steroids, with mineralocorticoid excess. Consequently, 46,XY fetuses are severely undervirilized while 46,XX sexual development is unaffected at birth (33). The typical presentation of this form of CAH is a phenotypic girl or adolescent with pubertal failure, including lack of breast development and primary amenorrhea, hypertension and hypokalemia (45). Alternatively, like in all other forms of DSD of adrenal origin, 46,XY individuals may present with ambiguous genitalia and testes present in the inguinal canals or intra-abdominally. In contrast with most forms of CAH, patients with P450c17D do not develop adrenal crises despite low cortisol levels, because corticosterone has glucocorticoid activity and mineralocorticoid synthesis is unaffected (33). Manifestations of mineralocorticoid excess due to the accumulation of DOC, such as hypertension and hypokalemia, usually appear later in childhood due to the relative kidney insensitivity to mineralocorticoids present in infancy.

2.2.2.2 Diagnosis

Like for other forms of steroid synthesis defects, DSD due to P450c17D are suspected in 46,XY girls or patients with ambiguous genitalia, absent uterus, testosterone above the female range but below the male range and AMH in the male range (**Table 2**). The distinctive feature of P450c17D is the elevation of pregnenolone, progesterone, DOC and corticosterone, associated to normal/low aldosterone and normal/low plasma renin activity, and decreased levels of steroids downstream P450c17 activity, i.e. 17-hydroxypregnenolone, 17-hydroxyprogesterone, 11-deoxycortisol and cortisol, as well as DHEA and androstenedione (33, 45). ACTH stimulation test may be necessary to evidence an increase in pregnenolone/17-hydroxypregenolone and progesterone/17-hydroxyprogesterone ratios (44). At pubertal age, gonadotropins are usually elevated reflecting gonadal dysfunction (45). Genetic analysis of *CYP17A1* confirms the diagnosis.

2.2.3 Isolated 17,20-Lyase Deficiency

2.2.3.1 Pathophysiology and Clinical Presentation

Isolated 17,20-lyase deficiency (MIM 202110) is a rare cause of CAH caused by mutations in any of three different genes: *CYP17A1*, *POR* or *CYB5A* (33). Missense mutations in *CYP17A1* (MIM 609300) affecting the redox partner binding site of the enzyme do not impair 17α-hydroxylase activity (44). The 17,20-lyase activity of P450c17 is also critical in the 'backdoor' pathway of dihydrotestosterone synthesis, through androstanediol, without going through DHEA, androstenedione and testosterone (**Figure 4**) (46, 47). Like other P450 enzymes, e.g. P450c21 (21OH) and P450aro (aromatase), P450c17 receives electrons from NADPH *via* P450 oxidoreductase (POR). Particularly human 17,20-lyase activity is stimulated by cytochrome b5 type A, acting as an allosteric factor. Therefore, impaired 17,20-lyase activity also results from mutations in *POR* (MIM 124015) and *CYB5A* (MIM 613218) (33, 44). POR defects result in a combined deficiency of 17,20-lyase, 21OH and aromatase, therefore likely to induce DSD in both 46,XX and 46,XY individuals; they will be addressed in a specific section below. Cytochrome b5 also reduces methemoglobin (ferric hemoglobin) to normal hemoglobin (ferrous hemoglobin); its defects result in associated methemoglobinemia (MIM 250790).

Clinically, 46,XY patients with 17,20-lyase deficiency present at birth with ambiguous genitalia, whereas 46,XX patients are usually detected when seeking attention for pubertal failure and primary amenorrhea. Infertility is the rule, and the first case of successful pregnancy and delivery in a 24-year-old woman after controlled ovarian stimulation and *in vitro* fertilization, has only recently been reported (48).

2.2.3.2 Diagnosis

Isolated 17,20-lyase deficiency leads to insufficient virilization of 46,XY fetuses and normal genitalia in 46,XX fetuses. Failure to enter puberty, primary amenorrhea and infertility the most common clinical presentation in females.

Biochemically, the blockage of 17,20-lyase activity due to *CYP17A1*, *POR* or *CYB5A* mutations leads to marked elevation of 17OH-pregnenolone and pregnenolone and mild elevation of progesterone and 17OH-progesterone, with low levels of DHEA, androstenedione and testosterone, low C19 steroids and poor response to hCG (33).

2.2.4 SF1 Defects

2.2.4.1 Pathophysiology and Clinical Presentation

SF1, encoded by *NR5A1* (MIM 184757), was first described as a key regulator of the P450 steroid hydroxylases in the adrenals and gonads (**Figure 3**), and subsequently found to be involved in embryonic morphogenesis of the ventromedial hypothalamic nucleus, the gonadotropes, the adrenal cortex and the testes and ovaries (49, 50). Pathogenic variants found in *NR5A1* are associated with DSD in 46,XY individuals due to testicular failure during early fetal development (MIM 612965); in some cases, primary adrenal insufficiency is associated (51).

Clinically, 46,XY patients with SF1 defects present with variable degrees of undervirilization of the external genitalia, reflecting androgen deficiency, and of persistence of Müllerian derivatives, indicating AMH deficiency associated with testicular dysgenesis (**Table 2**). Adrenal failure occurs in a minority of the cases, with glucocorticoid and mineralocorticoid deficiencies.

In 46,XX individuals, ovarian dysgenesis leading to primary ovarian insufficiency has been described (MIM 612964), but as

expected does not result in DSD. Recently, variants in *NR5A1* have been reported in virilized 46,XX patients with testicular or ovotesticular DSD (MIM 617480) (52); however, adrenal function does not seem affected.

2.2.4.2 Diagnosis

This is the only form of 46,XY DSD where AMH deficiency exists together with adrenal dysfunction. Therefore, apart from low androgen and high LH levels, low AMH and high FSH levels should alert of a SF1 defect in a patient with ambiguous genitalia and Müllerian remnants associated with adrenal insufficiency. The detection of a mutation in *NR5A1* confirms the diagnosis (51).

2.3 Adrenal Disorders Causing 46,XX and 46,XY DSD

2.3.1 3β-Hydroxysteroid Dehydrogenase Type 2 Deficiency (3βHSD2D)

2.3.1.1 Pathophysiology and Clinical Presentation

Classic 3β-hydroxysteroid dehydrogenase type 2 deficiency (3βHSD2D) is a rare form of CAH with estimated incidence < 1/1,000,000 live births, accounting for less than 0.5% of all cases of this condition (53). Two functional *HSD3B* genes are found in humans: *HSD3B1* encodes an isozyme expressed in peripheral tissue including brain, liver, skin, mammary glands and placenta, and *HSD3B2* encodes 3β-hydroxysteroid dehydrogenase type 2 found in the adrenals and gonads. This isoenzyme normally converts Δ5-steroids (pregnenolone, 17-hydroxypregnenolone, dehydroepiandrosterone and androstenediol) to the corresponding Δ4-steroids (progesterone, 17-hydroxyprogesterone, androstenedione and testosterone). Classic 3βHSD2D (MIM 201810) is caused by *HSD3B2* gene mutations (MIM 613890) and characterized by impaired steroidogenesis in both adrenals and gonads. Consequently, cortisol, aldosterone, and androstenedione concentrations are low and renin, ACTH, and dehydroepiandrosterone (DHEA) concentrations are increased with DHEA being converted to testosterone by extra-adrenal 3βHSD1. Clinical features include ambiguous genitalia in both 46,XX and 46,XY fetuses and adrenal insufficiency of both glucocorticoids and mineralocorticoids (54).

Genotypic females are generally born mildly virilized, presenting with enlarged clitoris, incomplete labial fusion and genital hyperpigmentation due to the shift from DHEA to testosterone by HSD3B1 (**Table 1**); however, they can present with normal external genitalia at birth. Preserved mineralocorticoid function and non-virilized genitalia may lead to underdiagnosis (55). Genotypic males are invariably undervirilized due to insufficient testicular conversion of DHEA to testosterone (**Table 2**). Phenotypic manifestations include severe hypospadias, micropenis, bifid scrotum, and undescended testis (53). There is no correlation between the impairment in male sexual differentiation and salt- wasting (54). Some patients experience spontaneous puberty while others fail to progress through puberty needing sex hormone replacement (53).

In adult 46,XX patients, hyperandrogenism becomes challenging due to both the increasing androgen production by the zona reticularis and the increased conversion of testosterone to DHT (56). In males, TART and gonadal dysfunction, leading to arrested spermatogenesis and azoospermia, have been reported which warrants the need of long-term follow-up of these patients through their lifespan. Very limited information exists regarding fertility in both females and males with 3βHSD2D (19, 25, 53).

Non-classic 3βHSD2D was originally suspected in children with premature pubarche and in young females with hirsutism and menstrual irregularities who presented exaggerated Δ5-steroid production after ACTH stimulation and elevated 17-hydroxyprogesterone to cortisol ratio. Alternatively, this group of patients is referred to as having "partial 3βHSD2D" (33). Interestingly, genetic testing was unable to identify mutations in *HSD3B2* gene in all these patients, which raises doubts about the real existence of non-classic forms (14).

2.3.1.2 Diagnosis

Primary biochemical abnormality in 3βHSD2D is the elevated Δ5 to Δ4 steroid ratio, including 17-hydroxypregnenolone/17-hydroxyprogesterone and DHEA/androstenedione ratios in serum, and pregnanetriol to pregnanediol ratio in urine, especially after ACTH stimulation. Diagnosis of the classic form of 3βHSD2D based on 17-hydroxypregnenolone levels above 100 nmol/L (3300 ng/dl) either basal or after ACTH stimulation is the best single biological criterion of 3βHSD2D. In addition, the baseline 1000-fold elevation of 17-hydroxypregnenolone to cortisol ratio and low 11-oxygenated androgens by liquid chromatography-tandem mass spectrometry (LC-MS/MS) provides an unequivocal biochemical diagnostic parameter (53, 55). Nonetheless, diagnosis at birth could be challenging due to HSD3B1 activity which can convert some of the elevated 17-hydroxypregnenolone to 17-hydroxyprogesterone, leading to false positives on neonatal screening for 21OHD (57). Genetic testing for *HSD3B2* mutations confirms the diagnosis of the classic form (53, 55).

2.3.2 POR Deficiency (PORD)

2.3.2.1 Pathophysiology and Clinical Presentation

As mentioned, POR is an important electron donor from NADPH to microsomal P450 enzymes, such as 17α-hydroxylase, 21-hydroxylase and P450 aromatase (33). POR deficiency (MIM 613571) due to mutations in the *POR* gene (MIM 124015) results in an unusual form of CAH first described in 2004, characterized by partially deficient P450c17 activity, with or without associated deficient activity of P450c21 and P450aro (58–60). Approximately 75 mutations have been reported to date in 140 individuals (16).

Due to the variability in enzymatic impairment, there is a wide spectrum of clinical phenotypes ranging from ambiguous genitalia in both 46,XX and 46,XY individuals with adrenal insufficiency to milder phenotypes in women who appear to have a form of polycystic ovary syndrome, or mildly affected men with gonadal insufficiency (33). Generally, 46,XY patients are born with undervirilization due to impaired of 17,20 lyase activity resulting in decreased androgen production (**Table 2**). On the other hand, 46,XX females present with virilized genitalia (**Table 1**), which depends on the causative *POR* mutation. One

possible explanation is that certain mutations (e.g. R457H) affect placental P450aro activity leading to maternal and 46,XX fetal virilization during pregnancy due to defective conversion of fetal adrenal C19 androgen precursors to estrogens (33, 61). An alternative explanation relies on the excess 17-hydroxyprogesterone conversion to DHT through the "backdoor pathway (**Figure 4**) (22). Interestingly, after birth, circulating androgen levels are low or normal, therefore, virilization in these patients does not progress (58).

Data on pubertal development in these patients is scarce. One study reported pubertal status in seven patients with POR deficiency: most female patients presented with significant pubertal impairment, hypergonadotropic hypogonadism and ovarian cysts, prone to rupture. Potential underlying mechanism of the cysts formation was an excessive LH-mediated ovarian stimulation as a consequence of primary hypogonadism (62).

Specific *POR* mutations can result in a phenotype similar to the Antley–Bixler syndrome (MIM 201750) in both sexes, characterized by craniosynostosis, brachycephaly, midface hypoplasia, proptosis and choanal stenosis, radio-humeral or radio-ulnar synostosis, bowed femora and arachnodactyly (60).

2.3.2.2 Diagnosis

Diagnosis of POR deficiency relies on the detection of a combined impairment of CYP21A2 and CYP17A1 activities, resulting in a combined mild elevation of pregnenolone, progesterone, 17-hydroxypregnenolone, 17-OHP and DOC, with variable cortisol response to ACTH. Genetic testing is usually needed to confirm the diagnosis (16, 60).

2.4 The Role of 11-Oxygenated Androgens in Hyperandrogenic Adrenal Disorders

An increasing interest has recently developed on the role of 11-oxygenated androgens (11-oxyandrogens) in hyperandrogenic adrenal disorders, especially CAH. 11-oxyandrogens are 19-carbon steroids primarily synthesized in the adrenal cortex: 11-hydroxyandrostenedione and 11-hydroxytestosterone are products of 11β-hydroxylase (CYP11B1) activity (**Figure 4**). On the other hand, 11-ketoandrostenedione and 11-ketotestosterone are produced in the kidneys from 11-hydroxyandrostenedione by 11β-hydroxysteroid dehydrogenase type 2 (63). The steroid 11-ketotestosterone is a potent androgen receptor agonist, showing an androgenic activity similar to that of testosterone (64). The specificity of 11-ketotestosterone as a biomarker of adrenal function is supported by the existence of higher concentrations in the adrenal vein than in the periphery (21), its rise during adrenarche (65) and after ACTH stimulation (66), and its complete decline in patients with adrenal insufficiency (21). In patients with CAH, high levels of 11-oxyandrogens correlate with adrenal volume and testicular adrenal rest tumors (67), and are particularly useful in the management of patients with discrepant 17-hydroxypreogesterone and androstenedione levels (63). On the other hand, 11-oxygenated androgens are not elevated in CAH due to 11β-hydroxylase or 3β-hydroxysteroid dehydrogenase deficiencies (18, 21).

3 MANAGEMENT OF PATIENTS WITH DSD ASSOCIATED TO ADRENAL DYSFUNCTION

The management of patients with DSD associated with adrenal dysfunction involves two main aspects: those related with genital and reproductive issues and those derived from the pathogenesis, frequently associated with adrenal insufficiency and steroid disorders.

3.1 Management of Genital and Reproductive Issues

Despite the significant societal changes observed in the last years vis-à-vis the importance of the sex of the newborn, gender assignment is still one of the major issues in patients with ambiguous genitalia. Decisions about the sex of rearing in babies with DSD can be particularly challenging, even if there is a growing comprehension that gender identity later in life may not correlate with the genetic, gonadal or genital sex of an individual. The karyotype and the degree of virilization are major drivers in the decision (68, 69). As already discussed, in the case of 46,XY DSD, the most severe forms of androgen deficiency result in completely female external genitalia, thus these individuals are assigned as girls. Conversely, those with less severe steroidogenic defects resulting in genital undervirilization are more frequently assigned male, given their good response to androgen replacement therapy. On the other hand, there is almost univocal consensus that newborns with 46,XX DSD benefit from female sex assignment (68), except for those with completely virilized external genitalia, where the decision may be controversial (69, 70).

3.1.1 46,XY Patients

Patients with completely female external genitalia, reared as girls, do not require any treatment of their genitalia before pubertal age. The extirpation of the testes, usually present in abdominal position, is most often performed despite the lack of information about their malignant transformation potential, to avoid virilization at pubertal age. Estrogen replacement is necessary to provoke breast development, pubertal growth spurt and adequate bone mineralization. The vagina is generally shorter than normal because its upper part derives from the Müllerian ducts that regress in fetal life due to AMH action. This may cause discomfort for sexual intercourse in the adolescent, but surgical procedures may prove challenging. The absence of uterus leads to permanent amenorrhea and impossibility of gestation. However, the recent development of sophisticated surgical procedures allowing uterine transplantation in young women (71) and oocyte donation give hope to those who do not consider adoption.

In undervirilized boys, surgical correction of hypospadias and cryptorchidism is usually performed in infancy. Although some androgenic activity may be conserved, testosterone therapy is most frequently needed in order to support an adequate development of secondary sexual characteristics, growth and muscle and bone trophism. These patients are generally

infertile: azoospermia results from insufficient intratesticular testosterone concentrations, which cannot be improved by exogenous testosterone treatment (72).

3.1.2 46,XX Patients

Historical practice characterized by surgery in infancy, including clitoroplasty, vaginoplasty and urogenital sinus division, has raised controversy in the past years (15, 18). Unfortunately, little evidence exists regarding long-term sexual function outcomes, owing to the lack of controlled studies with adequate design. Expert opinion recommends that parents should be clearly informed about surgical options, including delayed surgery (26, 69). Urinary disorders, with frequent infections, may require early surgery; otherwise, the decision may be delayed until the patient can participate. Special attention should receive the examination of the genital anatomy to determine whether adequate menstrual flow will require surgery before pubertal onset. At the age of puberty, besides corticoid replacement, estrogen therapy may be needed to induce breast development and bone maturation and mineralization in 46,XX patients with 3βHSD2D or PORD.

Because anxiety, substance abuse and gender dysphoria are more frequently observed in association with fetal and postnatal excessive exposure to androgens, which results in impaired reproductive outcomes (23), psychological support is important. In women desiring conception, progesterone levels should be below 0.6 ng/ml (or 2 nmol/l), which can be attained with the administration of adequate doses of hydrocortisone or prednisolone, but not dexamethasone, which crosses the placenta and reaches the fetus (26). Successful pregnancy has been reported in 46,XX patients with CAH due to 21OHD treated with 1-2 mg of prednisolone at bedtime (73). In patients with 11βOHD, spironolactone used for the treatment of hypertension should be discontinued, due to its teratogenic potential (19).

3.2 Management of Adrenal Steroidogenic Dysfunction

Glucocorticoid and frequently also mineralocorticoid therapy is needed to replace adrenal cortical insufficiency, as well as to reestablish the physiology of the hypothalamic-pituitary-gonadal axis disrupted by the androgen excess in the most frequent forms of DSD of adrenal origin.

3.2.1 Conventional Treatment

Glucocorticoid use for the treatment of CAH was introduced in the early 1950s by Wilkins, who was also the first to report that cortisone was able to suppress the elevated adrenal androgens (74). Since then, there has been little development in the way steroid hormone replacement therapy is conducted.

Glucocorticoids are currently the standard treatment for CAH associated to 9α-fludrocortisone, in cases of mineralocorticoid deficiency. A clinical practice guideline has recently been developed (26). The minimum dose that normalizes the excess of adrenal androgens and avoids cortisol insufficiency is recommended. Unfortunately, available preparations fail to suppress ACTH and to control adrenal androgen excess resulting often in glucocorticoid overtreatment (15). Therefore, management of CAH involves a challenging balance between glucocorticoid deficiency and hyperandrogenism, on one side, and hypercortisolism on the other, leading to short stature, obesity, hypertension, osteoporosis, and an adverse metabolic profile.

In growing children, hydrocortisone is the glucocorticoid of choice due to its short life, which allows childhood growth optimization. Recommended dose is 8-15 mg/m^2 daily divided in three doses (14, 26). However, in late adolescence and adults, there are no standard clinical guidelines for glucocorticoid therapy and multiple preparations are available. Patients are generally switched to intermediate-acting glucocorticoids, such as prednisolone at 5.0–7.5 mg/day divided in two doses or long-acting glucocorticoids, such as dexamethasone at 0.25–0.50 mg at bedtime to improve compliance (75).

Mineralocorticoid supplementation with 9α-fludrocortisone is necessary in patients with aldosterone deficiency, present in different degrees in approximately 75-90% of patients with DSD of adrenal origin. All newborn patients detected by neonatal screening programs receive 9α-fludrocortisone, typically 100-200 μg/day divided in 1-2 doses. Sodium chloride supplements are recommended usually along the first years of life. In childhood, fludrocortisone doses usually range between 50-200 μg/day. Due to its prolonged half-life (18-36 hours) low doses can be administered once a day, although doses above 200 μg/day may still be divided to be given twice daily (14).

3.2.2 Novel Treatment Options

Even though an adequate hormonal replacement would minimize complications and assure a normal quality of life, current therapies have failed to prevent co-morbidities, and adrenal crises still occur as a leading cause of death (15, 18, 76). This is partly due to the lack of adequate preparations, making it difficult to control the disease. For this reason, novel therapeutic options have been developed, and several clinical trials in adults and children are currently ongoing.

3.2.2.1 Modified-Release Hydrocortisone

Chronocort® is a modified release formulation of hydrocortisone (MR-HC) designed to mimic physiological cortisol secretion. Made of uniform multiparticulate beads with an inert core, a hydrocortisone drug layer and a delayed release enteric outer coat, it aims to mimic the cortisol circadian rhythm and control the overnight ACTH surge that leads to the increase androgens (75, 77, 78). MR-HC has shown to successfully lower androgen levels in patients and decrease the hydrocortisone equivalent dose. The larger evening dose reaches its peak in the early morning hours, and smaller morning dose peaks in the afternoon/evening thus providing glucocorticoid cover for the day with a more physiological cortisol profile. Chronocort® is currently under regulatory review for the treatment of adults with CAH (18).

3.2.2.2 Nevanimibe

Nevanimibe hydrochloride (ATR-101) inhibits acyl-coenzyme A: cholesterol O-acyltransferase (ACAT1/sterol O-acyltransferase 1 (SOAT1)), the main enzyme that catalyzes the esterification of free cholesterol to cholesteryl esters for storage in adrenal cortex cells. At lower concentrations, Nevanimibe selectively blocks

adrenal cortex function of all three steroidogenic pathways (mineralocorticoid, glucocorticoid, and androgens). In a phase II study, single-blind, multicentric, placebo-controlled study of adults with classic 21OHD, Nevanimibe given orally decreased 17-hydroxyprogesterone levels within 2 weeks of treatment in most patients. However, it failed to effectively suppress androstenedione levels, a more durable measure of adrenal control (79). This therapy would allow the use of lower glucocorticoid doses, minimizing adverse events as compared to standard therapy and might represent a promising addition to current treatment strategies. However, larger long-term studies with higher dose are needed to evaluate safety and efficacy.

3.2.2.3 Abiraterone

Abiraterone acetate (AA) is a prodrug that is metabolized to abiraterone, a potent CYP17A1 inhibitor. It is used to suppress circulating testosterone in the treatment of prostate cancer improving survival rates. As P450c17 activity is needed for the synthesis of all androgen, it has been hypothesized that by inhibiting it with abiraterone acetate, added to stable doses of physiological hydrocortisone and 9α-fludrocortisone acetate, androgen excess present in 21OHD might be controlled, thus eliminating the need for supraphysiological glucocorticoids doses (80). In a phase I study of adult women with inadequately controlled classic 21OHD, abiraterone acetate added to hydrocortisone was able to normalize androstenedione on days 6 and 7 in at least 80% of participants without causing hypertension or hypokalemia. In a recent study, abiraterone acetate has shown to effectively and consistently lower 11-oxygenated androgens in 21OHD (81). Abiraterone acetate might also be beneficial to suppress androgens and estrogens in prepubertal children with classic CAH until the anticipated age of puberty. A phase I trial testing this approach is underway (NCT02574910).

3.2.2.4 Corticotropin-Releasing Factor Receptor-1 (CRF1R) Antagonists

Corticotropin-Releasing Factor (CRF) is released from the hypothalamus into the hypophyseal portal system, acting directly on specific receptors on pituitary corticotropes. CRF type 1 (CRF1R), one of the two CRF receptors, is especially abundant in the pituitary and in the neocortex. CRF receptor antagonists reduce ACTH and adrenal steroid production. A phase Ib study including 8 women with classic CAH showed that the CRF1R antagonist NBI-77860 can effectively decrease the early morning rise of ACTH and 17-hydroxyprogesterone, eliminating the need for supraphysiologic doses of glucocorticoids (82). Tildacerfont (SPR001; LY2371712) is a second generation CRF1R antagonist that binds to pituitary receptors with high affinity, thus decreasing ACTH secretion. In two recent phase 2 clinical trials including adult patients with CAH, oral tildacerfont reduced ACTH, 17-hydroxyprogesterone and androstenedione for up to 12 weeks; normalization of ACTH and androstenedione was achieved in 40-60% of the patients according to dosage (83). Longer term multidose trials are needed to determine safety and effectiveness of this potential therapy.

3.2.3 Potential Options Based on Cell- or Gene-Therapy

Cellular reprogramming and gene therapy are theoretically viable options that are under current investigation. Different cell types of mouse or human origin have been used for cellular reprogramming to an adrenocortical phenotype, showing ultrastructural features typical of steroidogenic cells, expression of steroidogenic enzymes and secretion of steroids in response to ACTH. The reprogrammed human steroidogenic cells were viable when experimentally transplanted into the kidney capsule or in the adrenals of mice (84). These cells could be helpful to model adrenal defects and represent a potential therapy strategy.

An alternative strategy is gene therapy, which was tested in mice with a deletion encompassing the *Cyp21* locus who received an injection of a replication-deficient adenovirus containing the mouse gene extra-adrenally (85), or the human *CYP21A2* gene intra-adrenally (86) or intravenously (87). In all cases the adrenal function was restored, giving hope for the development of gene therapy in humans with CAH due to 21OHD.

4 CONCLUDING REMARKS

Disorders of adrenal function are the major cause of DSD. In 46,XX patients, it represents more than 90% of the underlying etiologies. In 46,XY individuals, the associated gonadal steroidogenic failure leads to undervirilization and ambiguous genitalia. DSD associated with adrenal dysfunction represent a challenging condition, given the risk of life associated with adrenal failure. Management requires a balanced supplementation of glucocorticoids –and mineralocorticoids in almost 75% of the cases–, together with consideration of the genital and reproductive disorders. Unfortunately, despite the long-lasting awareness of these conditions, evidence-based recommendations are still scarce, and adequately designed studies need to be carried out in order to provide a better standard of care for these relatively frequent disorders.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

REFERENCES

1. Lin YT, Capel B. Cell Fate Commitment During Mammalian Sex Determination. *Curr Opin Genet Dev* (2015) 32:144–52. doi: 10.1016/j.gde.2015.03.003
2. Makela JA, Koskenniemi JJ, Virtanen HE, Toppari J. Testis Development. *Endocr Rev* (2019) 40:857–905. doi: 10.1210/er.2018-00140
3. Freire AV, Ropelato MG, Rey RA. Ovaries and Testes. In: CS Kovacs and C Deal, editors. *Maternal-Fetal and Neonatal Endocrinology*, *1st ed.* Boston, MA, USA: Academic Press-Elsevier (2020). p. 625–41.

4. Rey R, Josso N, Racine C. Sexual Differentiation. In: KR Feingold, B Anawalt, A Boyce, G Chrousos, K Dungan, A Grossman and JM Hershman, editors. *Endotext*. South Dartmouth (MA), USA: MDText.com, Inc (2020).
5. Lee PA, Houk CP, Ahmed SF, Hughes IA. In Collaboration With the Participants in the International Consensus Conference on Intersex Organized by the Lawson Wilkins Pediatric Endocrine Society and the European Society for Paediatric Endocrinology. Consensus Statement on Management of Intersex Disorders. *Pediatrics* (2006) 118:e488–500. doi: 10.1542/peds.2006-0738
6. Rey RA, Grinspon RP. Normal Male Sexual Differentiation and Aetiology of Disorders of Sex Development. *Best Pract Res Clin Endocrinol Metab* (2011) 25:221–38. doi: 10.1016/j.beem.2010.08.013
7. Rey RA, Josso N. Diagnosis and Treatment of Disorders of Sexual Development. In: JL Jameson, LC De Groot, DM de Kretser, LC Giudice, A Grossman, S Melmed, JT Potts and GC Weir, editors. *Endocrinology: Adult and Pediatric, 7th edition*. Philadelphia: Elsevier Saunders (2016). p. 2086–118.
8. Prader A. Genital Findings in the Female Pseudo-Hermaphroditism of the Congenital Adrenogenital Syndrome; Morphology, Frequency, Development and Heredity of the Different Genital Forms. *Helv Paediatr Acta* (1954) 9:231–48.
9. Guercio G, Saraco N, Costanzo M, Marino R, Belgorosky A. Human Aromatase Deficiency. *Encyclopedia Endocr Dis* (2019) 5:532–49. doi: 10.1016/B978-0-12-801238-3.65212-1
10. Kaňová N, Bičíková M. Hyperandrogenic States in Pregnancy. *Physiol Res* (2011) 60:243–52. doi: 10.33549/physiolres.932078
11. Malinowski AK, Sen J, Sermer M. Hyperreactio Luteinalis: Maternal and Fetal Effects. *J Obstet Gynaecol Canada* (2015) 37:715–23. doi: 10.1016/s1701-2163(15)30176-6
12. Grinspon RP, Bergadá I, Rey RA. Male Hypogonadism and Disorders of Sex Development. *Front Endocrinol (Lausanne)* (2020) 11:211. doi: 10.3389/fendo.2020.00211
13. Auchus RJ, Miller WL. Defects in Androgen Biosynthesis Causing 46,XY Disorders of Sexual Development. *Semin Reprod Med* (2012) 30:417–26. doi: 10.1055/s-0032-1324726
14. El-Maouche D, Arlt W, Merke DP. Congenital Adrenal Hyperplasia. *Lancet* (2017) 390:2194–210. doi: 10.1016/s0140-6736(17)31431-9
15. Merke DP, Auchus RJ. Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency. *N Engl J Med* (2020) 383:1248–61. doi: 10.1056/NEJMra1909786
16. Baranowski ES, Arlt W, Idkowiak J. Monogenic Disorders of Adrenal Steroidogenesis. *Horm Res Paediatr* (2018) 89:292–310. doi: 10.1159/000488034
17. Finkielstain GP, Chen W, Mehta SP, Fujimura FK, Hanna RM, Van Ryzin C, et al. Comprehensive Genetic Analysis of 182 Unrelated Families With Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency. *J Clin Endocrinol Metab* (2011) 96:E161–72. doi: 10.1210/jc.2010-0319
18. Claahsen-van der Grinten HL, Speiser PW, Ahmed SF, Arlt W, Auchus RJ, Falhammar H, et al. Congenital Adrenal Hyperplasia - Current Insights in Pathophysiology, Diagnostics and Management. *Endocr Rev* (2022). doi: 10.1210/endrev/bnab016 (in press)
19. Guercio G, Costanzo M, Grinspon RP, Rey RA. Fertility Issues in Disorders of Sex Development. *Endocrinol Metab Clin North Am* (2015) 44:867–81. doi: 10.1016/j.ecl.2015.07.012
20. Claahsen-van der Grinten HL, Stikkelbroeck N, Falhammar H, Reisch N. Management of Endocrine Disease: Gonadal Dysfunction in Congenital Adrenal Hyperplasia. *Eur J Endocrinol* (2021) 184:R85–97. doi: 10.1530/EJE-20-1093
21. Turcu AF, Nanba AT, Chomic R, Upadhyay SK, Giordano TJ, Shields JJ, et al. Adrenal-Derived 11-Oxygenated 19-Carbon Steroids are the Dominant Androgens in Classic 21-Hydroxylase Deficiency. *Eur J Endocrinol* (2016) 174:601–9. doi: 10.1530/EJE-15-1181
22. Reisch N, Taylor AE, Nogueira EF, Asby DJ, Dhir V, Berry A, et al. Alternative Pathway Androgen Biosynthesis and Human Fetal Female Virilization. *Proc Natl Acad Sci USA* (2019) 116:22294–9. doi: 10.1073/pnas.1906623116
23. Hirschberg AL, Gidlof S, Falhammar H, Frisen L, Almqvist C, Nordenskiold A, et al. Reproductive and Perinatal Outcomes in Women With Congenital Adrenal Hyperplasia - A Population-Based Cohort Study. *J Clin Endocrinol Metab* (2020) 106:e957–65. doi: 10.1210/clinem/dgaa801
24. Reisch N. Pregnancy in Congenital Adrenal Hyperplasia. *Endocrinol Metab Clin North Am* (2019) 48:619–41. doi: 10.1016/j.ecl.2019.05.011
25. Gomes LG, Bachega T, Mendonca BB. Classic Congenital Adrenal Hyperplasia and its Impact on Reproduction. *Fertil Steril* (2019) 111:7–12. doi: 10.1016/j.fertnstert.2018.11.037
26. Speiser PW, Arlt W, Auchus RJ, Baskin LS, Conway GS, Merke DP, et al. Congenital Adrenal Hyperplasia Due to Steroid 21-Hydroxylase Deficiency: An Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab* (2018) 103:4043–88. doi: 10.1210/jc.2018-01865
27. Miller WL. Congenital Adrenal Hyperplasia: Time to Replace 17OHP With 21-Deoxycortisol. *Horm Res Paediatr* (2019) 91:416–20. doi: 10.1159/000501396
28. Yildiz M, Isik E, Abali ZY, Keskin M, Ozbek MN, Bas F, et al. Clinical and Hormonal Profiles Correlate With Molecular Characteristics in Patients With 11beta-Hydroxylase Deficiency. *J Clin Endocrinol Metab* (2021) 106:e3714–24. doi: 10.1210/clinem/dgab225
29. Mooij CF, Parajes S, Rose IT, Taylor AE, Bayraktaroglu T, Wass JA, et al. Characterization of the Molecular Genetic Pathology in Patients With 11beta-Hydroxylase Deficiency. *Clin Endocrinol (Oxf)* (2015) 83:629–35. doi: 10.1111/cen.12834
30. Khattab A, Haider S, Kumar A, Dhawan S, Alam D, Romero R, et al. Clinical, Genetic, and Structural Basis of Congenital Adrenal Hyperplasia Due to 11beta-Hydroxylase Deficiency. *Proc Natl Acad Sci USA* (2017) 114:E1933–40. doi: 10.1073/pnas.1621082114
31. Bulsari K, Falhammar H. Clinical Perspectives in Congenital Adrenal Hyperplasia Due to 11beta-Hydroxylase Deficiency. *Endocrine* (2017) 55:19–36. doi: 10.1007/s12020-016-1189-x
32. Simm PJ, Zacharin MR. Successful Pregnancy in a Patient With Severe 11-Beta-Hydroxylase Deficiency and Novel Mutations in CYP11B1 Gene. *Horm Res* (2007) 68:294–7. doi: 10.1159/000107651
33. Miller WL. Mechanisms in Endocrinology: Rare Defects in Adrenal Steroidogenesis. *Eur J Endocrinol* (2018) 179:R125–41. doi: 10.1530/EJE-18-0279
34. Miller WL. Disorders in the Initial Steps of Steroid Hormone Synthesis. *J Steroid Biochem Mol Biol* (2017) 165:18–37. doi: 10.1016/j.jsbmb.2016.03.009
35. Bose HS, Sugawara T, Strauss JF, Miller WL. The International Congenital Lipoid Adrenal Hyperplasia Consortium. The Pathophysiology and Genetics of Congenital Lipoid Adrenal Hyperplasia. *N Engl J Med* (1996) 335:1870–9. doi: 10.1056/NEJM199612193352503
36. Khoury K, Barbar E, Ainmelk Y, Ouellet A, Lehoux JG. Gonadal Function, First Cases of Pregnancy, and Child Delivery in a Woman With Lipoid Congenital Adrenal Hyperplasia. *J Clin Endocrinol Metab* (2009) 94:1333–7. doi: 10.1210/jc.2008-1694
37. Sertedaki A, Pantos K, Vrettou C, Kokkali G, Christofidou C, Kanavakis E, et al. Conception and Pregnancy Outcome in a Patient With 11-Bp Deletion of the Steroidogenic Acute Regulatory Protein Gene. *Fertil Steril* (2009) 91:934 e915–938. doi: 10.1016/j.fertnstert.2008.07.1770
38. Albarel F, Perrin J, Jegaden M, Roucher-Boulez F, Reynaud R, Brue T, et al. Successful IVF Pregnancy Despite Inadequate Ovarian Steroidogenesis Due to Congenital Lipoid Adrenal Hyperplasia (CLAH): A Case Report. *Hum Reprod* (2016) 31:2609–12. doi: 10.1093/humrep/dew239
39. Kim CJ. Congenital Lipoid Adrenal Hyperplasia. *Ann Pediatr Endocrinol Metab* (2014) 19:179–83. doi: 10.6065/apem.2014.19.4.179
40. Katharopoulos E, Di Iorgi N, Fernandez-Alvarez P, Pandey AV, Groessl M, Dubey S, et al. Characterization of Two Novel Variants of the Steroidogenic Acute Regulatory Protein Identified in a Girl With Classic Lipoid Congenital Adrenal Hyperplasia. *Int J Mol Sci* (2020) 21(17):6185. doi: 10.3390/ijms21176185
41. Zhang T, Ma X, Wang J, Jia C, Wang W, Dong Z, et al. Clinical and Molecular Characterization of Thirty Chinese Patients With Congenital Lipoid Adrenal Hyperplasia. *J Steroid Biochem Mol Biol* (2021) 206:105788. doi: 10.1016/j.jsbmb.2020.105788
42. Kolli V, Kim H, Torky A, Lao Q, Tatsi C, Mallappa A, et al. Characterization of the CYP11A1 Nonsynonymous Variant P.E314K in Children Presenting With Adrenal Insufficiency. *J Clin Endocrinol Metab* (2019) 104:269–76. doi: 10.1210/jc.2018-01661
43. Kallali W, Gray E, Mehdi MZ, Lindsay R, Metherell LA, Buonocore F, et al. Long-Term Outcome of Partial P450 Side-Chain Cleavage Enzyme Deficiency

in Three Brothers: The Importance of Early Diagnosis. *Eur J Endocrinol* (2020) 182:K15–24. doi: 10.1530/EJE-19-0696
44. Auchus RJ. Steroid 17-Hydroxylase and 17,20-Lyase Deficiencies, Genetic and Pharmacologic. *J Steroid Biochem Mol Biol* (2017) 165:71–8. doi: 10.1016/j.jsbmb.2016.02.002
45. Kurnaz E, Kartal Baykan E, Turkyilmaz A, Yarali O, Yavas Abali Z, Turan S, et al. Genotypic Sex and Severity of the Disease Determine the Time of Clinical Presentation in Steroid 17alpha-Hydroxylase/17,20-Lyase Deficiency. *Horm Res Paediatr* (2020) 93:558–66. doi: 10.1159/000515079
46. Miller WL, Auchus RJ. The "Backdoor Pathway" of Androgen Synthesis in Human Male Sexual Development. *PloS Biol* (2019) 17:e3000198. doi: 10.1371/journal.pbio.3000198
47. O'Shaughnessy PJ, Antignac JP, Le Bizec B, Morvan ML, Svechnikov K, Soder O, et al. Alternative (Backdoor) Androgen Production and Masculinization in the Human Fetus. *PloS Biol* (2019) 17:e3000002. doi: 10.1371/journal.pbio.3000002
48. Blumenfeld Z, Koren I. Successful Delivery in 17,20-Lyase Deficiency. *J Clin Endocrinol Metab* (2021) 106:1882–6. doi: 10.1210/clinem/dgab222
49. Parker KL. The Roles of Steroidogenic Factor 1 in Endocrine Development and Function. *Mol Cell Endocrinol* (1998) 145:15–20. doi: 10.1016/s0303-7207(98)00164-6
50. Morohashi K. Gonadal and Extragonadal Functions of Ad4BP/SF-1: Developmental Aspects. *Trends Endocrinol Metab* (1999) 10:169–73. doi: 10.1016/S1043-2760(98)00142-8
51. Suntharalingham JP, Buonocore F, Duncan AJ, Achermann JC. DAX-1 (NR0B1) and Steroidogenic Factor-1 (SF-1, NR5A1) in Human Disease. *Best Pract Res Clin Endocrinol Metab* (2015) 29:607–19. doi: 10.1016/j.beem.2015.07.004
52. Knarston IM, Robevska G, van den Bergen JA, Eggers S, Croft B, Yates J, et al. NR5A1 Gene Variants Repress the Ovarian-Specific WNT Signaling Pathway in 46,XX Disorders of Sex Development Patients. *Hum Mutat* (2019) 40:207–16. doi: 10.1002/humu.23672
53. Al Alawi AM, Nordenstrom A, Falhammar H. Clinical Perspectives in Congenital Adrenal Hyperplasia Due to 3beta-Hydroxysteroid Dehydrogenase Type 2 Deficiency. *Endocrine* (2019) 63:407–21. doi: 10.1007/s12020-018-01835-3
54. El-Maouche D, Hargreaves CJ, Sinaii N, Mallappa A, Veeraraghavan P, Merke DP. Longitudinal Assessment of Illnesses, Stress Dosing, and Illness Sequelae in Patients With Congenital Adrenal Hyperplasia. *J Clin Endocrinol Metab* (2018) 103:2336–45. doi: 10.1210/jc.2018-00208
55. Güran T, Kara C, Yildiz M, Bitkin EC, Haklar G, Lin JC, et al. Revisiting Classical 3beta-Hydroxysteroid Dehydrogenase 2 Deficiency: Lessons From 31 Pediatric Cases. *J Clin Endocrinol Metab* (2020) 105(3):dgaa022. doi: 10.1210/clinem/dgaa022
56. Auchus RJ, Chang AY. 46,XX DSD: The Masculinised Female. *Best Pract Res Clin Endocrinol Metab* (2010) 24:219–42. doi: 10.1016/j.beem.2009.11.001
57. Nordenstrom A, Forest MG, Wedell A. A Case of 3beta-Hydroxysteroid Dehydrogenase Type II (HSD3B2) Deficiency Picked Up by Neonatal Screening for 21-Hydroxylase Deficiency: Difficulties and Delay in Etiologic Diagnosis. *Horm Res* (2007) 68:204–8. doi: 10.1159/000102593
58. Arlt W, Walker EA, Draper N, Ivison HE, Ride JP, Hammer F, et al. Congenital Adrenal Hyperplasia Caused by Mutant P450 Oxidoreductase and Human Androgen Synthesis: Analytical Study. *Lancet* (2004) 363:2128–35. doi: 10.1016/S0140-6736(04)16503-3
59. Flück CE, Tajima T, Pandey AV, Arlt W, Okuhara K, Verge CF, et al. Mutant P450 Oxidoreductase Causes Disordered Steroidogenesis With and Without Antley-Bixler Syndrome. *Nat Genet* (2004) 36:228–30. doi: 10.1038/ng1300
60. Krone N, Reisch N, Idkowiak J, Dhir V, Ivison HE, Hughes BA, et al. Genotype-Phenotype Analysis in Congenital Adrenal Hyperplasia Due to P450 Oxidoreductase Deficiency. *J Clin Endocrinol Metab* (2012) 97:E257–267. doi: 10.1210/jc.2011-0640
61. Fluck CE, Parween S, Rojas Velazquez MN, Pandey AV. Inhibition of Placental CYP19A1 Activity Remains as a Valid Hypothesis for 46,XX Virilization in P450 Oxidoreductase Deficiency. *Proc Natl Acad Sci USA* (2020) 117:14632–3. doi: 10.1073/pnas.2003154117
62. Idkowiak J, O'Riordan S, Reisch N, Malunowicz EM, Collins F, Kerstens MN, et al. Pubertal Presentation in Seven Patients With Congenital Adrenal Hyperplasia Due to P450 Oxidoreductase Deficiency. *J Clin Endocrinol Metab* (2011) 96:E453–462. doi: 10.1210/jc.2010-1607
63. Jha S, Turcu AF, Sinaii N, Brookner B, Auchus RJ, Merke DP. 11-Oxygenated Androgens Useful in the Setting of Discrepant Conventional Biomarkers in 21-Hydroxylase Deficiency. *J Endocr Soc* (2021) 5:bvaa192. doi: 10.1210/jendso/bvaa192
64. Campana C, Rege J, Turcu AF, Pezzi V, Gomez-Sanchez CE, Robins DM, et al. Development of a Novel Cell Based Androgen Screening Model. *J Steroid Biochem Mol Biol* (2016) 156:17–22. doi: 10.1016/j.jsbmb.2015.11.005
65. Rege J, Turcu AF, Kasa-Vubu JZ, Lerario AM, Auchus GC, Auchus RJ, et al. 11-Ketotestosterone Is the Dominant Circulating Bioactive Androgen During Normal and Premature Adrenarche. *J Clin Endocrinol Metab* (2018) 103:4589–98. doi: 10.1210/jc.2018-00736
66. Rege J, Nakamura Y, Satoh F, Morimoto R, Kennedy MR, Layman LC, et al. Liquid Chromatography-Tandem Mass Spectrometry Analysis of Human Adrenal Vein 19-Carbon Steroids Before and After ACTH Stimulation. *J Clin Endocrinol Metab* (2013) 98:1182–8. doi: 10.1210/jc.2012-2912
67. Turcu AF, Mallappa A, Elman MS, Avila NA, Marko J, Rao H, et al. 11-Oxygenated Androgens Are Biomarkers of Adrenal Volume and Testicular Adrenal Rest Tumors in 21-Hydroxylase Deficiency. *J Clin Endocrinol Metab* (2017) 102:2701–10. doi: 10.1210/jc.2016-3989
68. Lee PA, Nordenstrom A, Houk CP, Ahmed SF, Auchus R, Baratz A, et al. Global Disorders of Sex Development Update Since 2006: Perceptions, Approach and Care. *Horm Res Paediatr* (2016) 85:158–80. doi: 10.1159/000442975
69. Cools M, Nordenström A, Robeva R, Hall J, Westerveld P, Flück C, et al. Group CABw. Caring for Individuals With a Difference of Sex Development (DSD): A Consensus Statement. *Nat Rev Endocrinol* (2018) 14:415–29. doi: 10.1038/s41574-018-0010-8
70. Mieszczak J, Houk CP, Lee PA. Assignment of the Sex of Rearing in the Neonate With a Disorder of Sex Development. *Curr Opin Pediatr* (2009) 21:541–7. doi: 10.1097/MOP.0b013e32832c6d2c
71. Brännström M, Johannesson L, Bokström H, Kvarnström N, Molne J, Dahm-Kähler P, et al. Livebirth After Uterus Transplantation. *Lancet* (2015) 385:607–16. doi: 10.1016/S0140-6736(14)61728-1
72. Guercio G, Rey RA. Fertility Issues in the Management of Patients With Disorders of Sex Development. *Endocr Dev* (2014) 27:87–98. doi: 10.1159/000363633
73. Casteràs A, De SP, Rumsby G, Conway GS. Reassessing Fecundity in Women With Classical Congenital Adrenal Hyperplasia (CAH): Normal Pregnancy Rate But Reduced Fertility Rate. *Clin Endocrinol (Oxf)* (2009) 70:833–7. doi: 10.1111/j.1365-2265.2009.03563.x
74. Wilkins L, Lewis RA, Klien R, Rosemberg E. The Suppression of Androgen Secretion by Cortisone in a Case of Congenital Adrenal Hyperplasia. *Bull Johns Hopkins Hosp* (1950) 86:249–52.
75. Verma S, Vanryzin C, Sinaii N, Kim MS, Nieman LK, Ravindran S, et al. A Pharmacokinetic and Pharmacodynamic Study of Delayed- and Extended-Release Hydrocortisone (Chronocort) vs. Conventional Hydrocortisone (Cortef) in the Treatment of Congenital Adrenal Hyperplasia. *Clin Endocrinol (Oxf)* (2010) 72:441–7. doi: 10.1111/j.1365-2265.2009.03636.x
76. Ali SR, Bryce J, Haghpanahan H, Lewsey JD, Tan LE, Atapattu N, et al. Real-World Estimates of Adrenal Insufficiency-Related Adverse Events in Children With Congenital Adrenal Hyperplasia. *J Clin Endocrinol Metab* (2021) 106:e192–203. doi: 10.1210/clinem/dgaa694
77. Mallappa A, Sinaii N, Kumar P, Whitaker MJ, Daley LA, Digweed D, et al. A Phase 2 Study of Chronocort, a Modified-Release Formulation of Hydrocortisone, in the Treatment of Adults With Classic Congenital Adrenal Hyperplasia. *J Clin Endocrinol Metab* (2015) 100:1137–45. doi: 10.1210/jc.2014-3809
78. Merke DP, Mallappa A, Arlt W, Brac de la Perriere A, Linden Hirschberg A, Juul A, et al. Modified-Release Hydrocortisone in Congenital Adrenal Hyperplasia. *J Clin Endocrinol Metab* (2021) 106:e2063–77. doi: 10.1210/clinem/dgab051
79. Speiser PW. Invited Commentary: A Phase 2, Multicenter Study of Nevanimibe for the Treatment of Congenital Adrenal Hyperplasia. *J Clin Endocrinol Metab* (2020) 105(10):dgaa509. doi: 10.1210/clinem/dgaa509
80. Auchus RJ, Buschur EO, Chang AY, Hammer GD, Ramm C, Madrigal D, et al. Abiraterone Acetate to Lower Androgens in Women With Classic 21-Hydroxylase Deficiency. *J Clin Endocrinol Metab* (2014) 99:2763–70. doi: 10.1210/jc.2014-1258

81. Wright C, O'Day P, Alyamani M, Sharifi N, Auchus RJ. Abiraterone Acetate Treatment Lowers 11-Oxygenated Androgens. *Eur J Endocrinol* (2020) 182:413–21. doi: 10.1530/EJE-19-0905
82. Turcu AF, Spencer-Segal JL, Farber RH, Luo R, Grigoriadis DE, Ramm CA, et al. Single-Dose Study of a Corticotropin-Releasing Factor Receptor-1 Antagonist in Women With 21-Hydroxylase Deficiency. *J Clin Endocrinol Metab* (2016) 101:1174–80. doi: 10.1210/jc.2015-3574
83. Sarafoglou K, Barnes CN, Huang M, Imel EA, Madu IJ, Merke DP, et al. Tildacerfont in Adults With Classic Congenital Adrenal Hyperplasia: Results From Two Phase 2 Studies. *J Clin Endocrinol Metab* (2021) 106:e4666–79. doi: 10.1210/clinem/dgab438
84. Ruiz-Babot G, Balyura M, Hadjidemetriou I, Ajodha SJ, Taylor DR, Ghataore L, et al. Modeling Congenital Adrenal Hyperplasia and Testing Interventions for Adrenal Insufficiency Using Donor-Specific Reprogrammed Cells. *Cell Rep* (2018) 22:1236–49. doi: 10.1016/j.celrep.2018.01.003
85. Naiki Y, Miyado M, Horikawa R, Katsumata N, Onodera M, Pang S, et al. Extra-Adrenal Induction of Cyp21a1 Ameliorates Systemic Steroid Metabolism in a Mouse Model of Congenital Adrenal Hyperplasia. *Endocr J* (2016) 63:897–904. doi: 10.1507/endocrj.EJ16-0112
86. Tajima T, Okada T, Ma XM, Ramsey W, Bornstein S, Aguilera G. Restoration of Adrenal Steroidogenesis by Adenovirus-Mediated Transfer of Human Cytochromep450 21-Hydroxylase Into the Adrenal Gland Of21-Hydroxylase-Deficient Mice. *Gene Ther* (1999) 6:1898–903. doi: 10.1038/sj.gt.3301018
87. Perdomini M, Dos Santos C, Goumeaux C, Blouin V, Bougneres P. An AAVrh10-CAG-CYP21-HA Vector Allows Persistent Correction of 21-Hydroxylase Deficiency in a Cyp21(-/-) Mouse Model. *Gene Ther* (2017) 24:275–81. doi: 10.1038/gt.2017.10

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Frequently Asked Questions in Patients With Adrenal Insufficiency in the Time of COVID-19

Chiara Sabbadin[1†], *Corrado Betterle*[2†], *Carla Scaroni*[1,2†] *and Filippo Ceccato*[1,2,3*†]

[1] Endocrine Disease Unit, University-Hospital of Padova, Padova, Italy, [2] Department of Medicine (DIMED), University of Padova, Padova, Italy, [3] Department of Neuroscience (DNS), University of Padova, Padova, Italy

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Maria Fragoso,
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Takeda Pharmaceutical Company Limited, Argentina

***Correspondence:**
Filippo Ceccato
filippo.ceccato@unipd.it

†ORCID:
Chiara Sabbadin
orcid.org/0000-0003-1526-6832
Corrado Betterle
orcid.org/0000-0002-8996-410X
Carla Scaroni
orcid.org/0000-0001-9396-3815
Filippo Ceccato
orcid.org/0000-0003-1456-8716

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Adrenal insufficiency (AI) is a life-threatening disorder, with increased morbidity and mortality, especially in case of an acute illness that can increase the requirement of cortisol. A novel infectious disease, termed Coronavirus Disease 2019 (COVID-19), appeared in 2020. Therefore, AI patients are experiencing a novel challenge: the risk of infection. In our experience, a prompt contact to the Endocrine center (with a telemedicine consultation) and a full awareness of diseases (cortisol deficiency, COVID-19 and the self-management of an adrenal crisis) are important to motivate patients. Vaccine is an effective treatment to prevent hospitalization and aggressive course of COVID-19. Some patients manifest challenges due to inequitable access and vaccine hesitancy, resulting in a delay in the acceptance of vaccines despite the availability of vaccination services. Therefore, an effort of all physicians must be conducted in order to advise patients with AI. In this short review, we try to answer some frequently asked questions regarding the management of patients with AI.

Keywords: COVID-19, glucocorticoid treatment, immune response, adrenal insufficiency, Addison disease

INTRODUCTION

In early 2020, the world experienced the global pandemic of the Severe Acute Respiratory Syndrome (SARS) Coronavirus 2 (SARS-CoV-2) (1). From a clinical point-of-view, the Coronavirus Disease 2019 (COVID-19) ranges from asymptomatic cases, to patients with mild/self-limiting respiratory tract illness, up to subjects with severe progressive disease with pneumonia and multi-organ failure (2, 3). Containment measurements were progressively expanded, combined with the use of personal protective equipment, enforcing social distancing, isolation and quarantine of all positive cases and their relatives. Nowadays, several vaccines (including those with novel mRNA technology) are validated by national and international drug regulation agencies (4), and are effective in the control of the progression from mild symptoms to severe disease (5).

Adrenal insufficiency (AI) is defined as an insufficient production/secretion of glucocorticoids (GC) and/or mineralocorticoids (6), especially in primary AI (6). On the contrary, central AI is characterized by inappropriate ACTH secretion (7). People with AI are facing their primary disease and the risk/fear of COVID-19 infection (8). Therefore, a complete awareness of diseases (AI and COVID-19) and motivation about self-management are of paramount importance in patients.

In this short review, we propose an updated state-of-art regarding the management of patients with AI.

IS MORTALITY AND INFECTIOUS RISK INCREASED IN PATIENTS WITH AI?

Conflicting data exist about the rate and the causes of mortality of patients with Addison Diseases (AD, the most common autoimmune AI) (9–12).

The first study, based on data from the National Swedish Hospital Register, reported that the risk for mortality in patients with AD was 2.19 in men and 2.86 in women, mainly due to cardiovascular diseases, cancers, infectious diseases and diabetes mellitus (DM) (9). An inappropriate GC replacement (both excess or inadequate increment of doses in response to stress conditions) may be responsible for the increased mortality. These results were confirmed in AD patients admitted to hospitals: mortality rate was 2.9 for women and 2.5 for men, up to 4.6 in autoimmune polyglandular syndrome type 1 (APS-1, a rare AD characterized by hypoparathyroidism and chronic mucocutaneous candidiasis, due to mutations in the autoimmune regulatory gene) (10). A subsequent Norway study did not confirm these data, except in patients with young onset of AD, probably affected by APS-1 (11). A critical evaluation of these studies revealed some bias, as the use of general mortality registers in which it was not possible to confirm the accuracy of the diagnosis of AD (12).

A study in 2017 reported that patients with AD had a reduced natural-killer cell cytotoxicity that impairs the early recognition of infected cells in the respiratory tract (13). This impairment in anti-viral immune defense may contribute to the increased rate of infections (not only SARS-CoV-2). However, a UK study with 1580 patients with AI (AD or congenital adrenal hyperplasia, CAH) showed that patients with GC treatment had an increased risk of respiratory, urinary or gastrointestinal infections and of prescription of antimicrobials respect to CAH without GC therapy (14). A recent paper demonstrated that patients on conventional GC therapy had a pro-inflammatory state and a weakened immune defense; a normalization of the immune cell profile and a reduction of infections was observed after the restoration of the physiological circadian cortisol rhythm with modified-release hydrocortisone (HC) (15). A Swedish cohort reported an increased relative risk of death (respectively 28% and 10%) in 226 patients with diabetes (Type 1 and 2) and AD matched with 1129 controls with only diabetes, especially due to diabetic complications and infectious diseases (16). The authors suggested that adrenal crisis could be a contributing factor to this increased mortality.

IS COVID-19 RISK AND MANAGEMENT IN PATIENTS WITH AI DIFFERENT FROM GENERAL POPULATION?

Patients with AI are considered "clinically vulnerable" for their increased risk of infections, that could lead to poor prognosis and death due to adrenal crisis (14). A recent Expert Opinion of the Italian Society of Endocrinology (SIE) suggested that not only the aetiology of the AI, the length of follow-up, the patient's age or the expected adherence to therapy, but also the comorbidities must be carefully evaluated during substitutive treatments (17), because mimicking cortisol rhythm can reduce recurrent infections (15). In the last months, a task force of the SIE (8) and the European Society of Endocrinology (ESE) (18) published some recommendations regarding GC replacement in patients with AD infected with SARS-COV-2 according to the stage of the disease. Others proposed strategies to improve patients' education to manage high-risk situations, to prevent adrenal crisis (19).

During the first COVID-19 wave, two cross-sectional studies reported a low prevalence of infection among AD patients, and COVID-19 disease severity similar to healthy controls in 393 patients with primary and secondary AI referring to Italian centers. An important emotional impact was found in some patients requiring an up-titration of the usual GC replacement: patients' education about infection-related risks and adequately self-adjustment of replacement therapy were fundamental to prevent acute events and complications (20, 21).

Another study reported that among 159 patients taking steroid replacement therapy for pituitary disease, 30 patients (18.9%) reported symptoms of COVID-19 infection, but only two of the seven patients tested for COVID-19 infection resulted to be positive (22). Finally, a recent longitudinal survey study performed in 2 tertiary medical centers of the US confirmed a lower prevalence of COVID-19 infection in AI patients compared with overall prevalence (1.8% versus 7.9%, respectively) (23). All infected patients reported mild symptoms and were managed at home.

Based on the available data, there is no evidence that patients with both primary and secondary AI have an increased risk of infection and disease. However, these conclusions could not be extended to patients with APS-1: their primary immunodeficiency lead to the development of young-onset multiple autoimmune disorders (24). APS-1 is associated with an increased mortality from infections and from cancers in comparison to the general population (24, 25). Considering the low mean prevalence of APS-1 (about 10 cases per million inhabitants) (26), only 24 cases of APS-1 patients from seven countries who were infected with SARS-CoV2 has been reported until now (27, 28). 20 patients were hospitalized, 15 showed severe complications requiring admission to an intensive care unit (ICU) and 4 of them died. Interestingly, beyond the underlying peculiar condition (genetic, age and AI), in APS-1 patients the pre-existence of autoantibodies (auto-Abs) neutralizing most type 1 interferons (IFNs), key immune regulators against viral infections, confer a very high risk of developing critical COVID-19. A recent study reported these auto-Abs in about 10% of cases of severe pneumonia in the general population (27). Infected patients with APS-1 should be always hospitalized promptly to evaluate the best management according to the severity of the disease and to their pre-existing risk factors.

In case of Sars-CoV-2 infection, the management of patients with AI should follow the sick-days rules, as reassumed in **Figure 1**. In case of signs or symptoms of infection, all patients with AI are encouraged to prompt contact their referral Endocrinologist in

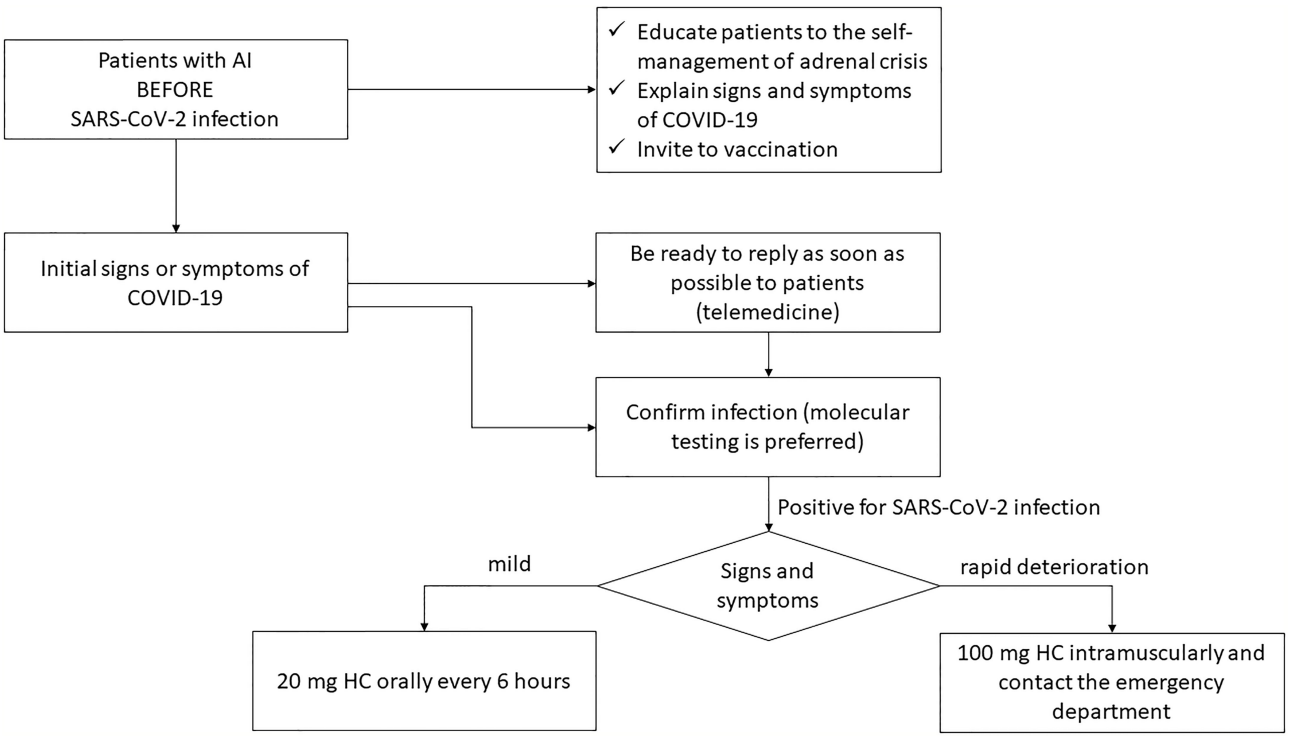

FIGURE 1 | Clinical management of patients with AI and SARS-CoV-2. SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2; COVID-19. Coronavirus Disease 2019; HC, hydrocortisone.

order to adjust GC dose and to being advise in case of hospital admission (29). The clearance of HC significantly drops during critical illness: the SIE/ESE recommendations are to administer 20 mg HC orally every 6 hours in case of asymptomatic disease or only uncomplicated mild symptomatic infection (sore throat, mild cough, without headache, vomit, diarrhoea, or fever <38°C) (8, 18). Then, in case of clinical deterioration (incoming hypotension, persistent cough, increased respiratory rate > 30 breaths/minute or SpO2 <93%), the self-administration of 100 mg HC and the contact with the emergency department are suggested (8, 18). In children, 2 mg/kg or 50 mg/m^2 of HC every 6–8 h intramuscularly, subcutaneous or intravenous, combined with the correction of hypovolemia (0.9% up to 60 mL/kg within 1 hour) and hypoglycaemia, are suggested (6, 30, 31). Beyond the adjustment of GC therapy during COVID-19, patients with AI should reduce their risk to get the SARS-Cov-2 infection through social distancing, the use of masks, hands cleaning with dedicate gel, the choice of work-from-home if possible (18, 32).

HOW SHOULD WE DIAGNOSE AI AT THE TIME OF COVID-19?

Several drugs and conditions can affect hypothalamic-pituitary-adrenal (HPA) axis and induce primary or secondary AI, during or after SARS-Cov-2 infection. In a real-life clinical setting, physicians tend to use GC in most critically ill patients, especially in those affected by acute respiratory distress syndrome (ARDS). Daily GC doses in patients with community-acquired pneumonia in ICU are 32–40 mg of methylprednisolone equivalent daily in the majority of randomized controlled trials reported in a Cochrane review (33). At these doses, GC inhibit immune responses and pathogen clearance, but also suppress lung inflammation. GC administration, titration, duration, or underlying disease are not able to predict AI after GC withdrawal (34). The rationale of GC use in COVID-19 infection is to reduce the abnormal immune reactivity that induce lung damage and progression to ARDS more than uncontrolled viral replication (35). In July 2020 the RECOVERY trial reported that 6 mg of dexamethasone (the most potent synthetic GC) for 10 days is effective in reducing 28-day mortality among patients who were receiving mechanical ventilation or oxygen alone (36).

In addition, the co-administration of antiretroviral drugs may trigger drug-interactions and enhance the exposure to GC, metabolized through the CYP3A pathway (37), the dominant isoenzyme of the hepatic cytochrome P450 system and the primary metabolic step for the degradation of GC (37). Several reports in HIV-infected patients have documented an impairment of HPA axis in patients treated with GC and ritonavir (38, 39): it reduce the activity of CYP3A4 enzymes, increasing GC levels. Most of ritonavir-associated AI have involved fluticasone, an inhaled GC (40–42). IFN-α is used to treat chronic viral infections: it suppresses CYP3A4 expression in human hepatoma cells (43) and alter the expression of constitutive and inducible CYP3A genes in well-differentiated

male rat hepatocytes in culture (44). In humans, a flat diurnal ACTH curve and cortisol slope has been observed after IFN-α/ribavirin administration (45). Finally, IFN-β has been shown to modulate the induction of cytochrome P450 enzyme in mice (46).

Viral infection can induce AI directly. During previous SARS outbreak in 2002-2003, up to 40% of the patients showed low basal and post-synacthen cortisol levels, suggesting a direct negative effect on corticotroph cells (47). A primary adrenal injury consistent with bilateral adrenal haemorrhage has been reported in several patients with Covid-19 infection, especially in those with positive antiphospholipid antibodies (APA) (48, 49). In an autopsy study that described 28 different patients, half adrenals presented microscopic alterations: 7 necrosis (generally ischemic), 4 cortical lipid degeneration, 2 hemorrhage and one unspecific focal adrenalitis (50). Despite exogenous GC, critically ill patients may present a relative AI termed Critical-illness Related Corticosteroid Insufficiency (CIRCI), secondary to higher levels of IL-6, IL-10 and TNF-α (51). CIRCI not indicates strictly a pituitary or adrenal injury, but rather a condition of relative cortisol insufficiency resulting from inadequate GC-mediated anti-inflammatory response (52).

Diagnosis of AI is based upon low morning cortisol levels and, in selected cases, to dynamic tests (6, 53). In case of a pandemic outbreak, out-patient visits or blood collection could be a source of viral spreading: during Sars-CoV-2 waves an effort to limit face-to-face consultations has been proposed (32). Salivary sample is a stress-free tool to measure cortisol, suitable for out-patients who can mail it to the referral center (54). A paper of the ESE reported some concerns regarding salivary cortisol, due to the potential contamination of laboratory staff (55), however no study are reported in this situation. Even if it has been never used, also dynamic tests can be performed without an Endocrine clinic, using salivary cortisol (56) and intramuscular ACTH administration (57).

CAN COVID-19 BE A TRIGGER FOR AUTOIMMUNE DISEASES?

Autoimmune diseases are multifactorial: the concomitant presence of genetic, epigenetic, exogenous and endogenous factors is required for their development (58).

The role of genetic factors derives from the observation that autoimmune diseases are more common in peculiar ethnic groups or in families: the genes involved are mainly those related to the major histocompatibility complex. However, the genetic predisposition is in general a condition "*sine qua non*" and a) the discordance in identical twins; b) the appearance of the disease in a minimal part of the "genetically susceptible" subjects; c) the diversity of frequency in individuals of the same race living in different geographical areas, argues in favour of the existence of other factors. The main endogenous factors are gender and age. It is a common observation that females have a greater predisposition to autoimmune diseases than males (from 2:1 up to 10:1), and it seems to depend both by the direct action of sex chromosome genes, and by the concomitant hormonal status. Regarding age, some autoimmune diseases favour adults (Hashimoto thyroiditis, systemic lupus erythematosus, Sjogren's syndrome, AD), the elderly population (pernicious anemia, polyarteritis nodosa) and some present a paediatric onset (type 1 DM, celiac disease, Kawasaki's disease [KD], type 1 autoimmune hepatitis, APS-1) (59).

The concept of "exogenous factor" can be attributed to infections, chemicals, iodine, radiations, drugs, foods, trauma, additives, smoke, pollution and socio-economic situations. There are many indirect data that support a relationship between viruses and autoimmune diseases; however, the direct data are limited to HBV and panarteritis nodosa; HCV and cryoglobulinemia; rotavirus infection and celiac disease, enteric viruses and type 1 DM, herpesviruses and systemic lupus erythematosus, rheumatoid arthritis or adult-onset Still's disease (59).

COVID-19 is a new condition and little is known about the immunological changes that occur in the infected individuals. Viral infections stimulate a vigorous immune response, with a cascade of events involving both the innate and adaptive immunity. In addition, viruses can break immunological tolerance and induce autoimmunity by bystander activation, epitope spreading or molecular mimicry. The last occurs when similarities between foreign- and self-peptides favour an activation of autoreactive T or B cells by foreign derived peptides in a genetic susceptible individual. Several studies have documented very high plasma levels of cytokines and chemokines during Sars-Cov-2 infection. IL-1β and TNFα promote Th-1 and Th-17 responses, contributing to high levels of pro-inflammatory cytokines in the context of a cytokine storm syndrome (59). SARS-CoV-2 shares some sequences (GSQASS, LNEVAK, and SAAEAS) with three proteins present in the brainstem respiratory pacemaker: it might account for an autoimmune disease with depression of respiratory pacemaker and it may induce an autoimmune pulmonary damage (59). Several studies demonstrated immunological (as spike protein) and clinical similarities between COVID-19 and hyperinflammatory diseases, leading to the hypothesis that SARS-CoV-2 infection might trigger autoimmune responses in genetically predisposed subjects (59, 60). In the first period of SARS-CoV-2 infection various autoimmune manifestations, including neurologic demyelinating syndromes, autoimmune cytopenias and thrombotic events, were reported (59, 61). APA and APA-related syndrome associated with SARS-CoV-2 infection was evaluated on overall 4273 patients and was found to be present in 515 cases, especially in ICU. On the other hands, most individuals with APA do not experience thrombotic events (62). Immune thrombocytopenia associated with SARS-CoV-2 infection was described in about 30% of the infected patients (62). Acute inflammatory neuropathies resembling Guillain-Barrè syndrome have been reported in 48 patients with COVID-19, a Miller-Fisher syndrome was developed in 4, few cases developed an acute disseminated encephalomyelitis or myelitis (62). Autoimmune hemolytic anemia (AIHA) was described in 14 patients with COVID-19: AIHA could be induced by a molecular mimicry between the viral spike protein and ankyrin-1, a membrane protein of erythrocytes (62). Systemic lupus erythematosus associated with SARS-CoV-2 infection was very

rare and described in 6 case reports. A vasculitis associated to anti-neutrophil cytoplasmic antibodies related to SARS-CoV-2 infection is reported in 3 cases (62). Skin lesions reported in COVID-19 patients were classified into 4 groups: exanthema, vascular, urticarial and acro-papular eruptions (62, 63).

The paediatric population appears to be less affected than adults that develop severe SARS-CoV-2 infection. This can be due both to the decreased level of maturity and function of ACE2, and differences in the immune response. Nonetheless, since 2020, paediatricians began reporting cases of children with fever and signs of systemic inflammation with features in common with KD. Compared with the classical KD, newly diagnosed KD-like patients were older and had more signs of cardiac involvement, shock and required more frequently higher steroid treatment. These patients manifested also gastrointestinal symptoms, which are uncommon in typical KD, and very high levels of procalcitonin. KD-like syndrome was confirmed in 1888 patients with an age from 4 months to 35 years (62).

IS THERE AN INCREASED RISK TO DEVELOP OTHER AUTOIMMUNE DISEASES AFTER COVID-19 INFECTION OR VACCINATION IN PATIENTS WITH AI? A PERSONAL EXPERIENCE

Patients with isolated autoimmune AD or with polyglandular diseases ask if they are at increased risk of developing new autoimmune disease after COVID-19 infection or after vaccination against COV-19. Patients affected by one or more autoimmune diseases are at risk to develop other autoimmune diseases (58). In addition, it is important to remember that COVID-19 infection can induce autoimmune diseases in the general population. Nevertheless, in patients with AI followed at the Endocrinology of Padova (202 primary AI and 134 central AI) we did not document new-onset autoimmune diseases during or after COVID-19 infection or 6-months after vaccination (we use only mRNA-based vaccine according to the recommendation of the Italian Institute of Health). Furthermore, to our knowledge, there are not so far published cases describing the development of new autoimmune disorders in patients with autoimmune AD after COVID-19 infection or vaccinations. Obviously, the post-vaccination observation period is limited to 6 months. Vaccinations started in Italy at the end of December 2020 (starting with health employers), in February 2021 it was proposed to frail patients (as AI). At the best of our knowledge, one case of vaccine-induced primary AI has been reported after a bilateral adrenal haemorrhagic infarction due to bilateral vein thrombosis in a patient with vaccine-induced immune thrombotic thrombocytopenia (64).

Regarding the management of substitutive treatment in patients with AI who will receive a COVID-19 vaccine, a recent survey of the Pituitary Society reported that 36% of physicians recommend an increase in GC dosage with the first injection; the others plan to increase replacement therapy in case of fever or vaccination-related symptoms (65).

CONCLUSIONS AND FUTURE PERSPECTIVES

Patients with AI could present an increased risk of COVID-19; however, the severity of the disease is mainly due to an inappropriate and prompt GC treatment rather than an increased infection susceptibility (which can be real only for the rare patients with APS-1).

Given the current state of the art, we think that vaccine is a safe procedure, and the patients with AI that hesitate to receive the COVID-19 vaccination should be carefully advised that viral infection or the vaccine can produce autoimmune diseases in rare cases, and on the contrary the vaccination is protective against a disease with a high-risk of hospitalization and mortality, especially in frail patients as those with AI.

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REFERENCES

1. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical Features of Patients Infected With 2019 Novel Coronavirus in Wuhan, China. *Lancet* (2020) 395(10223):497–506. doi: 10.1016/S0140-6736(20)30183-5
2. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, et al. Clinical Characteristics of 138 Hospitalized Patients With 2019 Novel Coronavirus–Infected Pneumonia in Wuhan, China. *JAMA* (2020) 323(11):1061. doi: 10.1001/jama.2020.1585
3. Bai Y, Yao L, Wei T, Tian F, Jin DY, Chen L, et al. Presumed Asymptomatic Carrier Transmission of COVID-19. *JAMA* (2020) 323(14):1406. doi: 10.1001/jama.2020.2565
4. Fenton C, Lamb YN. COVID-19: State of the Vaccination. *Drugs Ther Perspect Ration Drug Sel Use* (2021) 15:1–11. doi: 10.1007/s40267-021-00869-4
5. Fan Y-J, Chan K-H, Hung IF-N. Safety and Efficacy of COVID-19 Vaccines: A Systematic Review and Meta-Analysis of Different Vaccines at Phase 3. *Vaccines* (2021) 9(9):989. doi: 10.3390/vaccines9090989
6. Bornstein SR, Allolio B, Arlt W, Barthel A, Don-Wauchope A, Hammer GD, et al. Diagnosis and Treatment of Primary Adrenal Insufficiency: An Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab* (2016) 101(2):364–89. doi: 10.1210/jc.2015-1710
7. Ceccato F, Scaroni C. Central Adrenal Insufficiency: Open Issues Regarding Diagnosis and Glucocorticoid Treatment. *Clin Chem Lab Med* (2019) 57 (8):1125–35. doi: 10.1515/cclm-2018-0824
8. Isidori AM, Arnaldi G, Boscaro M, Falorni A, Giordano C, Giordano R, et al. COVID-19 Infection and Glucocorticoids: Update From the Italian Society of Endocrinology Expert Opinion on Steroid Replacement in Adrenal

Insufficiency. *J Endocrinol Invest* (2020) 43(8):1141–7. doi: 10.1007/s40618-020-01266-w
9. Bergthorsdottir R, Leonsson-Zachrisson M, Odén A, Johannsson G. Premature Mortality in Patients With Addison's Disease: A Population-Based Study. *J Clin Endocrinol Metab* (2006) 91(12):4849–53. doi: 10.1210/jc.2006-0076
10. Bensing S, Brandt L, Tabaroj F, Sjöberg O, Nilsson B, Ekbom A, et al. Increased Death Risk and Altered Cancer Incidence Pattern in Patients With Isolated or Combined Autoimmune Primary Adrenocortical Insufficiency. *Clin Endocrinol (Oxf)* (2008) 69(5):697–704. doi: 10.1111/j.1365-2265.2008.03340.x
11. Erichsen MM, Løvås K, Fougner KJ, Svartberg J, Hauge ER, Bollerslev J, et al. Normal Overall Mortality Rate in Addison's Disease, But Young Patients Are at Risk of Premature Death. *Eur J Endocrinol* (2009) 160(2):233–7. doi: 10.1530/EJE-08-0550
12. Falorni A, Minarelli V, Morelli S. Therapy of Adrenal Insufficiency: An Update. *Endocrine* (2013) 43(3):514–28. doi: 10.1007/s12020-012-9835-4
13. Bancos I, Hazeldine J, Chortis V, Hampson P, Taylor AE, Lord JM, et al. Primary Adrenal Insufficiency Is Associated With Impaired Natural Killer Cell Function: A Potential Link to Increased Mortality. *Eur J Endocrinol* (2017) 176(4):471–80. doi: 10.1530/EJE-16-0969
14. Tresoldi AS, Sumilo D, Perrins M, Toulis KA, Prete A, Reddy N, et al. Increased Infection Risk in Addison's Disease and Congenital Adrenal Hyperplasia. *J Clin Endocrinol Metab* (2020) 105(2):418–29. doi: 10.1210/clinem/dgz006
15. Isidori AM, Venneri MA, Graziadio C, Simeoli C, Fiore D, Hasenmajer V, et al. Effect of Once-Daily, Modified-Release Hydrocortisone Versus Standard Glucocorticoid Therapy on Metabolism and Innate Immunity in Patients With Adrenal Insufficiency (DREAM): A Single-Blind, Randomised Controlled Trial. *Lancet Diabetes Endocrinol* (2017) 6(3):173–85. doi: 10.1016/S2213-8587(17)30398-4
16. Chantzichristos D, Persson A, Eliasson B, Miftaraj M, Franzén S, Bergthorsdottir R, et al. Mortality in Patients With Diabetes Mellitus and Addison's Disease: A Nationwide, Matched, Observational Cohort Study. *Eur J Endocrinol* (2017) 176(1):31–9. doi: 10.1530/EJE-16-0657
17. Isidori AM, Arnaldi G, Boscaro M, Falorni A, Giordano C, Giordano R, et al. Towards the Tailoring of Glucocorticoid Replacement in Adrenal Insufficiency: The Italian Society of Endocrinology Expert Opinion. *J Endocrinol Invest* (2020) 43(5):683–96. doi: 10.1007/s40618-019-01146-y
18. Arlt W, Baldeweg SE, Pearce SHS, Simpson HL. Endocrinology in the Time of COVID-19: Management of Adrenal Insufficiency. *Eur J Endocrinol* (2020) 183(1):G25–32. doi: 10.1530/EJE-20-0361
19. Kienitz T, Hahner S, Burger-Stritt S, Quinkler M. Therapeutic Patient Education for Adrenal Insufficiency Under COVID-19 Pandemic Conditions. *Exp Clin Endocrinol Diabetes* (2021) 129(03):241–9. doi: 10.1055/a-1217-7208
20. Martino M, Aboud N, Cola MF, et al. Impact of COVID-19 Pandemic on Psychophysical Stress in Patients With Adrenal Insufficiency: The CORTI-COVID Study. *J Endocrinol Invest* (2021) 44(5):1075–84. doi: 10.1007/s40618-020-01422-2
21. Carosi G, Morelli V, Del Sindaco G, Serban AL, Cremaschi A, Frigerio S, et al. Adrenal Insufficiency at the Time of COVID-19: A Retrospective Study in Patients Referring to a Tertiary Center. *J Clin Endocrinol Metab* (2021) 106(3):e1354–61. doi: 10.1210/clinem/dgaa793
22. Graf A, Marcus HJ, Baldeweg SE. The Direct and Indirect Impact of the COVID-19 Pandemic on the Care of Patients With Pituitary Disease: A Cross Sectional Study. *Pituitary* (2021) 24(2):262–8. doi: 10.1007/s11102-020-01106-3
23. Li D, Suresh M, Abbondanza T, Vaidya A, Bancos I. The Impact of the COVID-19 Pandemic on Self-Reported Outcomes in Patients With Adrenal Insufficiency. *J Clin Endocrinol Metab* (2021) 106(7):e2469–79. doi: 10.1210/clinem/dgab334
24. Garelli S, Dalla Costa M, Sabbadin C, Barollo S, Rubin B, Scarpa R, et al. Autoimmune Polyendocrine Syndrome Type 1: An Italian Survey on 158 Patients. *J Endocrinol Invest* (2021) 44(11):2493–510. doi: 10.1007/s40618-021-01585-6
25. Borchers J, Pukkala E, Mäkitie O, Laakso S. Patients With APECED Have Increased Early Mortality Due to Endocrine Causes, Malignancies and Infections. *J Clin Endocrinol Metab* (2020) 105(6):e2207–13. doi: 10.1210/clinem/dgaa140
26. Husebye ES, Anderson MS, Kämpe O. Autoimmune Polyendocrine Syndromes. Ingelfinger JR, Ed. *N Engl J Med* (2018) 378(12):1132–41. doi: 10.1056/NEJMra1713301
27. Bastard P, Orlova E, Sozaeva L, Lévy R, James A, Schmitt MM, et al. Preexisting Autoantibodies to Type I IFNs Underlie Critical COVID-19 Pneumonia in Patients With APS-1. *J Exp Med* (2021) 218(7):e20210554. doi: 10.1084/jem.20210554
28. Carpino A, Buganza R, Matarazzo P, Tuli G, Pinon M, Calvo PL, et al. Autoimmune Polyendocrinopathy–Candidiasis–Ectodermal Dystrophy in Two Siblings: Same Mutations But Very Different Phenotypes. *Genes (Basel)* (2021) 12(2):169. doi: 10.3390/genes12020169
29. Husebye ES, Allolio B, Arlt W, Badenhoop K, Bensing S, Betterle C, et al. Consensus Statement on the Diagnosis, Treatment and Follow-Up of Patients With Primary Adrenal Insufficiency. *J Intern Med* (2014) 275(2):104–15. doi: 10.1111/joim.12162
30. Reznik Y, Barat P, Bertherat J, Bouvattier C, Castinetti F, Chabre O, et al. SFE/SFEDP Adrenal Insufficiency French Consensus: Introduction and Handbook. *Ann Endocrinol (Paris)* (2018) 79(1):1–22. doi: 10.1016/j.ando.2017.12.001
31. Yoo H-W. Diverse Etiologies, Diagnostic Approach, and Management of Primary Adrenal Insufficiency in Pediatric Age. *Ann Pediatr Endocrinol Metab* (2021) 26(3):149–57. doi: 10.6065/apem.2142150.075
32. Ceccato F, Voltan G, Sabbadin C, Camozzi V, Merante Boschin I, Mian C, et al. Tele-Medicine Versus Face-to-Face Consultation in Endocrine Outpatients Clinic During COVID-19 Outbreak: A Single-Center Experience During the Lockdown Period. *J Endocrinol Invest* (2021) 44(8):1689–98. doi: 10.1007/s40618-020-01476-2
33. Stern A, Skalsky K, Avni T, Carrara E, Leibovici L, Paul M. Corticosteroids for Pneumonia. *Cochrane Database Syst Rev* (2017) 12:CD007720. doi: 10.1002/14651858.CD007720.pub3
34. Broersen LHA, Pereira AM, Jørgensen JOL, Dekkers OM. Adrenal Insufficiency in Corticosteroids Use: Systematic Review and Meta-Analysis. *J Clin Endocrinol Metab* (2015) 100(6):2171–80. doi: 10.1210/jc.2015-1218
35. Isidori AM, Pofi R, Hasenmajer V, Lenzi A, Pivonello R. Use of Glucocorticoids in Patients With Adrenal Insufficiency and COVID-19 Infection. *Lancet Diabetes Endocrinol* (2020) 8(6):472–3. doi: 10.1016/S2213-8587(20)30149-2
36. RECOVERY Collaborative Group, Horby P, Lim WS, Emberson JR, Mafham M, Bell JL, Linsell L, et al. Dexamethasone in Hospitalized Patients With Covid-19 — Preliminary Report. *N Engl J Med* (2020) 384(8):693–704. doi: 10.1056/NEJMoa2021436
37. Ferraù F, Ceccato F, Cannavò S, Scaroni C. What We Have to Know About Corticosteroids Use During Sars-Cov-2 Infection. *J Endocrinol Invest* (2020) 44(4):693–701. doi: 10.1007/s40618-020-01384-5
38. Elliot ER, Theodoraki A, Jain LR, Marshall NJ, Boffito M, Baldeweg SE, et al. Iatrogenic Cushing's Syndrome Due to Drug Interaction Between Glucocorticoids and the Ritonavir or Cobicistat Containing HIV Therapies. *Clin Med (Northfield Il)* (2016) 16(5):412–8. doi: 10.7861/clinmedicine.16-5-412
39. Saberi P, Phengrasamy T, Nguyen DP. Inhaled Corticosteroid Use in HIV-Positive Individuals Taking Protease Inhibitors: A Review of Pharmacokinetics, Case Reports and Clinical Management. *HIV Med* (2013) 14(9):519–29. doi: 10.1111/hiv.12039
40. Epperla N, McKiernan F. Iatrogenic Cushing Syndrome and Adrenal Insufficiency During Concomitant Therapy With Ritonavir and Fluticasone. *Springerplus* (2015) 4(1):455. doi: 10.1186/s40064-015-1218-x
41. Foisy MM, Yakiwchuk EMK, Chiu I, Singh AE. Adrenal Suppression and Cushing's Syndrome Secondary to an Interaction Between Ritonavir and Fluticasone: A Review of the Literature. *HIV Med* (2008) 9(6):389–96. doi: 10.1111/j.1468-1293.2008.00579.x
42. Zubillaga I, Francés C, Nicolau J, Homar F, Masmiquel L. Adrenal Insufficiency and Exogenous Cushing's Syndrome in a Patient Receiving Inhaled Fluticasone and Ritonavir. *Endocrinol Diabetes y Nutr* (2017) 64(6):338–9. doi: 10.1016/j.endinu.2017.02.006
43. Flaman AS, Gravel C, Hashem AM, Tocchi M, Li X. The Effect of Interferon-α on the Expression of Cytochrome P450 3A4 in Human Hepatoma Cells. *Toxicol Appl Pharmacol* (2011) 253(2):130–6. doi: 10.1016/j.taap.2011.03.019
44. Tapner M, Liddle C, Goodwin B, George J, Farrell GC. Interferon Gamma Down-Regulates Cytochrome P450 3A Genes in Primary Cultures of Well-

Differentiated Rat Hepatocytes. *Hepatology* (1996) 24(2):367–73. doi: 10.1053/jhep.1996.v24.pm0008690406
45. Raison CL, Borisov AS, Woolwine BJ, Massung B, Vogt G, Miller AH. Interferon-α Effects on Diurnal Hypothalamic–Pituitary–Adrenal Axis Activity: Relationship With Proinflammatory Cytokines and Behavior. *Mol Psychiatry* (2010) 15(5):535–47. doi: 10.1038/mp.2008.58
46. Carelli M, Porras MC, Rizzardini M, Cantoni L. Modulation of Constitutive and Inducible Hepatic Cytochrome(s) P-450 by Interferon β in Mice. *J Hepatol* (1996) 24(2):230–7. doi: 10.1016/S0168-8278(96)80034-1
47. Leow MK-S, Kwek DS-K, Ng AW-K, Ong K-C, Kaw GJ-L, Lee LS-U. Hypocortisolism in Survivors of Severe Acute Respiratory Syndrome (SARS). *Clin Endocrinol (Oxf)* (2005) 63(2):197–202. doi: 10.1111/j.1365-2265.2005.02325.x
48. Álvarez-Troncoso J, Zapatero Larrauri M, Montero Vega MD, Gil Vallano R, Palmier Peláez E, Martín Rojas-Marcos P, et al. Case Report: COVID-19 With Bilateral Adrenal Hemorrhage. *Am J Trop Med Hyg* (2020) 103(3):1156–57. doi: 10.4269/ajtmh.20-0722
49. Machado IFR, Menezes IQ, Figueiredo SR, Coelho FMA, Terrabuio DRB, Ramos DV, et al. Primary Adrenal Insufficiency Due to Bilateral Adrenal Infarction in COVID-19. *J Clin Endocrinol Metab* (2021) 107(1):e394–400. doi: 10.1210/clinem/dgab557
50. Freire Santana M, Borba MGS, Baía-da-Silva DC, Val F, Alexandre MAA, Brito-Sousa JD, et al. Case Report: Adrenal Pathology Findings in Severe COVID-19: An Autopsy Study. *Am J Trop Med Hyg* (2020) 103(4):1604–7. doi: 10.4269/ajtmh.20-0787
51. Kwon YS, Suh GY, Jeon K, Park SY, Lim SY, Koh WJ, et al. Serum Cytokines and Critical Illness-Related Corticosteroid Insufficiency. *Intensive Care Med* (2010) 36(11):1845–51. doi: 10.1007/s00134-010-1971-9
52. Annane D, Pastores SM, Rochwerg B, Arlt W, Balk RA, Beishuizen A, et al. Guidelines for the Diagnosis and Management of Critical Illness-Related Corticosteroid Insufficiency (CIRCI) in Critically Ill Patients (Part I): Society of Critical Care Medicine (SCCM) and European Society of Intensive Care Medicine (ESICM) 2017. *Intensive Care Med* (2017) 43(12):1751–63. doi: 10.1007/s00134-017-4919-5
53. Fleseriu M, Hashim I, Karavitaki N, Melmed S, Murad MH, Salvatori R, et al. Hormonal Replacement in Hypopituitarism in Adults: An Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab* (2016) 101(11):3888–921. doi: 10.1210/jc.2016-2118
54. Raff H. Utility of Salivary Cortisol Measurements in Cushing's Syndrome and Adrenal Insufficiency. *J Clin Endocrinol Metab* (2009) 94(10):3647–55. doi: 10.1210/jc.2009-1166
55. Newell-Price J, Nieman L, Reincke M, Tabarin A. Endocrinology in the Time of COVID-19: Management of Cushing's Syndrome. *Eur J Endocrinol* (2020) 183(1):G1–7. doi: 10.1530/EJE-20-0352
56. Ceccato F, Selmin E, Antonelli G, Barbot M, Daniele A, Boscaro M, et al. Low-Dose Short Synacthen Test With Salivary Cortisol in Patients With Suspected Central Adrenal Insufficiency. *Endocr Connect* (2021) 10(9):1189–99. doi: 10.1530/EC-21-0404
57. George GS, Jabbar PK, Jayakumari C, John M, Mini M, Thekkumkara Surendran Nair A, et al. Long-Acting Porcine ACTH Stimulated Salivary Cortisol in the Diagnosis of Adrenal Insufficiency. *Clin Endocrinol (Oxf)* (2020) 93(6):652–60. doi: 10.1111/cen.14286
58. Olivieri B, Betterle C, Zanoni G. Vaccinations and Autoimmune Diseases. *Vaccines* (2021) 9(8):815. doi: 10.3390/vaccines9080815
59. Rodríguez Y, Novelli L, Rojas M, De Santis M, Acosta-Ampudia Y, Monsalve DM, et al. Autoinflammatory and Autoimmune Conditions at the Crossroad of COVID-19. *J Autoimmun* (2020) 114:102506. doi: 10.1016/j.jaut.2020.102506
60. Vojdani A, Kharrazian D. Potential Antigenic Cross-Reactivity Between SARS-CoV-2 and Human Tissue With a Possible Link to an Increase in Autoimmune Diseases. *Clin Immunol* (2020) 217:108480. doi: 10.1016/j.clim.2020.108480
61. Helms J, Tacquard C, Severac F, Leonard-Lorant I, Ohana M, Delabranche X, et al. High Risk of Thrombosis in Patients With Severe SARS-CoV-2 Infection: A Multicenter Prospective Cohort Study. *Intensive Care Med* (2020) 46(6):1089–98. doi: 10.1007/s00134-020-06062-x
62. Novelli L, Motta F, De Santis M, Ansari AA, Gershwin ME, Selmi C. The JANUS of Chronic Inflammatory and Autoimmune Diseases Onset During COVID-19 – A Systematic Review of the Literature. *J Autoimmun* (2021) 117:102592. doi: 10.1016/j.jaut.2020.102592
63. Gisondi P, PIaserico S, Bordin C, Alaibac M, Girolomoni G, Naldi L. Cutaneous Manifestations of SARS-CoV-2 Infection: A Clinical Update. *J Eur Acad Dermatol Venereol* (2020) 34(11):2499–504. doi: 10.1111/jdv.16774
64. Varona JF, Garcia-Isidro M, Moeinvaziri M, Ramos-López M, Fernández-Domínguez M. Primary Adrenal Insufficiency Associated With Oxford-AstraZeneca ChAdOx1 Ncov-19 Vaccine-Induced Immune Thrombotic Thrombocytopenia (VITT). *Eur J Intern Med* (2021) 91(January):90–2. doi: 10.1016/j.ejim.2021.06.025
65. Katznelson L, Gadelha M. Glucocorticoid Use in Patients With Adrenal Insufficiency Following Administration of the COVID-19 Vaccine: A Pituitary Society Statement. *Pituitary* (2021) 24(2):143–5. doi: 10.1007/s11102-021-01130-x

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Characteristics of In2G Variant in Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency

Mirjana Kocova[1*†], *Paola Concolino*[2†] *and Henrik Falhammar*[3,4†]

[1] Medical Faculty, University "Cyril & Methodius" Skopje, Skopje, North Macedonia, [2] Dipartimento di Scienze di Laboratorio e Infettivologiche, Unita' Operativa Complessa (UOC) Chimica, Biochimica e Biologia Molecolare Clinica, Fondazione Policlinico Universitario Agostino Gemelli Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Rome, Italy, [3] Department of Endocrinology, Karolinska University Hospital, Stockholm, Sweden, [4] Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden

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Maria Fragoso,
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University of São Paulo, Brazil
Gabriela Paula Finkielstain,
Takeda Pharmaceutical Company Limited, Argentina

***Correspondence:**
Mirjana Kocova
mirjanakocova@yahoo.com

†ORCID:
Mirjana Kocova
orcid.org/0000-0001-7097-3439
Paola Concolino
orcid.org/0000-0002-0523-5744
Henrik Falhammar
orcid.org/0000-0002-5622-6987

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Substantial research has been performed during the last decades on the clinical and genetic variability of congenital adrenal hyperplasia (CAH) and its most common form, 21-hydroxylase deficiency (21OHD). CAH is one of the most prevalent autosomal recessive diseases in humans, and it can be divided into classic—further subdivided into salt wasting (SW) and simple virilizing (SV)—and non-classic (NC) forms. Pathogenic variants of *CYP21A2* gene, encoding the 21-hydroxylase enzyme, have been reported with variable prevalence in different populations. NM_000500.9:c.293-13C/A>G (In2G) variant represents the most common *CYP21A2* gene changes related to the classic 21OHD form. However, the phenotype of In2G carriers is variable depending on the variant homozygous/heterozygous status and combination with other *CYP21A2* pathogenic variants. In addition, identical genotypes, harboring the homozygous In2G variant, can present with variable phenotypes including the SW and SV or rarely NC form of the disease. Here, we analyze and present the clinical aspects, genotype/phenotype correlations, and other characteristics related to the *CYP21A2* In2G variant.

Keywords: ***CYP21A2*, c.293-13C/A>G, splicing variant, CAH, genetic counselling**

INTRODUCTION

Congenital adrenal hyperplasia (CAH) is one of the most common autosomal recessive diseases in humans with an incidence, evaluated on neonatal screening in different populations during the last decade or so, of 1:6,084 to 1:26,727 live births (1). It comprises several steroid enzyme deficiencies, among which 21-hydroxylase deficiency (21OHD) (OMIM # 201910) is by far the most common, affecting about 95%–99% of all CAH patients (1–5). In 21OHD, the hormonal disbalances consist of variable low cortisol and aldosterone levels, and compensatory high levels of 17-hydroxyprogesterone (17OHP), which converts to androgens. CAH appears in two clinical forms, classic and non-classic (NC) phenotypes (6). The classic form is further classified as salt-wasting (SW) and simple-virilizing (SV) forms. Patients affected by the SW form have SW with severe dehydration, hypoglycemia, failure to thrive, and hyperandrogenism. This can be clinically recognized early in girls due to atypical genitalia, while boys may first be identified, if not neonatally

screened, when presenting with a life-threatening SW crisis within 2 weeks postnatally (2). The SV form has enough aldosterone production to avoid SW crisis and, prior to the introduction of neonatal screening, was identified due to atypical genitalia in girls and in male toddlers due to signs and symptoms of excessive androgen production, even though cases of diagnosis in adulthood occasionally happened (2, 7). The NC form is mild and can appear in numerous variants of the clinical picture, ranging from no signs in the newborn period, to mild virilization later in childhood, up to polycystic ovary syndrome or isolated hyperandrogenism and decreased fertility in adulthood (8). NC 21OHD is sometimes identified in neonatal screening (9), but most cases are identified due to symptoms and signs in adolescence or young adulthood, even though the majority probably never gets diagnosed. NC CAH shows an incidence of 1:200 to 1:1,000 (10, 11).

However, despite the traditional and generally accepted classification of CAH in different forms, due to a large variety of *CYP21A2* mutations, the phenotype can have many variants, and it is clear that CAH phenotype represents a continuum between non-classic and classic forms (12). This is important for tailoring appropriate therapy in individual patients. The 21-hydroxylase enzyme is encoded by *CYP21A2* gene, and the clinical 21OHD presentation depends upon the combination of pathogenic variants affecting this locus (13). Both *CYP21A2* gene and *CYP21A1P* pseudogene are located on the short arm of chromosome 6 (6p21.3), in the human leukocyte antigen (HLA) class III region of the major histocompatibility (MHC) locus. These genes contain ten exons spaced over 3.4 kb with a sequence homology reaching 98% (12, 14, 15). Intergenic recombination events represent more than 95% of pathogenic variants causing 21OHD. Approximately 75% of the deleterious variants are transferred by small conversions from the pseudogene during meiosis (16, 17). Of the cases of 21OHD, 20%–25% are due to gene deletions, gene duplications, and deletions involving *CYP21A2* and other contiguous genes (18). Finally, *CYP21A2* pathogenic variants that are not apparently gene conversions account for 5%–10% of CAH alleles in most populations (16, 17).

To date, more than 230 *CYP21A2* pathogenic variants have been identified (18). Most patients are compound heterozygous; and in this case, the phenotype correlates with the variant that predicts the higher residual enzyme activity (19). Based on the residual activity of the mutant enzyme, *CYP21A2* variants are classified into specific groups (null, A, B, and C) (20, 21). While variants of the null group show 0% enzyme activity during *in vitro* assay, group A variants preserve a minimal (<1%) residual activity. These two groups are both associated with the SW form of 21OHD. Differently, group B (1%–5% enzyme activity) and group C (20%–50% enzyme activity) variants are related to the SV and NC forms, respectively (12, 22–25). Although there is good agreement between clinical phenotype and patient genotype, it is well-known that some exceptions exist (18–29).

The prevalence of 21OHD is highly variable among populations; some countries show higher (China and India) (19, 20) or lower prevalence (Japan and New Zealand) (3, 21–27). However, in most of the analyzed studies, the In2G variant is found to be, with rare exceptions (30–50), among the most common *CYP21A2* pathogenic variants. It is usually related to the SW 21OHD; however, patients with a non-correspondent phenotype have been widely reported. In this review, we collected the most relevant evidence showing the phenotypic variability of the In2G variant.

CYP21A2 IN2G VARIANT

To date, 18 intronic splicing variants, representing 7.7% of all disease-causing variants, have been reported in *CYP21A2* gene (18, 51). *In silico* analysis or functional studies showed that all these variants are associated with the severe form of 21OHD due to the changed reading frame of the gene producing a non-functional enzyme (21, 52, 53). Generally, an intronic splicing variant causes the disruption of the acceptor/donor site, inducing activation of an intronic cryptic acceptor/donor site, retention of a whole intron or part of it, and exon skipping (51). Regarding intron 2 of *CYP21A2* gene, five pathogenic splicing variants have been reported (51). Two of these, c.292+1G>A and c.293-2A>G, cause the SW phenotype to disrupt the donor and acceptor splicing sites, respectively (54, 55). Differently, the c.292+5G>A and c.293-7C>G variants were described, in SW patients, as reducing the consensus value for the intron 2 splice donor and acceptor sites, respectively (52, 56).

c.293-13C/A>G (In2G) is the most common splicing variant in *CYP21A2* gene. It is usually transferred by microconversion from *CYP21A2* pseudogene. At the −13 position, before the end of intron 2, the wild-type nucleotide is A or C. Substitution to G creates an additional splice acceptor site, causing aberrant splicing of intron 2 with retention of 19 intronic nucleotides. This results in a shift in the translational reading frame (21) (**Figure 1**). The In2G variant is typically related to the SW form of CAH. It is by far the most common CAH mutation in the majority of countries and ethnicities, predominantly Europeans followed by Middle Easterners and Hispanics (27), although there are exceptions (**Table 1**). In different studies, the prevalence of the In2G variant was between 20.6% and 30.3% (25, 27, 61, 63). In the homozygous form, it is more common in those with European and Middle Eastern ancestries than in Hispanic Americans, Asians, or East Indians (27). Some authors even refer up to 60.4% prevalence of this variant in certain ethnicities (32) (**Table 1**).

In some populations, the In2G variant could be considered a founder variant. It is a unique finding in some enclosed populations such as Alaskan and one of the Roma populations from the Balkan region, although the number of explored individuals was rather small (62). Finally, in other populations, such as the Spanish population, the In2G variant appears to be related to recent conversion events (64).

Genotype/Phenotype Correlation in In2G Patients

There is a reasonable genotype/phenotype correlation in 21OHD despite disease-causing variant variability (3, 28–30). As mentioned previously, the phenotype is almost uniformly

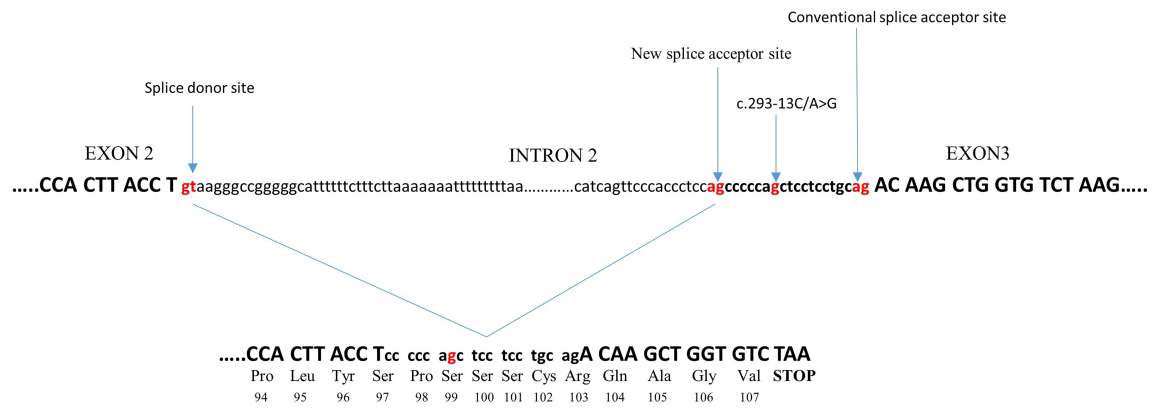

FIGURE 1 | Effect of the c.293-13C/A>G variant on splicing. The c.293-13C/A>G variant, at a –13 position before the end of intron 2, creates an additional splice acceptor site, causing aberrant splicing of intron 2 with retention of 19 intronic nucleotides. This causes a frameshift in the following third exon to generate a stop codon after 107 amino acids, resulting in the production of a truncated protein.

TABLE 1 | Prevalence of In2G variant in different countries.

Country	In2G (%)	No. of alleles	Reference
Argentina	35.2	866	(25)
Austria +Germany	29.2	1,320	(57)
Brazil	21.1	856	(20)
Chile	5.3	38	(35)
China	35	460	(58)
Czech Republic	45.4	174	(48)
Croatia	34.9	186	(49)
Cuba	24.5	110	(59)
Denmark	33.8	136	(36)
France	10.9	247	(37)
Finland	9.6	156	(44)
Greece	29.3	222	(50)
Italy	21.1	114	(38)
India	48	124	(47)
Iran	14.7	88	(39)
Japan	26.5	136	(43)
Turkey	22	112	(45)
Mexico	47.9	94	(60)
Netherlands	28.1	370	(61)
North Macedonia	60.4	48	(32)
Rome population	95	20	(62)
Romania	43.9	86	(40)
Serbia	18.5	122	(33)
Spain	17.5	354	(41)
Sweden	26.6	400	(63)
United Kingdom	30.3	284	(46)
United States	22.9	3,005	(27)
Mid-Europe	31.2	864	(42)

dependent on the milder variant in the genotype. However, some variants may occasionally confer an unexpected phenotype (27, 63, 65).

In the homozygous status or *in trans* with another null variant, the In2G variant usually causes severe SW CAH. When combined with a moderate or mild variant, it normally confers SV or NC CAH. However, a specific characteristic of the In2G variant is that the clinical picture might be less severe even in the homozygous form when it can present as an SV or NC phenotype (22, 24, 31, 32).

During the 1990s, the first evidences about the phenotypic heterogeneity related to the In2G variant were reported (66, 67).

Witchel et al. compared the clinical and molecular findings in 38 individuals from 21 families. All patients carried two deleterious variants *in trans*, with the In2G present on at least 1 allele. A comparison of the phenotypic features with the molecular genotypes showed phenotypic heterogeneity extending from classic SW 21OHD to be asymptomatic (28). The authors hypothesized that other sequence variations influenced the competitive splicing signals at the intron 2/exon 3 junctions. However, experimental testing did not support this hypothesis, and the molecular basis of the phenotypic heterogeneity associated with the In2G variant remained to be elucidated (28). A few years later, Schulze et al. suggested that the putative asymptomatic In2G homozygous individuals were incorrectly typed due to the dropout of one allele during PCR amplification (68). Effectively, in the 1990s, it was too challenging to accurately genotype *CYP21A2*, which still presents as one of the most difficult and error-prone genes, even today. In fact, many old *CYP21A2* genotyping results have been found to be incomplete or inaccurate by using up-to-date methodologies. For this reason, it could be necessary to re-evaluate the accuracy of some of the old literature. However, even with the use of more sophisticated techniques for genotyping, patients with the In2G variant and a non-correspondent phenotype have been reported. In a large study by New et al., out of 155 homozygous In2G patients, 143 (92.3%) had the SW form, 11 (7%) had the SV form, and 1 (0.6%) had the NC form (27). Even when the In2G variant was detected *in trans* with another severe mutation, such as p.(Gln319Ter), still 12% of patients (3/25) presented as SV (27). Finally, the genotype In2G/p.(Val282Leu) was related to the NC phenotype in 96.4% of patients, and only 4 (3.6%) subjects presented a severe phenotype (27). In a recent study by Riedl et al., of 62 In2G homozygous patients, 53 (85.5%) had the SW form, whereas 9 (14.5%) had the SV form (57). DumiK et al. described a comprehensive *CYP21A2* mutation analysis in a large cohort of 93 Croatian patients with classic 21OHD (49). The most frequently detected mutation in this population was the In2G variant (34.9%) (**Table 1**). The concordance between observed and predicted clinical phenotype in Group A (In2G variant) patients was 85% (49). In particular, the authors described two families with genotype–phenotype discordance (49). In the first family, three sisters carried the In2G/In2G genotype. Two of them displayed an SW phenotype and were on hydrocortisone and 9-alpha-fludrohydrocortisone therapy. In contrast, the middle sisters had ambiguous genitalia, high levels of 17OHP and androgens, but repeat measurements of electrolytes, aldosterone, and plasma renin activity (PRA) were within the normal range, excluding the SW phenotype. In the second family, two siblings showed the In2G/p.(Arg358Trp) genotype. The brother was diagnosed with SV 21OHD at 3 years of age due to precocious pseudopuberty, high levels of 17OHP and androgens, and a normal level of aldosterone and PRA. His sister was diagnosed with SW CAH at birth, as she showed ambiguous genitalia, low levels of sodium and aldosterone, and high levels of potassium and PRA. In this case, hydrocortisone and 9-alpha-fludrohydrocortisone were introduced 10 days after birth (49). Also, in Argentinean CAH patients, the In2G variant was reported as the most prevalent mutation (35.2%) (**Table 1**), and while 83.8% of patients in group A (In2G variant) presented with the SW form, 16.2% showed the SV form of the disease (25). In this regard, also these authors described two siblings with the same genotype (In2G/In2G) but a different phenotype (25). Similar data from Brazilian, Hellenic, and Chinese CAH populations were provided by Carvalho et al., Dracopoulou-Vabouli et al., and Wang et al., respectively (**Table 1**) (20, 50, 58).

The In2G variant, in homozygous or *in trans* with a severe *CYP21A2* mutation, was also related to the NC form of 21OHD. Bidet et al. analyzed the molecular spectrum of *CYP21A2* gene in a large cohort of French NC CAH patients (37). The In2G variant was present on 10.9% of all chromosomes (**Table 1**), making it the second most frequent mutation in this study. Interestingly, the authors described a mild clinical and biological phenotype, related to the NC form of 21OHD, in a patient homozygous for the In2G variant.

Finally, a peculiar case was reported by Kohn et al. (55). These authors described two affected boys, both carrying the In2G/In2G genotype, who thrived in early infancy but suffered SW crises unusually late in infancy, at 3.5 and 5.5 months. At the onset of symptoms, the children showed hyponatremia, hyperkalemia, dehydration, and acidosis; serum aldosterone was low in spite of markedly elevated PRA. Baseline 17OHP levels were only moderately elevated; however, stimulated levels were consistent with the classic form of 21OHD. The authors speculated that the In2G variant could sometimes be associated with the delayed phenotypic expression of SW CAH and that the variable splicing may modify the clinical manifestations of the disease (55).

Outcomes of In2G Patients

Although all variants causing SW 21OHD induce similar clinical picture, require similar therapy, and produce similar outcomes, some of the specificities of the In2G variant are being confirmed in a number of larger studies. Here, we will mention some of them.

Fertility

Fertility is significantly decreased in all genetic forms of SW CAH in women due to high levels of androgens, problems after genital surgery, decreased sex drive, social adaptation issues such as not having a partner, or non-willingness to bear children (69). Only approximately 25% of women with CAH conceive a child compared with 45% of matched controls (70, 71). They give birth mostly by cesarean section (72%) and are prone to gestational diabetes. Elevated androgen concentrations impair the ability of progesterone to lower the activity of gonadotrophin-releasing hormone/luteinizing hormone (GnRH/LH) pulse generator, causing increased frequency of pulse amplitude of LH over follicle-stimulating hormone (FSH) production and also disrupting endometrial thickening, making the cervical mucus thicker, disrupting ovulation, and impairing embryo implantation, which all lead to impaired fertilization (69). Psychosexual factors also have a role. Women with CAH frequently present with masculine behavior, and approximately

one-third do not have sexual interest and fantasies (72). Moreover, homosexuality is more common in women with CAH, and there is a direct relationship between the severity of the genotype and non-heterosexuality (73, 74). For example, in women with the null genotype, 50% had a non-heterosexual orientation; in the In2G genotype, 30%; and in matched controls, only 2% (74). Women with null and In2G genotypes were less often married and had fewer children than women with milder genotypes (75). However, fertility in women with the In2G genotype was better compared with the null genotype (71). Pregnancy in women with SW CAH was normal, and the offspring had a normal weight and development (76). The better fertility in females with In2G might have to do with the dose-dependent effects of prenatal androgens on the development of higher brain functions (74). Females with the null genotype scored lower on sexual function and satisfaction with their sexual life as well as had more genital surgical complications, compared with the In2G genotype (77).

Fertility is also compromised in males with CAH, mostly due to testicular adrenal rest tumor (TART) or sometimes hypogonadotropic hypogonadism (78). However, the remaining testicular tissue is larger, and the amount and quality of semen are better in patients with the In2G variant, although not reaching statistical difference (79), with male SW CAH having an increased number of adopted children (80). Nevertheless, the number of males with at least one biological child was equally low in both the null and In2G genotype groups (80).

Psychiatric Disorders

Research in animal models has demonstrated that sex differences in brain and behavior are induced by steroid hormones during specific, hormone-sensitive developmental periods (81). Steroid hormones permanently organize the brain for gender, including the pattern of sexuality, cognition, temperament, and specific interests according to sex, although these features can be modified by environmental and social factors (82). It has been demonstrated that typical male neural and behavioral characteristics develop under the influence of testosterone during perinatal development (81). The fetal hyperandrogenemia in females with CAH leads to male brain organization and subsequently to masculinized behavior and cognitive function (72, 83). Significant psychologic issues originate from these brain compositions in female patients with CAH, and they are dependent on the amount of prenatal and perinatal androgen levels. On the other hand, the disturbed hypothalamic–pituitary–adrenal (HPA) axis in patients with CAH may result in a hypersensitive stress system, making them vulnerable to addiction (84). Thus, three major psychiatric disturbances are present in female patients with CAH: high risk for psychiatric disorders, substance misuse disorders, and stress-adjustment disorders (85). Some authors find psychologic and psychiatric disturbances most expressed in patients with the null genotype (86). Having in mind the symbolic level of the 21-hydroxylase enzyme produced in some patients with the In2G genotype due to alternative splicing, it is expected that they will be less prone to psychologic or psychiatric disorders. In the study of Mueller et al. on a large sample of female patients with CAH, 44.4% met the criteria for at least one psychiatric diagnosis (87). Similar findings were reported in 221 adult females with CAH from six European countries (88). In the study of Engberg et al., females with CAH had high levels of psychiatric disorders as compared with matched controls, and interestingly, patients with the In2G mutation genotype were slightly more frequently affected than the null genotype (85). On the other hand, substance misuse, alcohol, drugs, and attention-deficit hyperactivity disorder were more frequent in patients with the null genotype. In contrast, in males with the In2G genotype, psychiatric disorders, personality disorders, and alcohol misuse were increased as compared with the null genotype (89). Similar findings were reported by Daae et al. (90). The reason for such discrepancy remains elusive. Interestingly, the parents of children with severe genotypes including null and In2G, i.e., obligate *CYP21A2* variant carriers, are at much lower risk of being diagnosed with psychiatric disorders (91).

Cardiovascular and Metabolic Disorders and Bone Health

As far as the metabolic outcomes and complications such as obesity, cardiovascular complications, and bone fragility, they are mostly associated with the therapy; therefore, delineating the influence of genotype is very complicated due to different treatment regimens and length of therapy. However, the risk is generally increased as compared with controls (92–95). In one large epidemiological study, only obesity and venous thromboembolism were significantly more common in patients with In2G than in controls, while patients with null variants had more cardiometabolic risk (96).

DISCUSSION

The In2G variant is frequent in patients with 21OHD. It normally causes severe disease; however, the clinical presentation can vary from the SW form, through the SV form and rarely the NC form. Thus, there is a difference in the severity of 21OHD within group A. The mechanism underlying the variation in the clinical phenotype of the In2G variant was widely discussed. The most accredited hypothesis is that a small number of transcripts avoid aberrant splicing, providing a small amount of the 21-hydroxylase enzyme, which is sufficient for a milder clinical presentation of the disease. For this reason, in some patients, the phenotype appears as SV or even NC. *In vitro* experiments showed that the *CYP21A2* intron 2 c.292+1G>A variant produces two different transcripts: the type I fragment lacked the entire introns 1 and 2 and exon 2, whereas the type II, representing approximately one-third of the mRNAs produced and generated by the use of a cryptic splice acceptor site downstream from exon 3, had a deletion of intron 1, entire exon 2, and part of intron 2 (97). These results supported the evidence that splicing is not a homogenous mechanism and that a single splicing variant can generate alternative transcripts, which might explain some unusual phenotypes (97, 98). In addition, the potential

influence of extra-adrenal 21-hydroxylation on the CAH patient's phenotype might be an additional cause to consider. In fact, it was demonstrated that hepatic CYP2C19 and CYP3A4 have the ability to 21-hydroxylate progesterone and thus may modulate mineralocorticoid deficiency (99).

In conclusion, although a good genotype–phenotype correlation exists in 21OHD, a disparity in phenotypic appearance is present in a portion of patients carrying the In2G/In2G or In2G/null genotypes. This evidence represents the most challenging issue in prenatal diagnosis and familiar counselling since the predictive value for different phenotypes can be uncertain.

AUTHOR CONTRIBUTIONS

MK drafted the manuscript and participated in writing and editing. PC participated in writing and editing. HF participated in writing and editing. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Speiser PW, Ng P, Sinaii N, Leschek EW, Green-Golan L, VanRyzin C, et al. Congenital Adrenal Hyperplasia Due to Steroid 21-Hydroxylase Deficiency: An Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab* (2018) 103(11):4043–88. doi: 10.1210/jc.2018-01865
2. El-Maouche D, Arlt W, Merke DP. Congenital Adrenal Hyperplasia. *Lancet* (2017) 390(10108):2194–210. doi: 10.1016/S0140-6736(17)31431-9
3. Gidlöf S, Falhammar H, Thilén A, von Döbeln U, Ritzén M, Wedell A, et al. One Hundred Years of Congenital Adrenal Hyperplasia in Sweden: A Retrospective, Population-Based Cohort Study. *Lancet Diabetes Endocrinol* (2013) 1(1):35–42. doi: 10.1016/S2213-8587(13)70007-X
4. Arlt W, Willis DS, Wild SH, Krone N, Doherty EJ, Hahner S, et al. Health Status of Adults With Congenital Adrenal Hyperplasia: A Cohort Study of 203 Patients. *J Clin Endocrinol Metab* (2010) 95(11):5110–21. doi: 10.1210/jc.2010-0917
5. Claahsen-van der Grinten HL, Speiser PW, Ahmed SF, Arlt W, Auchus RJ, Falhammar H, et al. Congenital Adrenal Hyperplasia - Current Insights in Pathophysiology, Diagnostics and Management. *Endocr Rev* (2021) 7: bnab016. doi: 10.1210/endrev/bnab016.
6. Falhammar H, Thorén M. Clinical Outcomes in the Management of Congenital Adrenal Hyperplasia. *Endocrine* (2012) 41(3):355–73. doi: 10.1007/s12020-011-9591-x
7. Falhammar H, Torpy DJ. Congenital Adrenal Hyperplasia due to 21-Hydroxylase Deficiency Presenting as Adrenal Incidentaloma: A Systematic Review and Meta-Analysis. *Endocr Pract* (2016) 22(6):736–52. doi: 10.4158/EP151085.RA
8. Witchel SF, Azziz R. Nonclassic Congenital Adrenal Hyperplasia. *Int J Pediatr Endocrinol* (2010) 2010:625105. doi: 10.1186/1687-9856-2010-625105
9. Zetterström RH, Karlsson L, Falhammar H, Lajic S, Nordenström A. Update on the Swedish Newborn Screening for Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency. *Int J Neonatal Screen* (2020) 6(3):71. doi: 10.3390/ijns6030071
10. Nordenström A, Falhammar H. Management of Endocrine Disease Diagnosis and Management of the Patient With Non-Classic CAH Due to 21-Hydroxylase Deficiency. *Eur J Endocrinol* (2019) 180(3):R127–45. doi: 10.1530/EJE-18-0712
11. Hannah-Shmouni F, Morissette R, Sinaii N, Elman M, Prezant TR, Chen W, et al. Revisiting the Prevalence of Nonclassic Congenital Adrenal Hyperplasia in US Ashkenazi Jews and Caucasians. *Genet Med* (2017) 19(11):1276–9. doi: 10.1038/gim.2017.46
12. Falhammar H, Wedell A, Nordenstrom A. Biochemical and Genetic Diagnosis of 21-Hydroxylase Deficiency. *Endocrine* (2015) 50(2):306–14. doi: 10.1007/s12020-015-0731-6
13. White PC, New MI, Dupont B. Structure of Human Steroid 21-Hydroxylase Genes. *Proc Natl Acad Sci USA* (1986) 83(14):5111–5. doi: 10.1073/pnas.83.14.5111
14. Higashi Y, Yoshioka H, Yamane M, Gotoh O, Fujii-Kuriyama Y. Complete Nucleotide Sequence of Two Steroid 21-Hydroxylase Genes Tandemly Arranged in Human Chromosome: A Pseudogene and a Genuine Gene. *Proc Natl Acad Sci U S A* (1986) 83(9):2841–5. doi: 10.1073/pnas.83.9.2841
15. Speiser PW, White PC. Congenital Adrenal Hyperplasia. *N Engl J Med* (2003) 349(8):776–88. doi: 10.1056/NEJMra021561
16. Simonetti L, Bruque CD, Fernández CS, Benavides-Mori B, Delea M, Kolomenski JE, et al. CYP21A2 Mutation Update: Comprehensive Analysis of Databases and Published Genetic Variants. *Hum Mutat* (2018) 39(1):5–22. doi: 10.1002/humu.23351
17. Concolino P, Mello E, Zuppi C, Capoluongo E. Molecular Diagnosis of Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency: An Update of New CYP21A2 Mutations. *Clin Chem Lab Med* (2010) 48 (8):1057–62. doi: 10.1515/CCLM.2010.239
18. Concolino P, Costella A. Congenital Adrenal Hyperplasia (CAH) Due to 21-Hydroxylase Deficiency: A Comprehensive Focus on 233 Pathogenic Variants of CYP21A2 Gene. *Mol Diagn Ther* (2018) 22(3):261–80. doi: 10.1007/s40291-018-0319-y
19. White PC, Speiser PW. Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency. *Endocr Rev* (2000) 21(3):245–91. doi: 10.1210/edrv.21.3.0398
20. de Carvalho DF, Miranda MC, Gomes LG, Madureira G, Marcondes JA, Billerbeck AE, et al. Molecular CYP21A2 Diagnosis in 480 Brazilian Patients With Congenital Adrenal Hyperplasia Before Newborn Screening Introduction. *Eur J Endocrinol* (2016) 175(2):107–16. doi: 10.1530/EJE-16-0171
21. Higashi Y, Hiromasa T, Tanae A, Miki T, Nakura J, Kondo T, et al. Effects of Individual Mutations in the P-450(C21) Pseudogene on the P-450(C21) Activity and Their Distribution in the Patient Genomes of Congenital Steroid 21-Hydroxylase Deficiency. *J Biochem* (1991) 109(4):638–44. doi: 10.1093/oxfordjournals.jbchem.a123433
22. Krone N, Arlt W. Genetics of Congenital Adrenal Hyperplasia. *Best Pract Res Clin Endocrinol Metab* (2009) 23(2):181–92. doi: 10.1016/j.beem.2008.10.014
23. Livadas S, Dracopoulou M, Dastamani A, Sertedaki A, Maniati-Christidi M, Magiakou AM, et al. The Spectrum of Clinical, Hormonal and Molecular Findings in 280 Individuals With Nonclassical Congenital Adrenal Hyperplasia Caused by Mutations of the CYP21A2 Gene. *Clin Endocrinol (Oxf)* (2015) 82(4):543–9. doi: 10.1111/cen.12543
24. Speiser PW, Dupont J, Zhu D, Serrat J, Buegeleisen M, Tusie-Luna MT, et al. Disease Expression and Molecular Genotype in Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency. *J Clin Invest* (1992) 90 (2):584–95. doi: 10.1172/JCI115897
25. Marino R, Ramirez P, Galeano J, Perez Garrido N, Rocco C, Ciaccio M, et al. Steroid 21-Hydroxylase Gene Mutational Spectrum in 454 Argentinean Patients: Genotype-Phenotype Correlation in a Large Cohort of Patients With Congenital Adrenal Hyperplasia. *Clin Endocrinol (Oxf)* (2011) 75 (4):427–35. doi: 10.1111/j.1365-2265.2011.04123.x
26. Kocova M, Anastasovska V, Falhammar H. Clinical Outcomes and Characteristics of P30L Mutations in Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency. *Endocrine* (2020) 69(2):262–77. doi: 10.1007/s12020-020-02323-3
27. New MI, Abraham M, Gonzalez B, Dumic M, Razzaghy-Azar M, Chitayat D, et al. Genotype-Phenotype Correlation in 1,507 Families With Congenital Adrenal Hyperplasia Owing to 21-Hydroxylase Deficiency. *Proc Natl Acad Sci USA* (2013) 110(7):2611–6. doi: 10.1073/pnas.1300057110
28. Witchel SF, Bhamidipati DK, Hoffman EP, Cohen JB. Phenotypic Heterogeneity Associated With the Splicing Mutation in Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency. *J Clin Endocrinol Metab* (1996) 81(11):4081–8. doi: 10.1210/jcem.81.11.8923864

29. Gong LF, Gao X, Yang N, Zhao JQ, Yang HH, Kong YY. A Pilot Study on Newborn Screening for Congenital Adrenal Hyperplasia in Beijing. *J Pediatr Endocrinol Metab* (2019) 32(3):253–8. doi: 10.1515/jpem-2018-0342
30. Maiti A, Chatterjee S. Congenital Adrenal Hyperplasia: An Indian Experience. *J Paediatr Child Health* (2011) 47(12):883–7. doi: 10.1111/j.1440-1754.2011.02104.x
31. Tsuji A, Konishi K, Hasegawa S, Anazawa A, Onishi T, Ono M, et al. Newborn Screening for Congenital Adrenal Hyperplasia in Tokyo, Japan From 1989 to 2013: A Retrospective Population-Based Study. *BMC Pediatr* (2015) 15:209. doi: 10.1186/s12887-015-0529-y
32. Anastasovska V, Kocova M. Genotype-Phenotype Correlation in CAH Patients With Severe CYP21A2 Point Mutations in the Republic of Macedonia. *J Pediatr Endocrinol Metab* (2010) 23(9):921–6. doi: 10.1515/jpem.2010.147
33. Milacic I, Barac M, Milenkovic T, Ugrin M, Klaassen K, Skakic A, et al. Molecular Genetic Study of Congenital Adrenal Hyperplasia in Serbia: Novel P.Leu129Pro and P.Ser165Pro CYP21A2 Gene Mutations. *J Endocrinol Invest* (2015) 38(11):1199–210. doi: 10.1007/s40618-015-0366-8
34. Wilson RC, Nimkarn S, Dumic M, Obeid J, Azar MR, Najmabadi H, et al. Ethnic-Specific Distribution of Mutations in 716 Patients With Congenital Adrenal Hyperplasia Owing to 21-Hydroxylase Deficiency. *Mol Genet Metab* (2007) 90(4):414–21. doi: 10.1016/j.ymgme.2006.12.005
35. Fardella CE, Poggi H, Soto J, Torrealba I, Cattani A, Ugarte F, et al. Mutations in the CYP21 B Gene in a Chilean Population With Simple Virilizing Congenital Adrenal Hyperplasia. *J Endocrinol Invest* (2000) 23(6):412–6. doi: 10.1007/BF03343746
36. Ohlsson G, Müller J, Skakkebaek NE, Schwartz M. Steroid 21-Hydroxylase Deficiency: Mutational Spectrum in Denmark, Three Novel Mutations, and *In Vitro* Expression Analysis. *Hum Mutat* (1999) 13(6):482–6. doi: 10.1002/(SICI)1098-1004(1999)13:6<482::AID-HUMU8>3.0.CO;2-0
37. Bidet M, Bellanné-Chantelot C, Galand-Portier MB, Tardy V, Billaud L, Laborde K, et al. Clinical and Molecular Characterization of a Cohort of 161 Unrelated Women With Nonclassical Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency and 330 Family Members. *J Clin Endocrinol Metab* (2009) 94(5):1570–8. doi: 10.1210/jc.2008-1582
38. Balsamo A, Cacciari E, Baldazzi L, Tartaglia L, Cassio A, Mantovani V, et al. CYP21 Analysis and Phenotype/Genotype Relationship in the Screened Population of the Italian Emilia-Romagna Region. *Clin Endocrinol (Oxf)* (2000) 53(1):117–25. doi: 10.1046/j.1365-2265.2000.01048.x
39. Ramazani A, Kahrizi K, Razaghiazar M, Mahdieh N, Koppens P. The Frequency of Eight Common Point Mutations in CYP21 Gene in Iranian Patients With Congenital Adrenal Hyperplasia. *Iran BioMed J* (2008) 12 (1):49–53.
40. Grigorescu Sido A, Weber MM, Grigorescu Sido P, Clausmeyer S, Heinrich U, Schulze E. 21-Hydroxylase and 11beta-Hydroxylase Mutations in Romanian Patients With Classic Congenital Adrenal Hyperplasia. *J Clin Endocrinol Metab* (2005) 90(10):5769–73. doi: 10.1210/jc.2005-0379
41. Ezquieta B, Oliver A, Gracia R, Gancedo PG. Analysis of Steroid 21-Hydroxylase Gene Mutations in the Spanish Population. *Hum Genet* (1995) 96(2):198–204. doi: 10.1007/BF00207379
42. Dolzan V, Sólyom J, Fekete G, Kovács J, Rakosnikova V, Votava F, et al. Mutational Spectrum of Steroid 21-Hydroxylase and the Genotype-Phenotype Association in Middle European Patients With Congenital Adrenal Hyperplasia. *Eur J Endocrinol* (2005) 153(1):99–106. doi: 10.1530/eje.1.01944
43. Asanuma A, Ohura T, Ogawa E, Sato S, Igarashi Y, Matsubara Y, et al. Molecular Analysis of Japanese Patients With Steroid 21-Hydroxylase Deficiency. *J Hum Genet* (1999) 44(5):312–7. doi: 10.1007/s100380050167
44. Jääskeläinen J, Levo A, Voutilainen R, Partanen J. Population-Wide Evaluation of Disease Manifestation in Relation to Molecular Genotype in Steroid 21-Hydroxylase (CYP21) Deficiency: Good Correlation in a Well Defined Population. *J Clin Endocrinol Metab* (1997) 82(10):3293–7. doi: 10.1210/jcem.82.10.4271
45. Baş F, Kayserili H, Darendeliler F, Uyguner O, Günöz H, Yüksel Apak M, et al. CYP21A2 Gene Mutations in Congenital Adrenal Hyperplasia: Genotype-Phenotype Correlation in Turkish Children. *J Clin Res Pediatr Endocrinol* (2009) 1(3):116–28. doi: 10.4008/jcrpe.v1i3.49
46. Lako M, Ramsden S, Campbell RD, Strachan T. Mutation Screening in British 21-Hydroxylase Deficiency Families and Development of Novel Microsatellite Based Approaches to Prenatal Diagnosis. *J Med Genet* (1999) 36(2):119–24.
47. Marumudi E, Sharma A, Kulshreshtha B, Khadgawat R, Khurana ML, Ammini AC. Molecular Genetic Analysis of CYP21A2 Gene in Patients With Congenital Adrenal Hyperplasia. *Indian J Endocrinol Metab* (2012) 16 (3):384–8. doi: 10.4103/2230-8210.95679
48. Kotaska K, Lisá L, Průsa R. Common CYP21 Gene Mutations in Czech Patients and Statistical Analysis of Worldwide Mutation Distribution. *Cent Eur J Public Health* (2003) 11(3):124–8.
49. Dumic KK, Grubic Z, Yuen T, Wilson RC, Kusec V, Barisic I, et al. Molecular Genetic Analysis in 93 Patients and 193 Family Members With Classical Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency in Croatia. *J Steroid Biochem Mol Biol* (2017) 165(Pt A):51–6. doi: 10.1016/j.jsbmb.2016.03.035
50. Dracopoulou-Vabouli M, Maniati-Christidi M, Dacou-Voutetakis C. The Spectrum of Molecular Defects of the CYP21 Gene in the Hellenic Population: Variable Concordance Between Genotype and Phenotype in the Different Forms of Congenital Adrenal Hyperplasia. *J Clin Endocrinol Metab* (2001) 86(6):2845–8. doi: 10.1210/jcem.86.6.7574
51. Concolino P, Rizza R, Costella A, Carrozza C, Zuppi C, Capoluongo E. CYP21A2 Intronic Variants Causing 21-Hydroxylase Deficiency. *Metabolism* (2017) 71:46–51. doi: 10.1016/j.metabol.2017.03.003
52. Rubtsov PM, Igudin EL, Pichugina M, Spirin PV, Prasolov VS, Tul'pakov AN. [Characterization of New Splicing Mutation in Steroid 21-Hydroxylase Gene]. *Bioorg Khim* (2011) 37(6):815–20. doi: 10.1134/S1068162011060124
53. Katsumata N, Shinagawa T, Horikawa R, Fujikura K. Novel Intronic CYP21A2 Mutation in a Japanese Patient With Classic Salt-Wasting Steroid 21-Hydroxylase Deficiency. *Metabolism* (2010) 59(11):1628–32. doi: 10.1016/j.metabol.2010.03.012
54. Lee HH, Chao HT, Lee YJ, Shu SG, Chao MC, Kuo JM, et al. Identification of Four Novel Mutations in the CYP21 Gene in Congenital Adrenal Hyperplasia in the Chinese. *Hum Genet* (1998) 103(3):304–10. doi: 10.1007/s004390050821
55. Kohn B, Day D, Alemzadeh R, Enerio D, Patel SV, Pelczar JV, et al. Splicing Mutation in CYP21 Associated With Delayed Presentation of Salt-Wasting Congenital Adrenal Hyperplasia. *Am J Med Genet* (1995) 57(3):450–4. doi: 10.1002/ajmg.1320570318
56. Friães A, Rêgo AT, Aragüés JM, Moura LF, Mirante A, Mascarenhas MR, et al. CYP21A2 Mutations in Portuguese Patients With Congenital Adrenal Hyperplasia: Identification of Two Novel Mutations and Characterization of Four Different Partial Gene Conversions. *Mol Genet Metab* (2006) 88(1):58–65. doi: 10.1016/j.ymgme.2005.11.015
57. Riedl S, Röhl FW, Bonfig W, Brämswig J, Richter-Unruh A, Fricke-Otto S, et al. Genotype/phenotype Correlations in 538 Congenital Adrenal Hyperplasia Patients From Germany and Austria: Discordances in Milder Genotypes and in Screened Versus Prescreening Patients. *Endocr Connect* (2019) 8(2):86–94. doi: 10.1530/EC-18-0281
58. Wang R, Yu Y, Ye J, Han L, Qiu W, Zhang H, et al. 21-Hydroxylase Deficiency-Induced Congenital Adrenal Hyperplasia in 230 Chinese Patients: Genotype-Phenotype Correlation and Identification of Nine Novel Mutations. *Steroids* (2016) 108:47–55. doi: 10.1016/j.steroids.2016.01.007
59. Espinosa Reyes TM, Collazo Mesa T, Lantigua Cruz PA, Agramonte Machado A, Domínguez Alonso E, Falhammar H. Genotype-Phenotype Correlation in Patients With Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency in Cuba. *Int J Endocrinol* (2021) 2021:9316284. doi: 10.1155/2021/9316284
60. Ordoñez-Sánchez ML, Ramírez-Jiménez S, López-Gutierrez AU, Riba L, Gamboa-Cardiel S, Cerrillo-Hinojosa M, et al. Molecular Genetic Analysis of Patients Carrying Steroid 21-Hydroxylase Deficiency in the Mexican Population: Identification of Possible New Mutations and High Prevalence of Apparent Germ-Line Mutations. *Hum Genet* (1998) 102(2):170–7. doi: 10.1007/s004390050672
61. Stikkelbroeck NM, Hoefsloot LH, de Wijs IJ, Otten BJ, Hermus AR, Sistermans EA. CYP21 Gene Mutation Analysis in 198 Patients With 21-Hydroxylase Deficiency in The Netherlands: Six Novel Mutations and a Specific Cluster of Four Mutations. *J Clin Endocrinol Metab* (2003) 88 (8):3852–9. doi: 10.1210/jc.2002-021681
62. Kocova M, Anastasovska V, Petlichkovski A, Falhammar H. First Insights Into the Genetics of 21-Hydroxylase Deficiency in the Roma Population. *Clin Endocrinol* (2021) 95(1):41–6. doi: 10.1111/cen.14447

63. Wedell A, Thilén A, Ritzén EM, Stengler B, Luthman H. Mutational Spectrum of the Steroid 21-Hydroxylase Gene in Sweden: Implications for Genetic Diagnosis and Association With Disease Manifestation. *J Clin Endocrinol Metab* (1994) 78(5):1145–52. doi: 10.1097/00006254-199410000-00020
64. Ezquieta B, Cueva E, Oyarzábal M, Oliver A, Varela JM, Jariego C. Gene Conversion (655G Splicing Mutation) and the Founder Effect (Gln318Stop) Contribute to the Most Frequent Severe Point Mutations in Congenital Adrenal Hyperplasia (21-Hydroxylase Deficiency) in the Spanish Population. *Clin Genet* (2002) 62(2):181–8. doi: 10.1034/j.1399-0004.2002.620213.x
65. Kocova M, Anastasovska V, Bitovska I. The Impact of CYP21A2 (P30L/I172N) Genotype on Female Fertility in One Family. *Eur J Med Res* (2019) 24 (1):21. doi: 10.1186/s40001-019-0379-4
66. Schulze E, Scharer G, Rogatzki A, Priebe L, Lewicka S, Bettendorf M, et al. Divergence Between Genotype and Phenotype in Relatives of Patients With the Intron 2 Mutation of Steroid-21-Hydroxylase. *Endocr Res* (1995) 21(1-2):359–64. doi: 10.3109/07435809509030452
67. Witchel SS, Lee PA, Trucco M. Who Is a Carrier? Detection of Unsuspected Mutations in 21-Hydroxylase Deficiency. *Am J Med Genet* (1996) 61(1):2–9. doi: 10.1002/(SICI)1096-8628(19960102)61:1<2::AID-AJMG1>3.0.CO;2-1
68. Schulze E, Bettendorf M, Maser-Gluth C, Decker M, Schwabe U. Allele-Dropout Using PCR-Based Diagnosis for the Splicing Mutation in Intron-2 of the CYP21B-Gene: Successful Amplification With a Taq/Pwo-Polymerase Mixture. *Endocr Res* (1998) 24(3-4):637–41. doi: 10.3109/07435809809032662
69. Gomes LG, Bachega TASS, Mendonca BB. Classic Congenital Adrenal Hyperplasia and Its Impact on Reproduction. *Fertil Steril* (2019) 111(1):7–12. doi: 10.1016/j.fertnstert.2018.11.037
70. Hirschberg AL, Gidlöf S, Falhammar H, Frisén L, Almqvist C, Nordenskjöld A, et al. Reproductive and Perinatal Outcomes in Women With Congenital Adrenal Hyperplasia: A Population-Based Cohort Study. *J Clin Endocrinol Metab* (2021) 106(2):e957–65. doi: 10.1210/clinem/dgaa801
71. Hagenfeldt K, Janson PO, Holmdahl G, Falhammar H, Filipsson H, Frisén L, et al. Fertility and Pregnancy Outcome in Women With Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency. *Hum Reprod* (2008) 23 (7):1607–13. doi: 10.1093/humrep/den118
72. Meyer-Bahlburg HF, Dolezal C, Baker SW, New MI. Sexual Orientation in Women With Classical or Non-Classical Congenital Adrenal Hyperplasia as a Function of Degree of Prenatal Androgen Excess. *Arch Sex Behav* (2008) 37 (1):85–99. doi: 10.1007/s10508-007-9265-1
73. Daae E, Feragen KB, Waehre A, Nermoen I. Falhammar H Sexual Orientation in Individuals With Congenital Adrenal Hyperplasia: A Systematic Review. *Front Behav Neurosci* (2020) 14:38. doi: 10.3389/fnbeh.2020.00038
74. Frisén L, Nordenström A, Falhammar H, Filipsson H, Holmdahl G, Janson PO, et al. Gender Role Behavior, Sexuality, and Psychosocial Adaptation in Women With Congenital Adrenal Hyperplasia Due to CYP21A2 Deficiency. *J Clin Endocrinol Metab* (2009) 94(9):3432–9. doi: 10.1210/jc.2009-0636
75. Strandqvist A, Falhammar H, Lichtenstein P, Hirschberg AL, Wedell A, Norrby C, et al. Suboptimal Psychosocial Outcomes in Patients With Congenital Adrenal Hyperplasia: Epidemiological Studies in a Nonbiased National Cohort in Sweden. *J Clin Endocrinol Metab* (2014) 99(4):1425–32. doi: 10.1210/jc.2013-3326
76. Bothou C, Anand G, Li D, Kienitz T, Seejore K, Simeoli C, et al. Current Management and Outcome of Pregnancies in Women With Adrenal Insufficiency: Experience From a Multicenter Survey. *J Clin Endocrinol Metab* (2020) 105(8):e2853–63. doi: 10.1210/clinem/dgaa266.
77. Nordenström A, Frisén L, Falhammar H, Filipsson H, Holmdahl G, Janson PO, et al. Sexual Function and Surgical Outcome in Women With Congenital Adrenal Hyperplasia Due to CYP21A2 Deficiency: Clinical Perspective and the Patients' Perception. *J Clin Endocrinol Metab* (2010) 95(8):3633–40. doi: 10.1210/jc.2009-2639
78. Claahsen-van der Grinten HL, Stikkelbroeck N, Falhammar H, Reisch N. MANAGEMENT OF ENDOCRINE DISEASE: Gonadal Dysfunction in Congenital Adrenal Hyperplasia. *Eur J Endocrinol* (2021) 184(3):R85–97. doi: 10.1530/EJE-20-1093
79. Falhammar H, Nyström HF, Ekström U, Granberg S, Wedell A, Thorén M. Fertility, Sexuality and Testicular Adrenal Rest Tumors in Adult Males With Congenital Adrenal Hyperplasia. *Eur J Endocrinol* (2012) 166(3):441–9. doi: 10.1530/EJE-11-0828
80. Falhammar H, Frisén L, Norrby C, Almqvist C, Hirschberg AL, Nordenskjöld A, et al. Reduced Frequency of Biological and Increased Frequency of Adopted Children in Males With 21-Hydroxylase Deficiency: A Swedish Population-Based National Cohort Study. *J Clin Endocrinol Metab* (2017) 102(11):4191–9. doi: 10.1210/jc.2017-01139
81. Bakker J. The Role of Steroid Hormones in the Sexual Differentiation of the Human Brain. *J Neuroendocrinol* (2021) p:e13050. doi: 10.1111/jne.13050
82. Jordan-Young RM. Hormones, Context, and "Brain Gender": A Review of Evidence From Congenital Adrenal Hyperplasia. *Soc Sci Med* (2012) 74 (11):1738–44. doi: 10.1016/j.socscimed.2011.08.026
83. Meyer-Bahlburg HFL. Brain Development and Cognitive, Psychosocial, and Psychiatric Functioning in Classical 21-Hydroxylase Deficiency. *Endocr Dev* (2011) 20:88–95. doi: 10.1159/000321225
84. Charmandari E, Merke DP, Negro PJ, Keil MF, Martinez PE, Haim A, et al. Endocrinologic and Psychologic Evaluation of 21-Hydroxylase Deficiency Carriers and Matched Normal Subjects: Evidence for Physical and/or Psychologic Vulnerability to Stress. *J Clin Endocrinol Metab* (2004) 89 (5):2228–36. doi: 10.1210/jc.2003-031322
85. Engberg H, Butwicka A, Nordenström A, Hirschberg AL, Falhammar H, Lichtenstein P, et al. Congenital Adrenal Hyperplasia and Risk for Psychiatric Disorders in Girls and Women Born Between 1915 and 2010: A Total Population Study. *Psychoneuroendocrinology* (2015) 60:195–205. doi: 10.1016/j.psyneuen.2015.06.017
86. Mueller SC, Grissom EM, Dohanich GP. Assessing Gonadal Hormone Contributions to Affective Psychopathologies Across Humans and Animal Models. *Psychoneuroendocrinology* (2014) 46:114–28. doi: 10.1016/j.psyneuen.2014.04.015
87. Mueller SC, Ng P, Sinaii N, Leschek EW, Green-Golan L, VanRyzin C, et al. Psychiatric Characterization of Children With Genetic Causes of Hyperandrogenism. *Eur J Endocrinol* (2010) 163(5):801–10. doi: 10.1530/EJE-10-0693
88. de Vries ALC, Roehle R, Marshall L, Frisén L, van de Grift TC, Kreukels BPC, et al. Mental Health of a Large Group of Adults With Disorders of Sex Development in Six European Countries. *Psychosom Med* (2019) 81(7):629–40. doi: 10.1097/PSY.0000000000000718
89. Falhammar H, Butwicka A, Landén M, Lichtenstein P, Nordenskjöld A, Nordenström A, et al. Increased Psychiatric Morbidity in Men With Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency. *J Clin Endocrinol Metab* (2014) 99(3):E554–60. doi: 10.1210/jc.2013-3707
90. Daae E, Feragen KB, Nermoen I, Falhammar H. Psychological Adjustment, Quality of Life, and Self-Perceptions of Reproductive Health in Males With Congenital Adrenal Hyperplasia: A Systematic Review. *Endocrine* (2018) 62 (1):3–13. doi: 10.1007/s12020-018-1723-0
91. Nordenström A, Butwicka A, Lindén Hirschberg A, Almqvist C, Nordenskjöld A, Falhammar H, et al. Are Carriers of CYP21A2 Mutations Less Vulnerable to Psychological Stress? A Population-Based National Cohort Study. *Clin Endocrinol (Oxf)* (2017) 86(3):317–24. doi: 10.1111/cen.13242
92. Tamhane S, Rodriguez-Gutierrez R, Iqbal AM, Prokop LJ, Bancos I, Speiser PW, et al. Cardiovascular and Metabolic Outcomes in Congenital Adrenal Hyperplasia: A Systematic Review and Meta-Analysis. *J Clin Endocrinol Metab* (2018) 103(11):4097–103. doi: 10.1210/jc.2018-01862
93. Li L, Bensing S, Falhammar H. Rate of Fracture in Patients With Glucocorticoid Replacement Therapy: A Systematic Review and Meta-Analysis. *Endocrine* (2021) 74(1):29–37. doi: 10.1007/s12020-021-02723-z
94. Rangaswamaiah S, Gangathimmaiah V, Nordenstrom A, Falhammar H. Bone Mineral Density in Adults With Congenital Adrenal Hyperplasia: A Systematic Review and Meta-Analysis. *Front Endocrinol (Lausanne)* (2020) 11:493. doi: 10.3389/fendo.2020.00493
95. Falhammar H, Frisén L, Hirschberg AL, Nordenskjöld A, Almqvist C, Nordenström A. Increased Prevalence of Fractures in Congenital Adrenal Hyperplasia: A Swedish Population-Based National Cohort Study. *J Clin Endocrinol Metab* (2021) 3:dgab712. doi: 10.1210/clinem/dgab712
96. Falhammar H, Frisén L, Hirschberg AL, Norrby C, Almqvist C, Nordenskjöld A, et al. Increased Cardiovascular and Metabolic Morbidity in Patients With 21-Hydroxylase Deficiency: A Swedish Population-Based National Cohort Study. *J Clin Endocrinol Metab* (2015) 100(9):3520–8. doi: 10.1210/JC.2015-2093
97. Lee HH, Chang SF. Multiple Transcripts of the CYP21 Gene Are Generated by the Mutation of the Splicing Donor Site in Intron 2 From GT to AT in 21-Hydroxylase Deficiency. *J Endocrinol* (2001) 171(3):397–402. doi: 10.1677/joe.0.1710397

98. Annalora AJ, Marcus CB, Iversen PL. Alternative Splicing in the Cytochrome P450 Superfamily Expands Protein Diversity to Augment Gene Function and Redirect Human Drug Metabolism. *Drug Metab Dispos* (2017) 45(4):375–89. doi: 10.1124/dmd.116.073254
99. Gomes LG, Huang N, Agrawal V, Mendonca BB, Bachega TA, Miller WL. Extraadrenal 21-Hydroxylation by CYP2C19 and CYP3A4: Effect on 21-Hydroxylase Deficiency. *J Clin Endocrinol Metab* (2009) 94:89–95. doi: 10.1210/jc.2008-1174

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Congenital Adrenal Hyperplasia and Ehlers-Danlos Syndrome

*Roxana Marino[1], Angélica Moresco[2], Natalia Perez Garrido[1], Pablo Ramirez[1] and Alicia Belgorosky[3,4]**

[1] Molecular Biology Laboratory, Endocrinology Service, Hospital de Pediatría Prof. Dr. Juan P. Garrahan, Buenos Aires, Argentina, [2] Genetics Service, Hospital de Pediatría Prof. Dr. Juan P. Garrahan, Buenos Aires, Argentina, [3] Endocrinology Service, Hospital de Pediatría Prof. Dr. Juan P. Garrahan, Buenos Aires, Argentina, [4] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina

Congenital adrenal hyperplasia (CAH) secondary to 21-hydroxylase deficiency is an autosomal recessive disorder. The 21-hydroxylase enzyme P450c21 is encoded by the *CYP21A2* gene located on chromosome 6p21.33 within the HLA major histocompatibility complex. This locus also contains the *CYP21A1P*, a non-functional pseudogene, that is highly homologous to the *CYP21A2* gene. Other duplicated genes are *C4A* and *C4B*, that encode two isoforms of complement factor C4, the *RP1* gene that encodes a serine/threonine protein kinase, and the *TNXB* gene that, encodes the extracellular matrix glycoprotein tenascin-X (TNX). TNX plays a role in collagen deposition by dermal fibroblasts and is expressed in the dermis of the skin and the connective tissue of the heart and skeletal muscle. During meiosis, misalignment may occur producing large gene deletions or gene conversion events resulting in chimeric genes. Chimeric recombination may occur between *TNXB* and *TNXA.* Three *TNXA/TNXB* chimeras have been described that differ in the junction site (CH1 to CH3) and result in a contiguous *CYP21A2* and *TNXB* gene deletion, causing CAH-X syndrome. *TNXB* deficiency is associated with Ehlers Danlos syndrome (EDS). EDS comprises a clinically and genetically heterogeneous group of connective tissue disorders. As molecular analysis of the *TNXB* gene is challenging, the TNX-deficient type EDS is probably underdiagnosed. In this minireview, we will address the different strategies of molecular analysis of the *TNXB*-gene, as well as copy number variations and genetic status of *TNXB* in different cohorts. Furthermore, clinical features of EDS and clinical recommendations for long-term follow-up are discussed.

Keywords: congenital adrenal hyperplasia, CAH-X, *CYP21A2*, *TNXB*, Ehlers-Danlos Syndrome

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Salvatore Savasta, University of Pavia, Italy

***Correspondence:**
Alicia Belgorosky
abelgo12345@gmail.com

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INTRODUCTION

Congenital adrenal hyperplasia (CAH) comprises a group of autosomal recessive enzymatic disorders, caused by a deficiency of one of the enzymes required for cortisol biosynthesis in the adrenal cortex. CAH is mostly associated with pathogenic variants in the 21-hydroxylase (*CYP21A2*) gene (1, 2). Residual enzyme activity defines the clinical severity of the disease. The most common form of CAH is 21-hydroxylase deficiency (21-OHD) accounting for 95% of cases. Prevalence of the most severe or classic forms is 1:16,000 live births in the Caucasian population,

while the non-classic or late-onset form is the most common, with a prevalence between 1:1000-1:500 live births depending on ethnicity and geographic area (1–3).

The *CYP21A2* gene is located on the long arm of chromosome 6, within the major human histocompatibility complex (HLA), a region with a complex gene organization (4–7) (**Figure 1**). There is a nonfunctional pseudogene (*CYP21A1P*), located approximately 30 kb from the *CYP21A2* gene. Both the functional gene and the pseudogene comprise ten exons that share a 98% nucleotide sequence identity (4–7). The close proximity and high level of homology between the functional and the non-functional gene facilitates misalignment resulting in recombination events that frequently produce large *CYP21A2* gene deletions and conversions as well as point pathogenic variants in the *CYP21A2* gene. The locus, is one of the most complex in the human genome since it contains three other genes, *RP1* also called *STK19* that encodes a serine/threonine protein kinase, *C4A* and *C4B* that encode two isoforms of complement factor C4, and *TNXB* that encodes an extracellular matrix glycoprotein tenascin-X (TNX), as well as two pseudogenes, *RP2* and *TNXA*, that together constitute a 30-kb genetic unit called the RCCX module (4–7). The genetic diversity of the RCCX module is highly attributable to nonallelic homologous recombination (NAHR). Unequal crossover during meiosis generates large structural rearrangements and copy number changes, whereas gene conversion mediates relatively short sequence transfers (8).

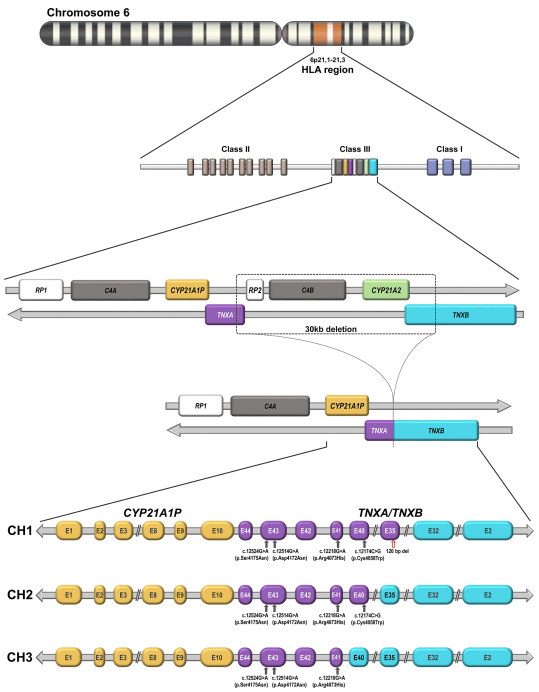

FIGURE 1 | Genomic organization of the CYP21 locus on chromosome 6p21.1-21.3: The functional *CYP21A2* gene and its non-functional *CYP21A1P* pseudogene are arranged in tandem repeat with the two *C4* genes that encode factor four of the complement system, the serine–threonine nuclear protein kinase active gene *RP1* (*STK19*) and a truncated pseudogene *RP2* (*STK19P*). The functional *TNXB* and the *TNXA* pseudogene are located on the complement strand. Schematic representation of a 30-kb deletion as a result of unequal crossover during meiosis and the formation of the three described *TNXA/TNXB* chimeras (CH1, CH2 and CH3), which differ in the junction site. CH1 is characterized by a 120-bp deletion in exon and intron 35 carried over from *TNXA* pseudogene sequences. CH2 lacks this deletion but contains a pseudogene-derived variant c.12174C>G (p.Cys4058Trp) in *TNXB* exon 40. CH3 is characterized by the presence of any variant of a cluster of three pseudogene-derived variants (exon 41: c.12218G>A, p.Arg4073His; exon 43: c.12514G>A, p.Asp4172Asn and c.12524G>A, p.Ser4175Asn). These three pseudogene-derived variants do not always co-segregate in the three chimeras. *TNXB* gene exons are represented in cyan and *TNXA* gene exons in purple. The red arrow indicates a 120-bp deletion in exon 35 and grey arrows indicate different pseudogene-derived variants. CH1: *TNXA/TNXB* chimera 1. CH2: *TNXA/TNXB* chimera 2. CH3: *TNXA/TNXB* chimera 3.

The *TNXA* and *TNXB* genes lie on the opposite strand of DNA from the other genes of the cluster and therefore have the opposite transcriptional direction. The last exon of *TNXA* and *TNXB* partially overlap the 3′ untranslated region of exon 10 in *CYP21A1P* and *CYP21A2*, respectively (9). *TNXB* is a large gene composed of 44 exons spanning 68.2 kb, whereas *TNXA* is a truncated gene of 4.5kb, homologous to exons 32 to 44 of *TNXB*.

As mentioned above, chimeric *CYP21A1P/CYP21A2* genes are caused by homologous recombination between *CYP21A2* and its pseudogene *CYP21A1P* as a result of unequal crossover and are found in 20-25% of alleles in CAH due to 21-OHD. To date, nine different *CYP21A1P/CYP21A2* genes have been characterized (10).

The unequal crossover may, in some cases, produce *TNXA/TNXB* chimeras from which the *CYP21A2* gene is completely removed (**Figure 1**). At present three different *TNXA/TNXB* chimeras have been described- CH1, CH2 and CH3-that differ in the junction site. CH1 is characterized by a 120-bp deletion in exon and intron 35 carried over from *TNXA* pseudogene sequences leading to haploinsufficiency (11). CH2 lacks this deletion but contains a pseudogene-derived variant-c.12174C>G (p.Cys4058Trp)- in *TNXB* exon 40 producing the loss of a critical disulfide bond in the tertiary structure of the TNX C-terminal fibrinogen-like domain (12). CH3 is characterized by the presence of any variant of a cluster of three pseudogene-derived variants -exon 41: c.12218G>A, (p.Arg4073His); exon 43: c.12514G>A (p.Asp4172Asn) and c.12524G>A (p.Ser4175Asn)-. The cluster of these three pseudogene-derived variants may differ in the haplotypes found in the three chimeras and they do not always co-segregate. Modeling and energy calculations suggest that the p.Arg4073His variant is detrimental to proper TNX folding while the remaining variants in the cluster did not significantly affect the folding energies in the models (13) . In addition, some CH1 haplotypes that harbor the 120-bp deletion in exon 35 but lack the p.Cys4058Trp variant in exon 40 have been found. This may be explained by the fact that the allele frequency of pseudogene derived-variants is not 100 percent. Since CH2 and CH3 produce altered proteins rather than reducing TNX expression, they are associated with a dominant-negative effect. The TNX protein belongs to a family of evolutionarily conserved large glycoproteins of the extracellular matrix. It plays a role in collagen deposition by dermal fibroblasts and is expressed in

the dermis of the skin and in the connective tissue of the heart and skeletal muscle. *TNXB* deficiency leads to Ehlers-Danlos Syndrome (EDS) and up to 10% of CAH patients also have EDS, an entity called CAH-X. EDS comprises a clinically and genetically heterogeneous group of connective tissue disorders characterized by joint hypermobility, skin hyperextensibility, and tissue fragility (14).

As molecular analysis of the *TNXB* gene is challenging, the TNX-deficient type EDS is probably underdiagnosed. In this minireview, we will address the different strategies of molecular analysis of the *TNXB* gene, as well as copy number variations and genetic status of *TNXB* in different cohorts. Furthermore, clinical features of EDS and clinical recommendations for long-term follow-up are discussed.

MOLECULAR ANALYSIS OF THE *TNXB* GENE

The first report of TNX deficiency was a description of a patient with CAH and EDS (15). In 2001, Schalkwijk et al. reported a subtype of EDS that is now known as classic-like EDS (clEDS) (16). clEDS is an autosomal recessive form of EDS and is caused by a deficiency of TNX encoded by the *TNXB* gene. The authors evaluated 151 patients with the classic hypermobility or vascular types of EDS, together with 168 patients with other conditions (psoriasis and rheumatoid arthritis) and 21 healthy individuals for the presence of TNX and tenascin-C by enzyme-linked immunosorbent assay. The patients were tested for the 30-kb deletion leading to a *TNXA/TNXB* chimeric gene by allele-specific PCR and for other point mutations by sequencing the coding sequence of the *TNXB* gene. Four of five TNX–deficient patients were identified to have homozygous *TNXB* mutations. Subsequently, the same authors reported an association between haploinsufficiency of the *TNXB* gene and the hypermobility-type EDS (hEDS) in 20 heterozygous family members; however, generalized joint hypermobility (GJH) was observed in only nine female patients (17).

The first evaluation of the potential clinical implications of *TNXB* heterozygosity in CAH patients was reported by Merke et al. in a large prospective observational study from the National Institutes of Health (NIH) (11). One hundred ninety-three unrelated patients with CAH were evaluated clinically for manifestations of EDS and genetically for *TNXB* mutations. DNA was analyzed for the presence of a contiguous gene deletion syndrome caused by deletion of *CYP21A2* and its flanking gene *TNXB* by PCR multiplex ligation-dependent probe amplification (MLPA) and confirmed by Southern blot. This deletion generated a *TNXA/TNXB* chimera characterized by a 120-bp deletion in exon 35 of the *TNXB* gene, which is replaced by *TNXA* sequences. In addition, *TNXB* sequencing was performed in a group of patients with one or more joint or skin manifestations. Heterozygosity for the *TNXB* deletion was observed in 7% of CAH patients, who were considered to have CAH-X syndrome. Here, the association between the hypermobility phenotype and *TNXB*-gene haploinsufficiency was established. In 2015, the same authors identified a pseudogene-derived variant- c.12174C>G (p.C4058W)-representing a novel *TNXA/TNXB* chimera that did not involve a 120-bp deletion in exon 35 in seven families with CAH-X (12). Interestingly, this variant did not affect the protein expression of tenascin in dermal fibroblasts and for this missense variant a dominant-negative mechanism was proposed, which is different from the haploinsufficiency caused by the above-described chimera. Of 246 CAH probands screened, 14 carried previously described *TNXA/TNXB* chimeras (CH1) while seven unrelated patients carried the novel *TNXB* variant (CH2) resulting in a prevalence of CAH-X of 8.5% (12). The same authors later reported three patients with biallelic CAH-X and identified a novel dominant-negative chimera (CH3) characterized by any of three *TNXB* variants [exon 41: c.12218G>A (p.Arg4073His); 191 exon 43: c.12514G>A (p.Asp4172Asn) and c.12524G>A (p.Ser4175Asn)] (**Figure 1**). This study presented evidence for disrupted TNX function, since by computational data the p.Arg4073His variant was predicted to reduce protein-folding energy by interfering with a cation-pi interaction between p.Arg4073 and p.Phe4080 (13).

Molecular analysis of the *TNXB* gene is challenging due to the presence of the pseudogene, which makes next-generation sequencing highly complicated in these cases. In 2019, Lao et al. reported a high-throughput CAH-X screening method based on allele-specific PCR to assess the copy numbers of *TNXB* exons 35 and 40. The method is compatible with either quantitative PCR or droplet digital PCR and allows detection of CH1 and CH2. Using this methodology, the authors found a 15.6% prevalence of CAH-X, which was higher than previously estimated. The prevalence was especially high (29.2%) in subjects with a 30-kb deletion genotype (18).

In 2020, Gao et al. assessed the prevalence of the chimeric *TNXA/TNXB* gene and clinical symptoms in a Chinese cohort of 424 21-OHD patients. MLPA analysis and Sanger sequencing was performed to genetically identify the CAH-X syndrome. In this cohort, 14% of the patients with 21-OHD were found to have a chimeric *TNXA/TNXB* gene (19). Finally, also in 2020, our group reported the molecular *TNXB*-gene status and clinical evaluation of the EDS phenotype in a cohort of 337 Argentine 21-OHD patients to assess the prevalence of this condition in our population. *TNXB* gene analysis was performed in 66 unrelated CAH patients that were carriers of the 30-kb *CYP21A2* gene deletion. A molecular strategy based on MLPA and Sanger sequencing analysis was developed for the detection of the three previously described *TNXA/TNXB* chimeras (20). The overall prevalence of CAH-X in 21-OHD patients in our cohort was 14%, which was similar to that previously found in the large cohort from the NIH and in the Chinese population (15% and 14%, respectively). In our population of 21-OHD patients carrying the 30-kb *CYP21A2* gene deletion in which the junction site was downstream exon 7 both in the homozygous or the heterozygous state, the incidence of *TNXA/TNXB* chimeras was 73% (48/66), similar to the prevalence of 62.8% (59/94) found in the Chinese population. On the other hand, in the NIH cohort a prevalence of 29.2% (21/72) was

reported. The reason for the lower prevalence found in the latter study is that the authors reported the presence of *TNXA/TNXB* chimeras in CAH patients that were carriers of all types of 30-kb *CYP21A2* deletions described.

In addition to *TNXA/TNXB* chimeras, pathogenic variants in the *TNXB* gene have been described as a less frequent cause of TNX deficiency. Pathogenic variants were detected in the coding region of the EGF-like repeats, the fibronectin type III domain or C-terminal domain structurally related to fibrinogen of the TNX protein. Moreover, recently a splice donor site variant has been described as a cause of the hEDS type (21). Finally, the variable prevalence of CAH-X reported in the different cohorts might be related to the molecular strategies used.

CLINICAL MANIFESTATIONS OF CAH-X PATIENTS

CAH-X patients are reported to have a wide range of connective tissue abnormalities, including generalized joint hypermobility, subluxations, chronic arthralgias, soft or velvety skin, mild skin hyperextensibility, and variable systemic manifestations. The severity of the phenotype may be correlated with the dosage of the dominant alleles, as monoallelic CAH-X is associated with hEDS, the mildest and most common EDS type, and biallelic CAH-X with the more severe clEDS subtype.

Biallelic CAH-X patients resemble the clEDS type, with extreme joint laxity, with or without recurrent joint dislocations, and skin hyperextensibility with a velvety skin texture and absence of atrophic scarring. Easily bruisable skin and soft-tissue injuries as well as organ prolapse, pes planus, piezogenic papules, chronic pain, arthralgias, and cardiac abnormalities have been described. Thus far, 12 patients with biallelic CAH-X syndrome have been reported (13, 15, 16, 19, 20); however, clinical information is not available for all cases. Our group reported four biallelic CAH-X patients (two with a CH1/CH1 and two with a CH1/CH2 combination) (20). Both CH1/CH2 patients had a more severe EDS phenotype, with greater skin involvement. Nevertheless, the low number of homozygous patients reported to date limits the possibility to draw robust conclusions based on these data. In addition, in the latter patients cardiac defects were detected; one had an atrial septal and the other a mild pulmonary valve defect. None of the patients developed either atrophic scarring, organ prolapse, or any other complications during the 3 years of follow-up; however, these observations are limited by the young age of our patients and the short-term follow-up. Chen et al. reported three biallelic patients of 14, 19, and 29 years of age, all displaying unique combinations of *TNXB* variants in both alleles; one of them was homozygous for CH2/CH2, the other was a CH2/CH3 compound, and the third a CH2/CH1 compound (13). All of them had skin hyperextensibility and significant joint hypermobility. Joint laxity was extreme and two patients had a history of joint dislocations, chronic arthralgias, and chronic tendinitis and/or bursitis. Unlike our findings, the authors reported widened atrophic scarring, rectal prolapse, severe gastroesophageal reflux, high palate, and elongated uvula in all three biallelic patients. Mild ventricular enlargement was detected in two patients. Currently, the limited number of cases reported and the heterogeneous combination of *TNXB* variants they display make it difficult to establish a certain genotype/phenotype correlation.

Long-term follow-up is needed to specifically evaluate quality of life in CAH-X patients. On the other hand, clEDS patients (without CAH) are affected by soft-tissue fragility and long-term complications (13, 15, 16, 19, 20).

Most patients with the monoallelic form of CAH-X syndrome present with the clinical spectrum of hEDS with variable expression at different stages of life, predominantly characterized by GJH, mild skin hyperextensibility, and soft velvety skin, without abnormal scarring. Related musculoskeletal complications, such as recurrent joint dislocations, pes planus, and chronic arthralgias, have been reported. Other associated features include functional gastrointestinal alterations and cardiac disorders. Although this type of EDS may cause chronic pain and reduced quality of life, life-threatening complications are uncommon (11, 12, 15, 17, 19, 20).

The underlying chimera translates into different degrees of hEDS phenotypes. Compared to *TNXB* haploinsufficiency caused by CAH-X CH1, a dominant negative effect related to CAH-X CH2 causes a more severe phenotype with increased joint and skin manifestations (12, 20). Gastrointestinal disorders, such as chronic gastroesophageal reflux and irritable bowel syndrome, hernias, and organ prolapse, are also more frequently reported in patients that are heterozygous for CAH-X CH2 than in those with CAH-X CH1. Data on the phenotype associated with the less frequent CAH-X CH3 are scarce (12, 19, 20, 22–24). Recently, Gao et al. reported the presence of CH3 in 11 patients; however, clinical information was available for only one of them, who had joint hypermobility and poor wound healing (19). In our cohort, CH3 was only found in one monoallelic patient who was not available for clinical evaluation (20).

Although the exact role of CAH-related hormonal factors and/or chronic glucocorticoid treatment in connective tissue dysplasia is not yet completely understood, it has been shown that CAH-X patients are consistently more severely affected than patients with homozygous or heterozygous TNX-deficient-type EDS without CAH (11, 12, 16, 19, 25).

Furthermore, a phenotype that is varied and usually milder than that of monoallelic CAH-X patients has been reported in the relatives of CAH-X patients, who were carriers of one CAH-X allele, but not affected by CAH. Some carriers were observed to have less joint, heart, or gastrointestinal symptoms, while others were asymptomatic (12, 13, 17, 19, 20). On the other hand, connective tissue dysplasia has been described in CAH patients without demonstrated *TNXB* deficiency (11, 12).

Recently, Lao et al. reported a novel cause of CAH-X syndrome, not associated with pathogenic *TNXA/TNXB* chimeras but due to a *TNXB* splice donor site variant (21). As currently *TNXB* testing remains challenging, at least in the

routine diagnostic approach, the diagnosis of CAH-X still relies on clinical evaluation.

Connective tissue dysplasia should be evaluated in all CAH patients, especially in those harboring a deletion in the *CYP21A2* gene. Screening for GJH and other soft tissue features presents age-related difficulties and should therefore be adapted to age, systematically evaluated, and retrospectively asked for. The Beighton Score (shown in **Figure 2**) remains the most objective assessment tool to measure GJH (22, 26).

Knowledge of the *TNXB* status in CAH will not only offer patients a better understanding of their symptomatology with the burden of the diagnosis of a second genetic disease, but also assures specific clinical management with a focus on preventing musculoskeletal manifestations and complications. Cardiovascular alterations may be underreported in CAH-X patients, probably related to the difficulties patients without cardiac symptoms may have to access specific diagnostic studies, such as cardiac magnetic resonance imaging (MRI), outside the context of a research study protocol, as observed in different series. A variable prevalence of cardiac abnormalities was found in different studies using different diagnostic methods.

Currently, no specific medical or genetic therapies are available for CAH-X patients. Management consists of interdisciplinary medical care, rehabilitation, and monitoring of major and organ-specific complications (24, 27, 28). Further *TNXB* and CAH-X studies are necessary to define detailed surveillance guidelines for these and other long-term complications and to develop prevention strategies.

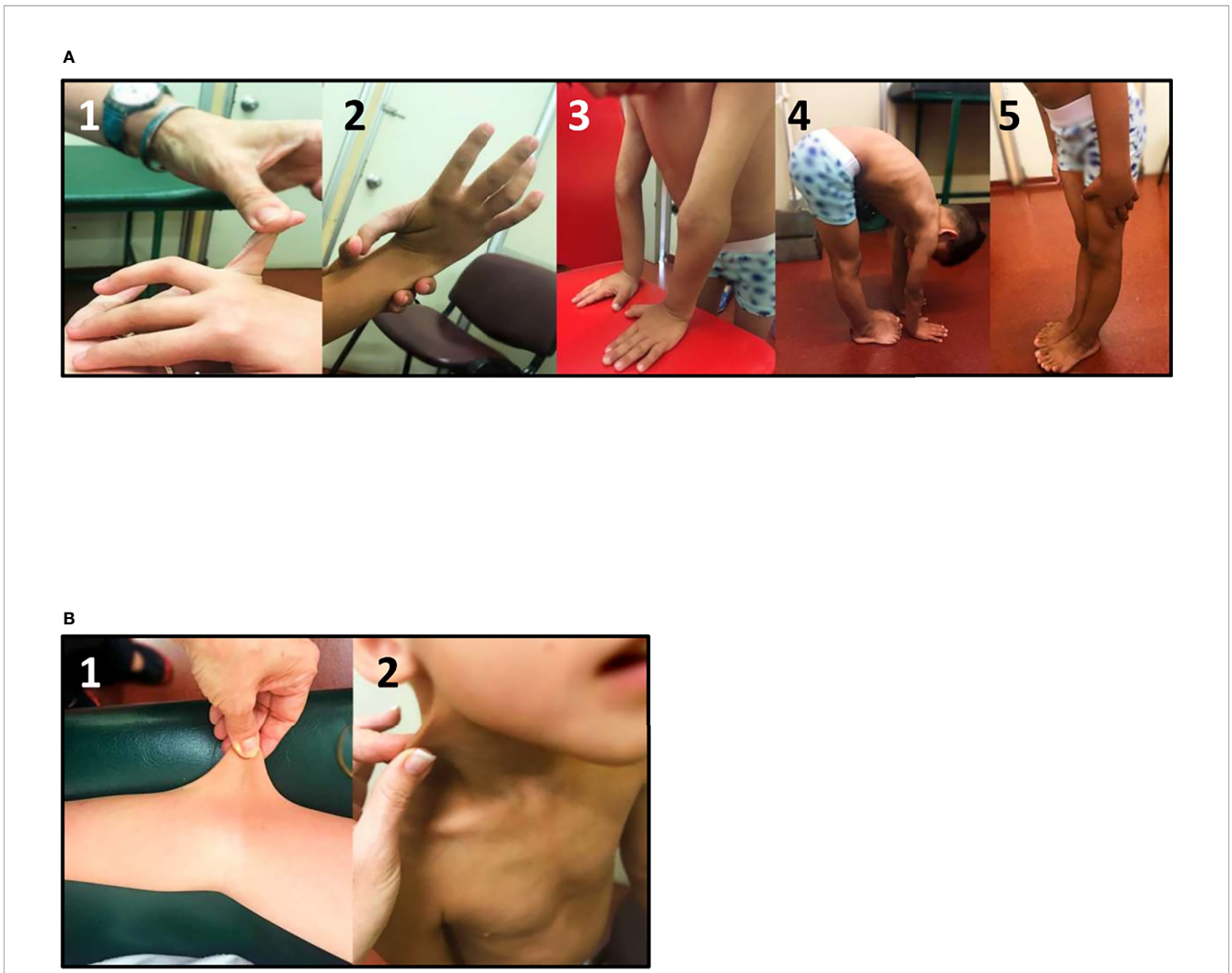

FIGURE 2 | Clinical evaluation of CAH-X Syndrome patients: **(A)** Generalized joint hypermobility Beighton score: 1) fifth finger extension test, 2) wrist flexion thumb abduction test, 3) elbow extension test, 4) trunk and hip flexion test, and 5) knee extension test. **(B)** Skin extensibility can be measured by lifting the cutaneous and subcutaneous layers of the skin and is considered hyperextensible if it can be stretched over 1.5 cm at the forearm and the dorsum of hands, and 3 cm for neck, elbow, and knees. Photographs shown belong to a biallelic CAH-X patient (compound heterozygous for CH1 and CH2).

CONCLUSIONS

Different studies on the *CYP21A2* gene have improved our knowledge on *TNXB*-related disorders. The *TNXB* gene encodes an extracellular matrix glycoprotein named TNX. EDS may be due to *TNXB* deficiency and up to 10% of 21-OHD CAH patients also have CAH-X. Chimeric recombination of the *TNXB* and *TNXA* genes may occur, and three *TNXA/TNXB* chimeras that differ in the junction site (CH1 to CH3) resulting in a contiguous *CYP21A2* and *TNXB* gene deletion, named CAH-X syndrome, have been described. Molecular studies are the gold standard to assess the presence of *TNXA/TNXB* chimeras. On the other hand, molecular analysis of the *TNXB* gene is challenging due to the presence of a pseudogene and next generation sequencing is highly complicated in these cases. For this reason, among others, TNX-deficient type EDS may be underdiagnosed. The variable prevalence of CAH-X reported in different cohorts may be related to the molecular strategies applied. Systematic study of *TNXB* status in individuals with a previous diagnosis of CAH and carriers of the complete 30-kb deletion of *CYP21A2* is highly recommended. Moreover, molecular genetic testing of CAH-21OHD should include *TNXA/TNXB* chimera analysis (29).

EDS comprises a clinically and genetically heterogeneous group of connective tissue disorders characterized by joint hypermobility, skin hyperextensibility, and tissue fragility as well as cardiovascular alterations. Cardiac disorders, in particular heart valve abnormalities, may be underdiagnosed, probably because of the young age of the majority of reported CAH-X patients and EDS-related cardiac abnormalities may appear with aging. In addition, it is unlikely that patients without heart symptoms are routinely checked for cardiac abnormalities with echocardiogram and/or cardiac MRI and currently only data from patients participating in specific CAH-X research studies are available. Severity of the phenotype may be correlated with the dosage of the dominant alleles, as monoallelic CAH-X patients have the mildest and most common EDS type and biallelic CAH-X patients the more severe clEDS subtype. CAH-X patients are consistently more severely affected than patients with homozygous or heterozygous TNX-deficient type EDS without CAH; however, the impact of the hormonal milieu on TNX and its role in connective-tissue pathophysiology is still poorly understood. In this line, carriers of one CAH-X allele who are not affected with CAH have a varied and milder EDS phenotype than monoallelic CAH-X patients.

Once the diagnosis of CAH-X has been established, it is advisable to guarantee long-term follow-up of these patients by medical specialists with a focus on preventing musculoskeletal manifestations and complications.

Finally, in order to prevent long-term musculoskeletal disorders, timely diagnosis of CAH-X is important and physical therapy for joint instability is recommended. In addition, molecular characterization of CAH-X is relevant for genetic counseling.

ETHICS STATEMENT

Written informed consent was obtained from the participant's legal guardians for the publication of any identifiable material in this study.

AUTHOR CONTRIBUTIONS

All authors contributed equally to design of the manuscript. RM, AM, and AB wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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REFERENCES

1. Speiser PW, White PC. Congenital Adrenal Hyperplasia. *N Engl J Med* (2003) 349(8):776–88. doi: 10.1056/NEJMra021561
2. Speiser PW, Arlt W, Auchus RJ, Baskin LS, Conway GS, Merke DP, et al. Congenital Adrenal Hyperplasia Due to Steroid 21-Hydroxylase Deficiency: An Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab* (2018) 103(11):4043–88. doi: 10.1210/jc.2018-01865
3. Witchel SF, Azziz R. Nonclassic Congenital Adrenal Hyperplasia. *Int J Pediatr Endocrinol* (2010) 2010:1–11. doi: 10.1155/2010/625105
4. Higashi Y, Yoshioka H, Yamane M, Gotoh O, Fujii-Kuriyama Y. Complete Nucleotide Sequence of Two Steroid 21-Hydroxylase Genes Tandemly Arranged in Human Chromosome: A Pseudogene and a Genuine Gene. *Proc Natl Acad Sci USA* (1986) 387 83(9):2841–5. doi: 10.1073/pnas.83.9.2841
5. Yang Z, Mendoza AR, Welch TR, Zipf WB, Yu CY. Modular Variations of the Human Major Histocompatibility Complex Class III Genes for Serine/Threonine Kinase RP, Complement Component C4, Steroid 21-Hydroxylase CYP21, and Tenascin TNX (the RCCX Module). A Mechanism for Gene Deletions and Disease Associations. *J Biol Chem* (1999) 274(17):12147–56. doi: 10.1074/jbc.274.17.12147
6. Pignatelli D, Carvalho BL, Palmeiro A, Barros A, Guerreiro SG and Macut D. The Complexities in Genotyping of Congenital Adrenal Hyperplasia: 21-Hydroxylase 395 Deficiency. *Front Endocrinol* (2019) 10:432. doi: 10.3389/fendo.2019.00432
7. Blanchong CA, Zhou B, Rupert KL, Chung EK, Jones KN, Sotos JF, et al. Deficiencies of Human Complement Component C4A and C4B and Heterozygosity in Length Variants of RP-C4-CYP21-TNX (RCCX) Modules in Caucasians. The Load of RCCX Genetic Diversity on Major Histocompatibility Complex-Associated Disease. *J Exp Med* (2000) 191 (12):2183–96. doi: 10.1084/jem.191.12.2183
8. Carrozza C, Foca L, De Paolis E, Concolino P. Genes and Pseudogenes: Complexity of the RCCX Locus and Disease. *Front Endocrinol (Lausanne)* (2021) 12:709758:709758. doi: 10.3389/fendo.2021.709758
9. Morel Y, Bristow J, Gitelman SE, Miller WL. Transcript Encoded on the Opposite Strand of the Human Steroid 21-Hydroxylase Complement Component C4 Gene Locus. *Proc Natl Acad Sci U.S.A.* (1989) 86(17):6582–6. doi: 10.1073/pnas.86.17.6582

10. Chen W, Xu Z, Sullivan A, Finkielstain GP, Van Ryzin C, Merke DP, et al. Junction Site Analysis of Chimeric CYP21A1P/CYP21A2 Genes in 21-Hydroxylase Deficiency. *Clin Chem* (2012) 58(2):421–30. doi: 10.1373/clinchem.2011.174037
11. Merke DP, Chen W, Morissette R, Xu Z, Van Ryzin C, Sachdev V, et al. Tenascin- X Haploinsufficiency Associated With Ehlers-Danlos Syndrome in Patients With Congenital Adrenal Hyperplasia. *J Clin Endocrinol Metab* (2013) 98(2):E379–87. doi: 10.1210/jc.2012-3148
12. Morissette R, Chen W, Perritt AF, Dreiling JL, Arai AE, Sachdev V, et al. Broadening the Spectrum of Ehlers Danlos Syndrome in Patients With Congenital Adrenal Hyperplasia. *J Clin Endocrinol Metab* (2015) 100(8): E1143–52. doi: 10.1210/jc.2015-2232
13. Chen W, Perritt A, Morissette R, Dreiling J, Bohn M, Mallappa A, et al. Ehlers-Danlos Syndrome Caused by Biallelic TNXB Variants in Patients With Congenital Adrenal Hyperplasia. Brief Report. *Hum Mutat* (2016) 37 (9):893–7. doi: 10.1002/humu.23028
14. Bristow J, Carey W, Egging D, Schalkwijk J. Tenascin-X, Collagen, Elastin, and the Ehlers-Danlos Syndrome. *Am J Med Genet C Semin Med Genet* (2005) 139C(1):24–30. doi: 10.1002/ajmg.c.30071
15. Burch GH, Gong Y, Liu W, Dettman RW, Curry CJ, Smith L, et al. Tenascin-X Deficiency is Associated With Ehlers-Danlos Syndrome. *Nat Genet* (1997) 17 (1):104–8. doi: 10.1038/ng0997-104
16. Schalkwijk J, Zweers MC, Steijlen PM, Dean WB, Taylor G, van Vlijmen IM, et al. A Recessive Form of the Ehlers-Danlos Syndrome Caused by Tenascin-X Deficiency. *N Engl J Med* (2001) 345(16):1167–75. doi: 10.1056/NEJMoa002939
17. Zweers MC, Bristow J, Steijlen PM, Dean WB, Hamel BC, Otero M, et al. Haploinsufficiency of TNXB is Associated With Hypermobility Type of Ehlers-Danlos Syndrome. *Am J Hum Genet* (2003) 73(1):214–7. doi: 10.1086/376564
18. Lao Q, Brookner B, Merke DP. High-Throughput Screening for CYP21A1P-TNXA/TNXB Chimeric Genes Responsible for Ehlers-Danlos Syndrome in Patients With Congenital Adrenal Hyperplasia. *J Mol Diagn* (2019) 21 (5):924–31. doi: 10.1016/j.jmoldx.2019.06.001
19. Gao Y, Lu L, Yu B, Mao J, Wang X, Nie M, et al. The Prevalence of the Chimeric TNXA/TNXB Gene and Clinical Symptoms of Ehlers-Danlos Syndrome With 21- Hydroxylase Deficiency. *Clin Endocrinol Metab* (2020) 105(7):dgaa199. doi: 10.1210/clinem/dgaa199
20. Marino R, Perez Garrido N, Ramirez P, Notaristéfano G, Moresco A, Touzon MS, et al. Ehlers-Danlos Syndrome: Molecular and Clinical Characterization of TNXA/TNXB Chimeras in Congenital Adrenal Hyperplasia. *J Clin Endocrinol Metab* (2021) 106(7):e2789–802. doi: 10.1210/clinem/dgab033
21. Lao Q, Mallappa A, Rueda Faucz F, Joyal E, Veeraraghavan P, Chen W, et al. A TNXB Splice Donor Site Variant as a Cause of Hypermobility Type Ehlers-Danlos Syndrome in Patients With Congenital Adrenal Hyperplasia. *Mol Genet Genomic Med* (2021) 9:e1556. doi: 10.1002/mgg3.1556
22. Malfait F, Francomano C, Byers P, Belmont J, Berglund B, Black J, et al. The 2017 International Classification of the Ehlers–Danlos Syndromes. *Am J Med Genet Part C Semin Med Genet* (2017) 175C:8–26. doi: 10.1002/ajmg.c.31552
23. Forghani I. Updates in Clinical and Genetics Aspects of Hypermobile Ehlers Danlos Syndrome. *Balkan Med J* (2019) 36(1):12–6. doi: 10.4274/balkanmedj.2018.1113
24. Brady AF, Demirdas S, Fournel-Gigleux S, Ghali N, Giunta C, Kapferer-Seebacher I, et al. The Ehlers-Danlos Syndromes, Rare Types. *Am J Med Genet C Semin Med Genet* (2017) 175(1):70–115. doi: 10.1002/ajmg.c.31550
25. Demirdas S, Dulfer E, Robert L, Kempers M, van Beek D, Micha D, et al. Recognizing the Tenascin-X Deficient Type of Ehlers-Danlos Syndrome: A Cross-Sectional Study in 17 Patients. *Clin Genet* (2017) 91(3):411–25. doi: 10.1111/cge.12853
26. Beighton P, De Paepe A, Steinmann B, Tsipouras P, Wenstrup RJ. Ehlers-Danlos Syndromes: Revised Nosology, Villefranche, 1997. Ehlers-Danlos National Foundation (USA) and Ehlers-Danlos Support Group (Uk). *Am J Med Genet* (1998) 77(1):31–7. doi: 10.1002/(sici)1096-8628(19980428)77:1<31::aid-ajmg8>3.0.co;2-o
27. Bowen JM, Sobey GJ, Burrows NP, Colombi M, Lavallee ME, Malfait F, et al. Ehlers-Danlos Syndrome, Classical Type. *Am J Med Genet C Semin Med Genet* (2017) 175(1):27–39. doi: 10.1002/ajmg.c.31548
28. Engelbert RH, Juul-Kristensen B, Pacey V, de Wandele I, Smeenk S, Woinarosky N, et al. The Evidence-Based Rationale for Physical Therapy Treatment of Children, Adolescents, and Adults Diagnosed With Joint Hypermobility Syndrome/Hypermobile Ehlers Danlos Syndrome. *Am J Med Genet C Semin Med Genet* (2017) 175(1):158–67. doi: 10.1002/ajmg.c.31545
29. Lao Q, Merke DP. Molecular Genetic Testing of Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency Should Include CAH-X Chimeras. *Eur J Hum Genet* (2021) 29(7):1047–8. doi: 10.1038/s41431-021-00870-5

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Components of Metabolic Syndrome in Youth With Classical Congenital Adrenal Hyperplasia

Mimi S. Kim[1,2,3]*, *Nicole R. Fraga*[1], *Nare Minaeian*[1,2] *and Mitchell E. Geffner*[1,2,3]

[1] Center for Endocrinology, Diabetes and Metabolism, Children's Hospital Los Angeles, Los Angeles, CA, United States, [2] Keck School of Medicine of University of Southern California, Los Angeles, CA, United States, [3] The Saban Research Institute at Children's Hospital Los Angeles, Los Angeles, CA, United States

Classical congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency is the most common primary adrenal insufficiency in children, involving cortisol deficiency, hyperandrogenism, and cardiometabolic risk. Prior studies have reported that youth with classical CAH have a higher prevalence of the components of metabolic syndrome: obesity, hypertension, elevated fasting blood glucose, and dyslipidemia. Yet, the incidence of the complete metabolic syndrome itself in children and adolescents with CAH is relatively rare. Traditional cardiometabolic risk factors can surface early in children with classical CAH, and continue to present and evolve over the lifetime, although it is only recently that reports of Type 2 diabetes and adverse cardiac events have begun to surface in adults affected by this condition. The pathophysiology underlying the increased prevalence of cardiometabolic risk factors in patients with CAH is not well-understood, with disease treatments and androgen excess having been studied to date. The aim of this review is to evaluate the recent literature on traditional cardiometabolic risk factors in youth with classical CAH, and to consider non-traditional risk factors/biomarkers for subclinical atherosclerosis, inflammation, and insulin resistance. A better understanding of these traditional and non-traditional risk factors in youth with CAH could help guide treatment options and prevent the onset of metabolic syndrome in adulthood, reducing overall patient morbidity.

Keywords: congenital adrenal hyperplasia, cardiovascular disease risk, metabolic syndrome, pediatrics, children, adolescents, pediatric obesity

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***Correspondence:**
Mimi S. Kim
mskim@chla.usc.edu

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INTRODUCTION

Classical congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency is the most common primary adrenal insufficiency in youth, affecting ~1 in 15,000 live births. CAH is characterized primarily by inadequate production of cortisol and aldosterone, along with overproduction of androgens (1, 2). Youth with CAH are not only affected by these hormone imbalances, but also exhibit an increased prevalence of cardiometabolic risk factors, which constitute the components of metabolic syndrome (**Figure 1**). Metabolic syndrome in youth is defined by having at least three or more of the following criteria: obesity, hypertension, elevated fasting blood glucose, and dyslipidemia (3).

Youth with classical CAH exhibit a higher prevalence of obesity across several countries (4–9) compared to their unaffected peers (10). Additionally, youth with CAH exhibit a heightened risk of hypertension (9, 11–13), and recently have been reported to have an increased prevalence of elevated fasting glucose, as well as dyslipidemia that worsens with increasing age (14).

Although youth with CAH manifest these individual components of metabolic syndrome, there have been fewer reports of the complete metabolic syndrome in youth with CAH than might be expected until recently (5, 14, 15). As a result, it would be enlightening to also consider non-traditional cardiometabolic risk factors in youth with CAH, during adolescence and to examine how these factors could potentially help to identify those patients who are at higher risk of developing metabolic syndrome. Non-traditional cardiometabolic risk factors to consider that have been studied in CAH include: subclinical atherosclerosis (16–18), inflammatory markers (19), and insulin resistance (5, 17, 19) (**Figure 1**). Importantly, a large, retrospective matched cohort study in Sweden showed that patients with CAH not only have increased prevalence of cardiometabolic risk factors, but that older adults demonstrate increased cardiovascular mortality (20), signaling a demand for further research to better understand cardiometabolic health in CAH.

In this short review, we provide an overview of current knowledge regarding the individual components of the metabolic syndrome in youth with CAH, with a focus on evidence from 2015 onwards for both traditional and non-traditional cardiometabolic risk factors (**Table 1**). As well, we discuss gaps in knowledge and areas for future research.

TRADITIONAL CARDIOMETABOLIC RISK FACTORS

Obesity

Compared to unaffected controls, youth with CAH present a higher prevalence of obesity overall, with one large study finding

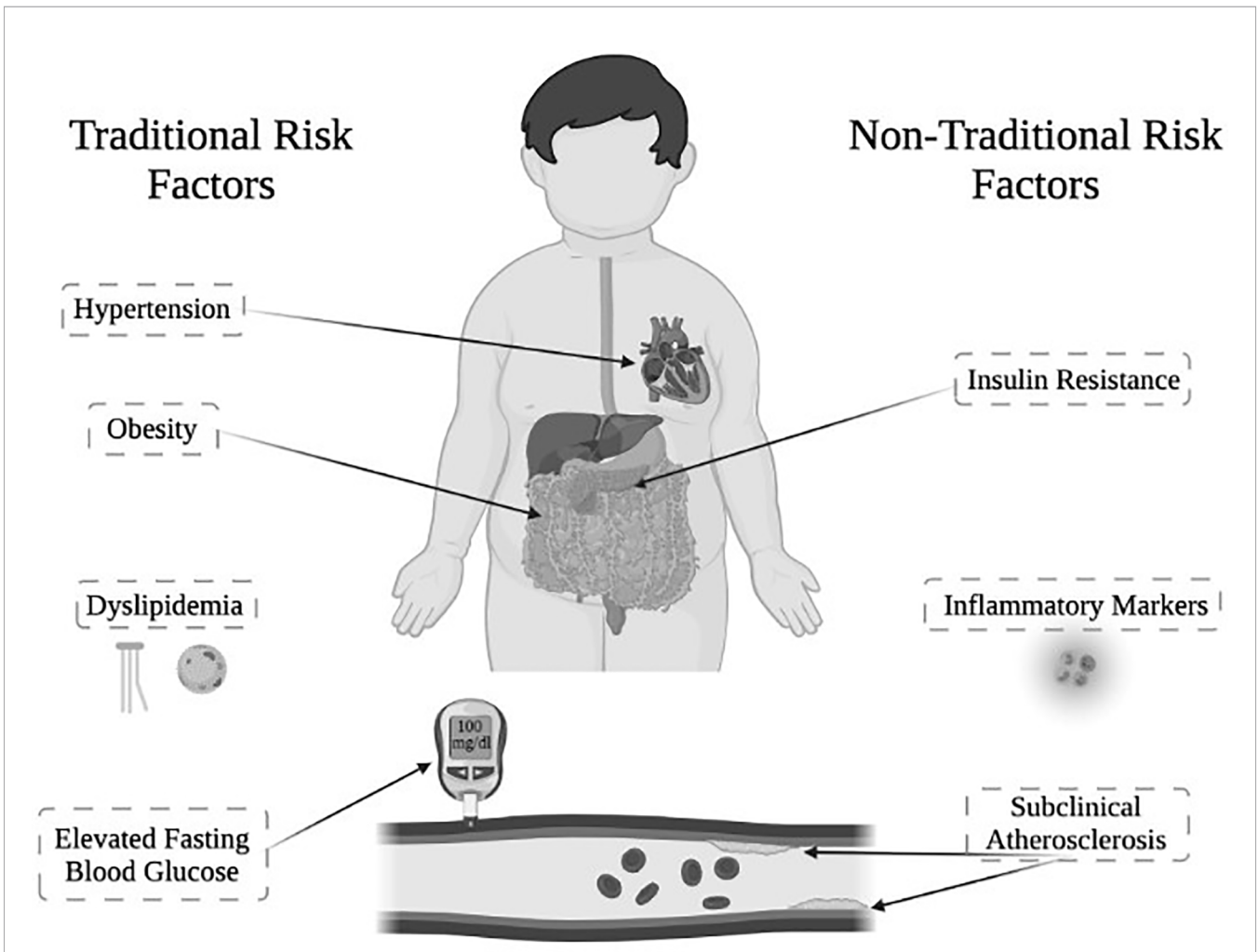

FIGURE 1 | Cardiometabolic risk factors in youth with classical CAH due to 21-hydroxylase deficiency. Traditional and non-traditional cardiometabolic risk factors observed in youth with classical CAH. *Figure created with Biorender.

TABLE 1 | Cardiometabolic risk factors and classical congenital adrenal hyperplasia: references from 2015 to present.

First Author Year	CAH Study Population	Age, Sex	Main Outcomes	Conclusions
Akyürek, N. 2015 (21)	N = 25	5-15 years 64% Female	CAH patients had increased BMI, insulin resistance, diastolic blood pressure (DBP) and carotid intima-media thickness (cIMT). 24% of patients exhibited arterial hypertension, and 20% had nocturnal hypertension. CIMT was higher in patients with nocturnal hypertension.	Classical CAH patients exhibit subclinical cardiovascular disease (CVD) with associations with hypertension.
Falhammar, H. 2015 (20)	N = 588	0-40 years 57% Female	Increased prevalence of hypertension, obesity, hyperlipidemia, and diabetes observed in CAH patients vs. controls.	CAH was associated with higher rates of cardiovascular and metabolic morbidity.
Kim, M.S. 2015 (15)	N = 28	15.6 ± 3.2 years 54% Female	Visceral adipose tissue (VAT), subcutaneous adipose tissue (SAT), and VAT : SAT were higher in CAH patients vs. controls.	Increased prevalence of unfavorable abdominal fat distribution could affect CVD risk in CAH.
Marra, A.M. 2015 (22)	N = 20	13.6 ± 2.5 years 50% Female	CAH patients had increased BMI, waist-to-height ratio and HOMA index vs. controls, and high systolic blood pressure (SBP) and decreased workload at peak exertion.	CAH patients can exhibit decreased exercise tolerance due to subclinical cardiovascular abnormalities.
Rodrigues, T.M.2015 (23)	N = 40	5-20 years 80% Female	Increased cIMT was observed in CAH youth, who also presented with increased BMI and SBP compared to controls.	Increased cIMT, BMI and SBP from a young age suggests increased CVD risk in CAH.
Bonfig, W. 2016 (11)	N = 716	3-18 years	Prevalence of hypertension in the study population was 12% and was more prominent in younger CAH patients.	CAH patients have increased risk for hypertension. However, the prevalence decreases with age.
Kim, M.S. 2016 (24)	N = 20	16 ± 3.3 years 50% Female	Mean cIMT was correlated with serum 17-hydroxyprogesterone (17-OHP) and androstenedione levels in CAH patients. No cIMT differences observed between CAH patients and controls.	Findings suggest a link between hyperandrogenism and subclinical atherosclerosis in CAH patients.
Metwalley, K.A. 2016 (25)	N = 32	13.6 ± 2.5 years 56% Female	Higher levels of highly-sensitive C-reactive protein (hs-CRP) and circulating endothelial cells in CAH patients, as well as left ventricular hypertrophy and prolonged mitral deceleration time.	Children with CAH present with markers of endothelial damage, subclinical atherosclerosis and left ventricular dysfunction.
Takishima, S. 2016 (26)	N = 29	Pediatric 52% Female	Adiposity rebound (AR) in CAH patients occurred before the age of 4 years, which is earlier than the general Japanese population.	Lower BMI at birth is associated with earlier AR in CAH patients.
Ariyawatkul, K. 2017 (27)	N = 21	15.2 ± 5.8 years 81% Female	Increased waist-to-hip ratio in patients with classical CAH.	Adolescents with CAH have increased risk of visceral obesity and cardiometabolic risk factors.
Mooij, C.F. 2017 (12)	N = 27	8-16 years	Elevated BMI and blood pressure observed in CAH patients, with seven patients categorized as overweight and four as obese.	Elevated BMI and blood pressure in CAH patients from a young age increases their CVD risk.
Sarafoglou, K. 2017 (7)	N = 194	≥ 2 years 52% Female	Children with CAH had increased risk for early onset obesity. AR occurred earlier at 3.3 years old.	Careful monitoring of hydrocortisone dosing during early childhood is needed to prevent increased weight gain and early AR in CAH.
Wierzbicka-Chimel, J. 2017 (28)	N = 19	23.7 ± 3.8 years 37% Female	CAH patients had decreased flow mediated dilation (FMD), cIMT, and common femoral artery IMT (fIMT).	CAH patients on long-term glucocorticoid therapy demonstrate decreased FMD and subclinical changes in left ventricular diastolic function.
Metwalley, K.A. 2018 (29)	N = 36	5-12 years 72% Female	CAH patients had elevated serum homocysteine levels, thicker cIMT, and high left ventricular mass.	Elevated homocysteine levels in CAH patients suggests risk for subclinical atherosclerosis.
Tamhane, S. 2018 (30)	Meta-Analysis	Pediatric and Adult	CAH patients had increased SBP, DBP, insulin resistance, and cIMT, but no evidence of morbidity or mortality due to cardiac events.	CAH patients have a high prevalence of cardiometabolic risk factors, but evidence has been lacking for actual morbidity or mortality.
Improda, N. 2019 (31)	Review Paper	Children and Adolescents	CAH patients presented with obesity, insulin resistance, hypertension, increased IMT and subclinical cardiac dysfunction from a young age.	Exposure to excess glucocorticoids, mineralocorticoids, and androgens may contribute to the development of cardiovascular changes.
Metwalley, K.A. 2019 (32)	N = 36	13.7 ± 2.4 years	CAH patients had greater epicardial fat thickness (EFT), cIMT, and left ventricular mass vs. controls.	Increased EFT suggests an increased risk of developing left ventricular dysfunction and subclinical atherosclerosis in CAH.

(Continued)

TABLE 1 | Continued

First Author Year	CAH Study Population	Age, Sex	Main Outcomes	Conclusions
		69% Female		
Vijayan, R. 2019 (33)	N = 52	3-21 years (Median 12y) 73% Female	CAH patients had a higher BMI, mean DBP, and greater insulin resistance vs. controls.	CAH youth have higher CVD risk and reduced quality of life despite adequate management.
Bhullar, G. 2020 (34)	N = 42	45.2% Female	CAH patients had earlier AR at 3.4 ± 1.3 years overall, and patients with obesity had an earlier AR vs. lean patients. Earlier AR predicted higher BMI-z during childhood, as well as increased central obesity and total body fat in adolescence.	Early AR can be used as a marker for disease severity and cardiometabolic risk in youth with classical CAH.
Gomes, L.G. 2020 (35)	Review Paper	Pediatric and Adult	Several studies showed increased prevalence of obesity, abnormal body composition, insulin resistance, and hypertension in CAH patients.	Despite an increased prevalence of cardiovascular markers, CVD remains unknown, and comparison of varying glucocorticoid regimens is needed.
Paizoni, L. 2020 (36)	N = 90	18-62 years (Median 29y) 57% Female	IMT was the same between CAH patients and controls. Only one patient in the cohort fulfilled the criteria for metabolic syndrome.	Though there is a high prevalence of insulin resistance and obesity in CAH patients, rarely do adults with CAH develop metabolic syndrome.
Farghaly, H.S. 2021 (37)	N = 40	14.8 ± 2.6 years 70% Female	CAH patients had elevated serum neopterin levels, decreased brachial FMD %, and normal cIMT vs. controls.	CAH patients have endothelial dysfunction as noted by elevated serum neopterin levels, which can explain vascular pathology seen in CAH.
Hasemi Dehkordi, E. 2021 (38)	N = 78	9.40 ± 4.09 years 53% Female	17-OHP serum concentrations were positively correlated with DBP and BMI in CAH patients.	Elevated 17-OHP, a marker of poor disease management, may be correlated to increased prevalence of CVD risk factors in CAH patients.
Torky, A. 2021 (14)	N = 57	Pediatric andAdult (longitudinal)	CAH patients exhibited a higher prevalence of obesity, hypertension, insulin resistance, and low HDL that began prior to age 10. 23 patients fit metabolic syndrome criteria at 1+ visits. Increased obesity in childhood was seen with maternal obesity.	Higher prevalence of CVD risk factors is seen in CAH patients at a young age and is associated with treatment and familial factors.

the median age of onset to be 8 years old (14). Not only is obesity more prevalent in youth with CAH, but a centralized fat distribution and increased waist-to-height ratio have also been observed, suggesting a more unfavorable distribution of body fat (17, 22, 27, 35). In youth with CAH, the fat mass-to-lean mass ratio was also recently shown to be significantly higher compared to controls (36). Central obesity or an 'apple-shape' indicates increased abdominal adipose tissue, which is of particular concern as visceral abdominal adipose tissue (VAT) is highly proinflammatory in individuals with obesity and metabolic syndrome (39). Youth with CAH exhibit increased VAT and subcutaneous adipose tissue (SAT) compared to BMI-matched controls, with an increased VAT-to-SAT ratio compared to controls as well, which constitutes an adverse metabolic phenotype in obese adolescents (15).

In addition to fat distribution, youth with CAH also exhibit an earlier age at adiposity rebound compared to their unaffected peers. Adiposity rebound (AR) is known as the second rise in BMI during childhood that corresponds to an increase in number of adipocytes (40, 41). In normative populations, AR takes place between 5 and 7 years old; however, for youth with CAH, age at AR has been shown in the U.S. and Japan to occur at approximately 3 years (7, 26, 34), with children in the U.K. exhibiting an even earlier AR at 1.7 years old (4). Youth with CAH and obesity had an even earlier age at AR in the U.S., at 2.8 years, with an earlier AR predicting a higher BMI z-score and central obesity in later childhood (34). Thus, early AR in patients with CAH could help identify youth at risk for cardiometabolic disease.

As well, familial factors such as maternal obesity during childhood can contribute to the increased incidence of obesity seen in this cohort (14).

Hypertension

High blood pressure is another major risk factor for cardiovascular disease, and in CAH youth, there is an increased frequency of hypertension overall observed across age groups, although more prevalent in younger children compared to adolescents (11). Youth with CAH exhibit higher systolic blood pressure compared to controls (30, 31) and have been shown again recently to exhibit an impaired or absent nocturnal drop in blood pressure compared to controls (21, 36).

Hypertension has been found to occur more frequently in patients with CAH who receive fludrocortisone therapy compared to those who are not taking fludrocortisone (42).

There has also been some recent evidence that the negative correlation between blood pressure and age could be explained by an overall reduction in fludrocortisone dose as patients with CAH become older (31). Suppressed plasma renin activity levels have also been shown to be correlated with high blood pressure (5, 14, 35).

In terms of contributing factors, among youth with CAH there is a positive correlation between BMI and blood pressure (27, 33), suggesting a meaningful relationship between prevalence of obesity and hypertension in this population. As well, 17-hydroxyprogesterone (17-OHP) levels have also been noted to be positively correlated with diastolic blood pressure and BMI (38). Conversely, a higher 17-OHP has been found to be protective against hypertension in a large study of children, while suppressed androstenedione was noted to be associated with hypertensive BP, perhaps indirectly representing an effect of excess glucocorticoid dosing on blood pressure (14).

Finally, sexual dimorphism has been noted in pubertal adolescents ages 12-18 years old, with high blood pressure found to be more prevalent in females compared to males with CAH (11).

Elevated Fasting Blood Glucose

Recent studies have shown that patients with CAH may exhibit a higher prevalence of fasting hyperglycemia during childhood compared to controls (14, 29). In a large longitudinal study of patients with CAH, the prevalence of elevated fasting blood glucose was shown to increase during school age and adolescence, but to decrease in young adulthood (14). Elevated fasting plasma glucose levels have been observed in adult patients with CAH (43), with emerging reports that this also may occur during childhood; however, it has been more common to see insulin resistance than hyperglycemia reported in youth with CAH.

Dyslipidemia

There has been a relatively small number of studies reporting dyslipidemia in youth with CAH, with higher triglycerides, lower HDL cholesterol, and higher small dense-LDL having recently been reported (14, 31). Nonetheless, dyslipidemia in youth with CAH has been shown to worsen with age, in particular the prevalence of low HDL in adulthood (14). Elevated levels of the androgen precursor, 17-OHP, used as a marker of disease severity and/or hormonal control, appear to negatively correlate with incidence of hypercholesterolemia and are associated with low HDL levels (14). Worse hormonal control (higher 17-OHP) could be relatively protective for dyslipidemia if those patients exhibiting tighter hormonal control (*i.e.*, lower 17-OHP) are therefore on higher glucocorticoid replacement (14). Overall, the evidence supporting an increased risk of dyslipidemia in youth with CAH has been variable.

NON-TRADITIONAL CARDIOMETABOLIC RISK FACTORS

Subclinical Atherosclerosis

Both flow-mediated dilation (FMD) of the brachial artery and intima-media thickness of the carotid artery (cIMT) are early surrogate markers of atherosclerosis that have been studied in youth with CAH. Vascular endothelial and smooth muscle dysfunction, as measured by a decreased FMD, has been shown in youth with CAH (17, 37) even after correcting for age, sex, BMI, and doses of glucocorticoid and fludrocortisone (28). Although endothelial dysfunction is a critical early step in the development of atherosclerosis and can serve as a potential predictor of cIMT (44), there have been mixed results in youth and young adults with CAH in terms of group differences in cIMT compared to controls (16, 17, 23, 24, 32, 45). Among youth with CAH, however, cIMT has been positively correlated with androgen levels (24, 29).

Additionally, markers for endothelial dysfunction have been studied such as neopterin, a novel inflammatory biomarker for endothelial damage that has been notably elevated in patients with CAH (37, 46–48). Elevated high-sensitivity C-reactive protein (hs-CRP) and circulating endothelial cell levels in serum are also seen in youth with CAH, suggesting endothelial damage and subclinical atherosclerosis (25).

Epicardial fat thickness is another emerging early marker of atherosclerosis and has also been noted to be higher in youth with CAH (32). Epicardial fat thickness was also positively correlated with waist circumference, 17-OHP, and insulin resistance, suggesting relationships with other cardiometabolic risk factors (32).

Further study is merited to understand the contribution of increased vascular endothelial injury and endothelial dysfunction to the development of higher blood pressure seen in youth with CAH.

Inflammatory Markers

Youth with CAH exhibit increased circulating concentrations of inflammatory markers compared to unaffected youth, which is important given they are surrogate markers of future cardiovascular disease (49). There could be several reasons for increased inflammation in youth with CAH, including increased VAT which produces more inflammatory substances associated with cardiovascular disease, promotes inflammation in the body, and is associated with risk for metabolic disease independent of total body adiposity (50). Youth with CAH have been shown to have significantly higher leptin concentrations compared to controls (15, 19, 31), potentially caused by epinephrine deficiency (19), and/or an altered leptin axis related to decreased soluble leptin receptor (51). Leptin levels are also positively correlated with obesity (27, 31) and abdominal fat (15) in youth with CAH. The inflammatory markers, PAI-1 and hs-CRP were correlated with abdominal fat as well (15). Lastly, homocysteine levels, an inflammatory marker for atherosclerosis and coronary artery disease, have also been shown to be increased in patients with CAH (29).

Insulin Resistance

In youth with CAH, a higher prevalence of insulin resistance has been found compared to their unaffected peers, with significantly higher insulin concentrations and homeostasis model assessment for insulin resistance index (HOMA-IR), even after adjusting for BMI (14, 22, 32). The prevalence of insulin resistance in youth with CAH increases with age (14). Among youth with CAH,

insulin resistance has been related to hydrocortisone dose, BMI-SDS, and plasma renin activity levels, but not with hyperandrogenism (12, 35). Some suggest that lower hydrocortisone doses could lead to a reduction of insulin resistance (8); however, this may only be true when the doses are supraphysiologic (14). Although there is increased insulin resistance and fasting hyperglycemia in youth with CAH, there has not been an increase in type 2 diabetes yet noted (35).

DISCUSSION

Traditional cardiometabolic risk factors may occur in youth with classical CAH due to 21-hydroxylase deficiency and continue to be present throughout childhood, although the metabolic syndrome itself has not been as commonly reported as might be expected. However, a longitudinal natural history study recently identified 23 cases of metabolic syndrome with a median age of onset of 9.6 years (14). A higher prevalence of obesity during childhood and adulthood, along with hormone replacements over the lifetime, could be contributing factors for hypertension and insulin resistance across all ages. Combined with emerging reports of type 2 diabetes, gestational diabetes, and adverse cardiac events in adults with classical CAH (20, 52, 53), further longitudinal study of this high-risk cohort is merited to assess risk factors from childhood through adulthood, to better understand the development of longer-term adverse outcomes. As well, the examination of non-traditional cardiometabolic risk factors as potential early biomarkers for subclinical atherosclerosis, inflammation, and insulin resistance could be useful in patients with CAH.

The pathophysiology underlying the increased prevalence of cardiometabolic risk factors in patients with CAH is not yet fully understood. However, both disease- and treatment-related factors should be considered. Decreased cortisol production in CAH necessitates lifelong glucocorticoid replacement, with studies pointing to the *supraphysiologic* glucocorticoid doses needed to suppress excess ACTH signaling to the adrenal gland, as contributing to the development of cardiometabolic risk factors (1, 31). The management of hyperandrogenism in patients with CAH is often a challenge with many patients having persistent elevations in circulating androgens despite attempts at optimization of glucocorticoid dosing, although there may be a trade-off between hormonal control and glucocorticoid dosing, in terms of cardiometabolic risk (1, 14). Multiple adjunctive therapies are currently under investigation, including CRH and corticotropin-releasing factor receptor antagonists, along with extended-release formulations of hydrocortisone, to decrease overall daily glucocorticoid dosing in patients with the goal of minimizing side effects related to the currently used supraphysiologic doses of glucocorticoid. As well, mineralocorticoid supplementation may potentially add to risk for hypertension and lipid abnormalities in youth with CAH.

Disease-related contributing factors to consider in youth with classical CAH include hyperandrogenism and adrenomedullary dysfunction. The contribution of hyperandrogenism is complicated to assess in patients with CAH who are already on hormone replacement therapies, presenting a need for more biomarkers with which to study prenatal and cumulative androgen exposure over the lifetime in these patients (54). We know that hyperandrogenic females with polycystic ovarian syndrome (PCOS) exhibit the traditional and non-traditional cardiometabolic risk factors that are present in patients with CAH and can occur at an early age (55–58). In addition, transgender men utilizing gender-affirming testosterone therapy are another important cohort that is chronically exposed to androgens and can exhibit an increase in BMI, dyslipidemia, and vascular dysfunction (59–61). Androgen exposure in females is associated with endothelial dysfunction and can directly contribute to vascular dysfunction and high blood pressure (60, 62). It should be noted though that these related natural human models of hyperandrogenism do not involve the additional inherent hormone imbalances found in patients with classical CAH (*e.g.*, lower cortisol, aldosterone, and epinephrine production). Patients with classical CAH have an additional deficiency in epinephrine (19, 63–65), which could lead to a lack of stimulated lipolysis of triglyceride stores, and dysregulation of insulin and adipokines (19). It is also interesting to consider the implications of lower epinephrine levels and disturbed adrenomedullary function under fasting and feeding conditions that have been noted in unaffected adults with obesity (66). Future research is needed to study the role of adrenomedullary dysfunction in the pathogenesis of cardiometabolic risk in patients with CAH.

Given the early onset of associated cardiometabolic risk factors, and prolonged hormone imbalances already present *in utero*, the assessment of children with CAH from an early age is merited to better understand prenatal and early postnatal origins of cardiometabolic disease in patients with CAH. Key components of the metabolic syndrome, *i.e.*, obesity and hypertension, can arise early in childhood in patients with CAH, with obesity itself linked to the development of hypertension, insulin resistance, type 2 diabetes, dyslipidemia, and long-term vascular complications. In patients with CAH, obesity is associated with the development of cardiometabolic risk factors in adults (14). Therefore, it will be particularly important to further understand the mechanism driving the increased prevalence of obesity in children with CAH and to provide appropriate interventions at an early age. While healthy lifestyle counseling should commence early in childhood as part of routine clinical practice guidelines (67), the development of medical therapeutics to treat obesity and insulin resistance in these high-risk youth with CAH may also be useful to prevent cardiometabolic sequelae and metabolic syndrome in adulthood.

CONCLUSIONS AND PERSPECTIVES

There is a need for the longitudinal study of patients with classical CAH from diagnosis at infancy through older adulthood to better characterize the natural history of the metabolic syndrome and its components, along with cardiovascular disease. While there is an underlying relationship between treatment-related factors and cardiometabolic risk factors, more needs to be understood about

the contribution of disease-related factors in CAH amidst the challenges of studying a cohort on hormone replacement from an early age. Gaining a better understanding of both traditional and non-traditional risk factors and their effects on youth with CAH could ultimately lead to the improved treatment and prevention of metabolic syndrome and cardiovascular disease in adulthood.

AUTHOR CONTRIBUTIONS

MK, NF, and NM performed an extensive literature search and drafted the manuscript. All authors critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Merke DP, Auchus RJ. Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency. *N Engl J Med* (2020) 383(13):1248–61. doi: 10.1056/NEJMra1909786
2. Witchel SF. Congenital Adrenal Hyperplasia. *J Pediatr Adolesc Gynecol* (2017) 30(5):520–34. doi: 10.1016/j.jpag.2017.04.001
3. Weiss R, Dziura J, Burgert TS, Tamborlane WV, Taksali SE, Yeckel CW, et al. Obesity and the Metabolic Syndrome in Children and Adolescents. *N Engl J Med* (2004) 350(23):2362–74. doi: 10.1056/NEJMoa031049
4. Cornean RE, Hindmarsh PC, Brook CG. Obesity in 21-Hydroxylase Deficient Patients. *Arch Dis Child* (1998) 78(3):261–3. doi: 10.1136/adc.78.3.261
5. Finkielstain GP, Kim MS, Sinaii N, Nishitani M, Van Ryzin C, Hill SC, et al. Clinical Characteristics of a Cohort of 244 Patients With Congenital Adrenal Hyperplasia. *J Clin Endocrinol Metab* (2012) 97(12):4429–38. doi: 10.1210/jc.2012-2102
6. Mooij CF, Kroese JM, Claahsen-van der Grinten HL, Tack CJ, Hermus AR. Unfavourable Trends in Cardiovascular and Metabolic Risk in Paediatric and Adult Patients With Congenital Adrenal Hyperplasia? *Clin Endocrinol* (2010) 73(2):137–46. doi: 10.1111/j.1365-2265.2009.03690.x
7. Sarafoglou K, Forlenza GP, Yaw Addo O, Kyllo J, Lteif A, Hindmarsh PC, et al. Obesity in Children With Congenital Adrenal Hyperplasia in the Minnesota Cohort: Importance of Adjusting Body Mass Index for Height-Age. *Clin Endocrinol* (2017) 86(5):708–16. doi: 10.1111/cen.13313
8. Subbarayan A, Dattani MT, Peters CJ, Hindmarsh PC. Cardiovascular Risk Factors in Children and Adolescents With Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency. *Clin Endocrinol* (2014) 80(4):471–7. doi: 10.1111/cen.12265
9. Volkl TM, Simm D, Dotsch J, Rascher W, Dorr HG. Altered 24-Hour Blood Pressure Profiles in Children and Adolescents With Classical Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency. *J Clin Endocrinol Metab* (2006) 91(12):4888–95. doi: 10.1210/jc.2006-1069
10. Ogden CL, Yanovski SZ, Carroll MD, Flegal KM. The Epidemiology of Obesity. *Gastroenterology* (2007) 132(6):2087–102. doi: 10.1053/j.gastro.2007.03.052
11. Bonfig W, Roehl FW, Riedl S, Dörr HG, Bettendorf M, Brämswig J, et al. Blood Pressure in a Large Cohort of Children and Adolescents With Classic Adrenal Hyperplasia (CAH) Due to 21-Hydroxylase Deficiency. *Am J Hypertens* (2016) 29(2):266–72. doi: 10.1093/ajh/hpv087
12. Mooij CF, van Herwaarden AE, Sweep FCGJ, Roeleveld N, de Korte CL, Kapusta L, et al. Cardiovascular and Metabolic Risk in Pediatric Patients With Congenital Adrenal Hyperplasia Due to 21 Hydroxylase Deficiency. *J Pediatr Endocrinol Metab* (2017) 30(9):957–66. doi: 10.1515/jpem-2017-0068
13. Nebesio TD, Eugster EA. Observation of Hypertension in Children With 21-Hydroxylase Deficiency: A Preliminary Report. *Endocrine* (2006) 30(3):279–82. doi: 10.1007/s12020-006-0005-4
14. Torky A, Sinaii N, Jha S, Desai J, El-Maouche D, Mallappa A, et al. Cardiovascular Disease Risk Factors and Metabolic Morbidity in a Longitudinal Study of Congenital Adrenal Hyperplasia. *J Clin Endocrinol Metab* (2021) 106(12):e5247–57. doi: 10.1210/clinem/dgab133
15. Kim MS, Ryabets-Lienhard A, Dao-Tran A, Mittelman SD, Gilsanz V, Schrager SM, et al. Increased Abdominal Adiposity in Adolescents and Young Adults With Classical Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency. *J Clin Endocrinol Metab* (2015) 100(8):E1153–1159. doi: 10.1210/jc.2014-4033
16. Amr NH, Ahmed AY, Ibrahim YA. Carotid Intima Media Thickness and Other Cardiovascular Risk Factors in Children With Congenital Adrenal Hyperplasia. *J Endocrinol Invest* (2014) 37(10):1001–8. doi: 10.1007/s40618-014-0148-8
17. Harrington J, Peña AS, Gent R, Hirte C, Couper J. Adolescents With Congenital Adrenal Hyperplasia Because of 21-Hydroxylase Deficiency Have Vascular Dysfunction. *Clin Endocrinol* (2012) 76(6):837–42. doi: 10.1111/j.1365-2265.2011.04309.x
18. Sartorato P, Zulian E, Benedini S, Mariniello B, Schiavi F, Bilora F, et al. Cardiovascular Risk Factors and Ultrasound Evaluation of Intima-Media Thickness at Common Carotids, Carotid Bulbs, and Femoral and Abdominal Aorta Arteries in Patients With Classic Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency. *J Clin Endocrinol Metab* (2007) 92(3):1015–8. doi: 10.1210/jc.2006-1711
19. Charmandari E, Weise M, Bornstein SR, Eisenhofer G, Keil MF, Chrousos GP, et al. Children With Classic Congenital Adrenal Hyperplasia Have Elevated Serum Leptin Concentrations and Insulin Resistance: Potential Clinical Implications. *J Clin Endocrinol Metab* (2002) 87(5):2114–20. doi: 10.1210/jcem.87.5.8456
20. Falhammar H, Frisen L, Hirschberg AL, Norrby C, Almqvist C, Nordenskjold A, et al. Increased Cardiovascular and Metabolic Morbidity in Patients With 21-Hydroxylase Deficiency: A Swedish Population-Based National Cohort Study. *J Clin Endocrinol Metab* (2015) 100(9):3520–8. doi: 10.1210/JC.2015-2093
21. Akyürek N, Atabek ME, Eklioğlu BS, Alp H. Ambulatory Blood Pressure and Subclinical Cardiovascular Disease in Patients With Congenital Adrenal Hyperplasia: A Preliminary Report. *J Clin Res Pediatr Endocrinol* (2015) 7(1):13–8. doi: 10.4274/jcrpe.1658
22. Marra AM, Improda N, Capalbo D, Salzano A, Arcopinto M, De Paulis A, et al. Cardiovascular Abnormalities and Impaired Exercise Performance in Adolescents With Congenital Adrenal Hyperplasia. *J Clin Endocrinol Metab* (2015) 100(2):644–52. doi: 10.1210/jc.2014-1805
23. Rodrigues TM, Barra CB, Santos JL, Goulart EM, Ferreira AV, Silva IN. Cardiovascular Risk Factors and Increased Carotid Intima-Media Thickness in Young Patients With Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency. *Arch Endocrinol Metab* (2015) 59(6):541–7. doi: 10.1590/2359-3997000000119
24. Kim MS, Dao-Tran A, Davidowitz E, Tseng T, Gilsanz V, Ryabets-Lienhard A, et al. Carotid Intima-Media Thickness Is Associated With Increased Androgens in Adolescents and Young Adults With Classical Congenital Adrenal Hyperplasia. *Horm Res Paediatr* (2016) 85(4):242–9. doi: 10.1159/000444169
25. Metwalley KA. Left Ventricular Dysfunction and Subclinical Atherosclerosis in Children With Classic Congenital Adrenal Hyperplasia: A Single-Center Study From Upper Egypt. *Eur J Pediatr* (2016) 175(3):415. doi: 10.1007/s00431-015-2678-2

26. Takishima S, Nakajima K, Nomura R, Tsuji-Hosokawa A, Matsuda N, Matsubara Y, et al. Lower Body Weight and BMI at Birth Were Associated With Early Adiposity Rebound in 21-Hydroxylase Deficiency Patients. *Endocr J* (2016) 63(11):983–90. doi: 10.1507/endocrj.EJ16-0194
27. Ariyawatkul K, Tepmongkol S, Aroonparkmongkol S, Sahakitrungruang T. Cardio-Metabolic Risk Factors in Youth With Classical 21-Hydroxylase Deficiency. *Eur J Pediatr* (2017) 176(4):537–45. doi: 10.1007/s00431-017-2875-2
28. Wierzbicka-Chmiel J, Chmiel A, Rychlik S, Ogrodowczyk-Bobik M, Marek B, Kajdaniuk D. Vascular and Cardiac Function in Young Adults With Classical Congenital Adrenal Hyperplasia. *Endokrynol Pol* (2017) 68(5):505–11. doi: 10.5603/EP.a2017.0046
29. Metwalley KA, Farghaly HS, Abdelhamid A. Homocysteine Level in Children With Classic Congenital Adrenal Hyperplasia: Relationship to Carotid Intimal Wall Thickness and Left Ventricular Function. *Horm Res Paediatr* (2018) 90 (4):228–35. doi: 10.1159/000492900
30. Tamhane S, Rodriguez-Gutierrez R, Iqbal AM, Prokop LJ, Bancos I, Speiser PW, et al. Cardiovascular and Metabolic Outcomes in Congenital Adrenal Hyperplasia: A Systematic Review and Meta-Analysis. *J Clin Endocrinol Metab* (2018) 103(11):4097–103. doi: 10.1210/jc.2018-01862
31. Improda N, Barbieri F, Ciccarelli GP, Capalbo D, Salerno M. Cardiovascular Health in Children and Adolescents With Congenital Adrenal Hyperplasia Due to 21-Hydroxilase Deficiency. *Front Endocrinol* (2019) 10:212. doi: 10.3389/fendo.2019.00212
32. Metwalley KA, Farghaly HS, Abdelhamid A. Epicardial Fat Thickness in Children With Classic Congenital Adrenal Hyperplasia. *J Clin Res Pediatr Endocrinol* (2019) 11(1):61–9. doi: 10.4274/jcrpe.galenos.2018.2018.0153
33. Vijayan R, Bhavani N, Pavithran PV, Nair V, Menon UV, Menon AS, et al. Metabolic Profile, Cardiovascular Risk Factors and Health-Related Quality of Life in Children, Adolescents and Young Adults With Congenital Adrenal Hyperplasia. *J Pediatr Endocrinol Metab* (2019) 32(8):871–7. doi: 10.1515/jpem-2019-0079
34. Bhullar G, Tanawattanacharoen VK, Yeh MY, Kim WS, Vidmar AP, Geffner ME, et al. Early Adiposity Rebound Predicts Obesity and Adiposity in Youth With Congenital Adrenal Hyperplasia. *Horm Res Paediatr* (2020) 93(11-12):609–15. doi: 10.1159/000514130
35. Gomes LG, Mendonca BB, Bachega TASS. Long-Term Cardio-Metabolic Outcomes in Patients With Classical Congenital Adrenal Hyperplasia: Is the Risk Real? *Curr Opin Endocrinol Diabetes Obes* (2020) 27(3):155–61. doi: 10.1097/MED.0000000000000545
36. Paizoni L, Auer MK, Schmidt H, Hübner A, Bidlingmaier M, Reisch N. Effect of Androgen Excess and Glucocorticoid Exposure on Metabolic Risk Profiles in Patients With Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency. *J Steroid Biochem Mol Biol* (2020) 197:105540. doi: 10.1016/j.jsbmb.2019.105540
37. Farghaly HS, Metwalley KA, Raafat DM, Saied GM, Gabri MF, Algowhary M. Association Between Vascular Endothelial Dysfunction and the Inflammatory Marker Neopterin in Patients With Classic Congenital Adrenal Hyperplasia. *Atherosclerosis* (2021) 328:38–43. doi: 10.1016/j.atherosclerosis.2021.05.017
38. Hashemi Dehkordi E, Khaheshi S, Mostofizadeh N, Hashemipour M. Cardiovascular Risk Factors in Children and Adolescents With Congenital Adrenal Hyperplasia. *Adv Biomed Res* (2021) 10:19. doi: 10.4103/abr.abr_219_20
39. Alexopoulos N, Katritsis D, Raggi P. Visceral Adipose Tissue as a Source of Inflammation and Promoter of Atherosclerosis. *Atherosclerosis* (2014) 233 (1):104–12. doi: 10.1016/j.atherosclerosis.2013.12.023
40. Knittle JL, Timmers K, Ginsberg-Fellner F, Brown RE, Katz DP. The Growth of Adipose Tissue in Children and Adolescents. Cross-Sectional and Longitudinal Studies of Adipose Cell Number and Size. *J Clin Invest* (1979) 63(2):239–46. doi: 10.1172/JCI109295
41. Rolland-Cachera MF, Deheeger M, Bellisle F, Sempé M, Guilloud-Bataille M, Patois E. Adiposity Rebound in Children: A Simple Indicator for Predicting Obesity. *Am J Clin Nutr* (1984) 39(1):129–35. doi: 10.1093/ajcn/39.1.129
42. Maccabee-Ryaboy N, Thomas W, Kyllo J, Lteif A, Petryk A, Gonzalez-Bolanos MT, et al. Hypertension in Children With Congenital Adrenal Hyperplasia. *Clin Endocrinol* (2016) 85(4):528–34. doi: 10.1111/cen.13086
43. Arlt W, Willis DS, Wild SH, Krone N, Doherty EJ, Hahner S, et al. Health Status of Adults With Congenital Adrenal Hyperplasia: A Cohort Study of 203 Patients. *J Clin Endocrinol Metab* (2010) 95(11):5110–21. doi: 10.1210/jc.2010-0917
44. Halcox JP, Donald AE, Ellins E, Witte DR, Shipley MJ, Brunner EJ, et al. Endothelial Function Predicts Progression of Carotid Intima-Media Thickness. *Circulation* (2009) 119(7):1005–12. doi: 10.1161/CIRCULATIONAHA.108.765701
45. Wasniewska M, Balsamo A, Valenzise M, Manganaro A, Faggioli G, Bombaci S, et al. Increased Large Artery Intima Media Thickness in Adolescents With Either Classical or Non-Classical Congenital Adrenal Hyperplasia. *J Endocrinol Invest* (2013) 36(1):12–5. doi: 10.3275/8194
46. Fuchs D, Avanzas P, Arroyo-Espliguero R, Jenny M, Consuegra-Sanchez L, Kaski JC. The Role of Neopterin in Atherogenesis and Cardiovascular Risk Assessment. *Curr Med Chem* (2009) 16(35):4644–53. doi: 10.2174/092986709789878247
47. Hoffmann G, Wirleitner B, Fuchs D. Potential Role of Immune System Activation-Associated Production of Neopterin Derivatives in Humans. *Inflamm Res* (2003) 52(8):313–21. doi: 10.1007/s00011-003-1181-9
48. Pacileo M, Cirillo P, De Rosa S, Ucci G, Petrillo G, Musto D'Amore S, et al. The Role of Neopterin in Cardiovascular Disease. *Monaldi Arch Chest Dis* (2007) 68(2):68–73. doi: 10.4081/monaldi.2007.454
49. Willerson JT, Ridker PM. Inflammation as a Cardiovascular Risk Factor. *Circulation* (2004) 109(21 Suppl 1):II2–10. doi: 10.1161/01.CIR.0000129535.04194.38
50. Tchernof A, Després JP. Pathophysiology of Human Visceral Obesity: An Update. *Physiol Rev* (2013) 93(1):359–404. doi: 10.1152/physrev.00033.2011
51. Volkl TM, Simm D, Korner A, Kiess W, Kratzsch J, Dorr HG. Adiponectin Levels Are High in Children With Classic Congenital Adrenal Hyperplasia (CAH) Due to 21-Hydroxylase Deficiency. *Acta Paediatr* (2009) 98(5):885–91. doi: 10.1111/j.1651-2227.2009.01231.x
52. Falhammar H, Filipsson H, Holmdahl G, Janson PO, Nordenskjöld A, Hagenfeldt K, et al. Metabolic Profile and Body Composition in Adult Women With Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency. *J Clin Endocrinol Metab* (2007) 92(1):110–6. doi: 10.1210/jc.2006-1350
53. Hagenfeldt K, Janson PO, Holmdahl G, Falhammar H, Filipsson H, Frisén L, et al. Fertility and Pregnancy Outcome in Women With Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency. *Hum Reprod* (2008) 23 (7):1607–13. doi: 10.1093/humrep/den118
54. AbdAlmageed W, Mirzaalian H, Guo X, Randolph LM, Tanawattanacharoen VK, Geffner ME, et al. Assessment of Facial Morphologic Features in Patients With Congenital Adrenal Hyperplasia Using Deep Learning. *JAMA Netw Open* (2020) 3(11):e2022199. doi: 10.1001/jamanetworkopen.2020.22199
55. Alpañés M, Luque-Ramírez M, Martínez-García M, Fernández-Durán E, Álvarez-Blasco F, Escobar-Morreale HF. Influence of Adrenal Hyperandrogenism on the Clinical and Metabolic Phenotype of Women With Polycystic Ovary Syndrome. *Fertil Steril* (2015) 103(3):795–801.e792. doi: 10.1016/j.fertnstert.2014.12.105
56. Azziz R. Polycystic Ovary Syndrome. *Obstet Gynecol* (2018) 132(2):321–36. doi: 10.1097/AOG.0000000000002698
57. Lo JC, Feigenbaum SL, Yang J, Pressman AR, Selby JV, Go AS. Epidemiology and Adverse Cardiovascular Risk Profile of Diagnosed Polycystic Ovary Syndrome. *J Clin Endocrinol Metab* (2006) 91(4):1357–63. doi: 10.1210/jc.2005-2430
58. Orio F, Muscogiuri G, Nese C, Palomba S, Savastano S, Tafuri D, et al. Obesity, Type 2 Diabetes Mellitus and Cardiovascular Disease Risk: An Uptodate in the Management of Polycystic Ovary Syndrome. *Eur J Obstet Gynecol Reprod Biol* (2016) 207:214–9. doi: 10.1016/j.ejogrb.2016.08.026
59. Maraka S, Singh Ospina N, Rodriguez-Gutierrez R, Davidge-Pitts CJ, Nippoldt TB, Prokop LJ, et al. Sex Steroids and Cardiovascular Outcomes in Transgender Individuals: A Systematic Review and Meta-Analysis. *J Clin Endocrinol Metab* (2017) 102(11):3914–23. doi: 10.1210/jc.2017-01643
60. Stone T, Stachenfeld NS. Pathophysiological Effects of Androgens on the Female Vascular System. *Biol Sex Differ* (2020) 11(1):45. doi: 10.1186/s13293-020-00323-6
61. Velho I, Fighera TM, Ziegelmann PK, Spritzer PM. Effects of Testosterone Therapy on BMI, Blood Pressure, and Laboratory Profile of Transgender Men: A Systematic Review. *Andrology* (2017) 5(5):881–8. doi: 10.1111/andr.12382
62. Liu PY, Death AK, Handelsman DJ. Androgens and Cardiovascular Disease. *Endocr Rev* (2003) 24(3):313–40. doi: 10.1210/er.2003-0005

63. Kim MS, Ryabets-Lienhard A, Bali B, Lane CJ, Park AH, Hall S, et al. Decreased Adrenomedullary Function in Infants With Classical Congenital Adrenal Hyperplasia. *J Clin Endocrinol Metab* (2014) 99(8):E1597–601. doi: 10.1210/jc.2014-1274
64. Merke DP, Chrousos GP, Eisenhofer G, Weise M, Keil MF, Rogol AD, et al. Adrenomedullary Dysplasia and Hypofunction in Patients With Classic 21-Hydroxylase Deficiency. *N Engl J Med* (2000) 343(19):1362–8. doi: 10.1056/NEJM200011093431903
65. Weise M, Mehlinger SL, Drinkard B, Rawson E, Charmandari E, Hiroi M, et al. Patients With Classic Congenital Adrenal Hyperplasia Have Decreased Epinephrine Reserve and Defective Glucose Elevation in Response to High-Intensity Exercise. *J Clin Endocrinol Metab* (2004) 89(2):591–7. doi: 10.1210/jc.2003-030634
66. Reimann M, Qin N, Gruber M, Bornstein SR, Kirschbaum C, Ziemssen T, et al. Adrenal Medullary Dysfunction as a Feature of Obesity. *Int J Obes* (2017) 41(5):714–21. doi: 10.1038/ijo.2017.36
67. Speiser PW, Arlt W, Auchus RJ, Baskin LS, Conway GS, Merke DP, et al. Congenital Adrenal Hyperplasia Due to Steroid 21-Hydroxylase Deficiency: An Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab* (2018) 103(11):4043–88. doi: 10.1210/jc.2018-01865

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Molecular Diagnosis of Steroid 21-Hydroxylase Deficiency: A Practical Approach

María Arriba[1,2*†] *and Begoña Ezquieta*[1,2*]

[1] *Molecular Diagnostics Laboratory, Department of Laboratory Medicine, Hospital General Universitario Gregorio Marañón, Madrid, Spain,* [2] *Gregorio Marañón Health Research Institute (IiSGM), Madrid, Spain*

Adrenal insufficiency in paediatric patients is mostly due to congenital adrenal hyperplasia (CAH), a severe monogenic disease caused by steroid 21-hydroxylase deficiency (21-OHD, encoded by the *CYP21A2* gene) in 95% of cases. *CYP21A2* genotyping requires careful analyses that guaranty gene-specific PCR, accurate definition of pseudogene-gene chimeras, gene duplications and allele dropout avoidance. A small panel of well-established disease-causing alterations enables a high diagnostic yield in confirming/discarding the disorder not only in symptomatic patients but also in those asymptomatic with borderline/positive results of 17-hydroxyprogesterone. Unfortunately, the complexity of this locus makes it today reluctant to high throughput techniques of massive sequencing. The strong relationship existing between the molecular alterations and the degree of enzymatic deficiency has allowed genetic studies to demonstrate its usefulness in predicting/classifying the clinical form of the disease. Other aspects of interest regarding molecular studies include its independence of physiological variations and analytical interferences, its usefulness in the diagnosis of simple virilizing forms in males and its inherent contribution to the genetic counseling, an aspect of great importance taking into account the high carrier frequency of CAH in the general population. Genetic testing of *CYP21A2* constitutes an irreplaceable tool to detect severe alleles not just in family members of classical forms but also in mild late-onset forms of the disease and couples. It is also helpful in areas such as assisted reproduction and preimplantation diagnosis. Molecular diagnosis of 21-OHD under expert knowledge definitely contributes to a better management of the disease in every step of the clinical course.

Keywords: ccongenital adrenal hyperplasia (CAH), 21-hydroxylase deficiency, *CYP21A2* gene, classical forms of congenital adrenal hyperplasia, non-classical forms of congenital adrenal hyperplasia, molecular diagnosis

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***Correspondence:**
Begoña Ezquieta
begona.ezquieta@salud.madrid.org
María Arriba
maria.arriba@salud.madrid.org

†ORCID:
María Arriba
orcid.org/0000-0001-6516-643X

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1 INTRODUCTION

Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency (21-OHD)(OMIM #201910) is an inherited autosomal recessive disorder responsible of 95% of CAH cases (1, 2). It has its origin in a defect of steroid 21-hydroxylase (21-OH), an enzyme encoded by the *CYP21A2* gene. Alterations in *CYP21A2* cause an impairment of the enzymatic activity and leads to the accumulation of 17-hydroxyprogesterone (17-OHP), which is diverted towards formation of

androgens (1, 3). As an actionable, non-infrequent and life-threatening disease, CAH is included in the neonatal screening of several countries (4).

Although 17-OHP is the metabolic marker of the deficiency, *CYP21A2* genotyping contributes as a diagnostic tool due to its independence on physiology and its strong relationship with clinical severity (4). The high carrier frequency (5, 6) and the recurrent impaired fertility in patients (7–9) further evidence the important contribution that genotyping does. Molecular studies provide valuable information in prevention and contribute to a better management of the disease (10, 11).

CAH is related to a wide range of clinical behaviors, with phenotypes varying from severe classical forms (CLF) to moderate late-onset non-classical forms (NCF). As a highly penetrant monogenic disease, 21-OHD shows a strong, although not complete, genotype-phenotype relationship in which the clinical features correspond to the less severely impaired allele (1, 12, 13). Variants causing null or minimal enzymatic activity in both alleles result in salt-wasting forms (SW), whereas their compound heterozygosity with variants causing residual activity result in simply virilizing forms (SV). NCF are due to mild alterations in homozygosity or a compound heterozygosity of either two mild alterations or a severe and a mild one (1, 3, 12, 14–16) (**Supplementary Tables 1**, **2**). Some lacks of genotype-phenotype relationship may result from extraadrenal 21-hydroxylation mediated by liver P450 cytochromes (17).

2 GENE LOCUS STRUCTURE AND NATURE OF *CYP21A2* ALTERATIONS

CYP21A2 is arranged in tandem with its inactive pseudogene (*CYP21A2P*) within a genetic unit designated as RCCX module, where also the genes *TNXA/B*, *C4A/B* and *RP* are harbored (18). Most chromosomes have two RCCX modules, although mono-, tri- or even quadrimodular arrangements have been described (19, 20). The high homology existing between gene and pseudogene (98% in coding and 96% in non-coding regions) together with that existing between RCCX modules favor unequal cross-overs during meiosis making that most pathological alleles in CAH arise from mechanisms of asymmetric recombination (25-30%) and gene conversion events (70%). Consequently, *CYP21A2* genotyping requires careful analyses that guaranty gene-specific PCR with allele dropout avoidance, and accurate definition of pseudogene-gene chimeras and gene duplications. Of course, an expert interpretation of the results is needed.

2.1 Alterations Due to Intrinsic Locus-Derived Mechanisms

2.1.1 Point Pathological Variants: Microconversions

Around 70% of the disease causing alterations in CAH are pseudogene-deleterious-variants that have been transferred to the gene by small gene conversion events. As a result, a limited group of pathogenic variants with well-known phenotypic effects is present in all populations (3) (**Figure 1**).

2.1.2 Gene Chimeras

Asymmetric recombination between *CYP21A2* and *CYP21A2P* is responsible of about 25-30% of all deficient alleles (3, 22, 23). This mechanism results in the appearance of pseudogene-gene chimeras (traditionally named "gene deletions") usually extending from somewhere between exons 3 and 8 of *CYP21A2P* to the corresponding point in *CYP21A2*, yielding a non-functional gene in which the 5'-end corresponds to *CYP21A2P* and the 3'-end corresponds to *CYP21A2*. It is important to mention a subset of patients in which the deletion is extended into the *TNXB* gene resulting in a contiguous gene syndrome named CAH-X consisting in CAH and Ehlers-Danlos Syndrome (24) that should also be investigated (25).

Chimeras are usually categorized into classic and attenuated depending on the location of the junction site, having been reported nine different types (26). Classic types contain the c.293-13C>G region and produce non-functional alleles whereas attenuated ones have the junction upstream of that region and associate a less severe phenotype (26) (see *Avoidable Pitfalls Upon Complementary Characterization of Alleles*).

2.1.3 *De Novo* Alterations

De novo alterations (1-2% of all 21OH-deficient alleles) are usually derived from gene recombination processes (27–30), being therefore detectable in the basic screening of recurrent variants.

2.2 Alterations Due to Conventional Mechanisms

Alterations other than those derived from recombinant events are less frequent and usually involve functional residues, generate frameshifts or stop codons (16, 21, 30, 31). The number of splicing pathological variants described so far is small (30), with a new candidate recently reported (32). Alterations in regulatory regions are controversial and difficult to demonstrate but tend to be mild changes. To date, more than 200 different pathogenic variants in *CYP21A2* have been described (16, 30, 31).

3 DISEASE FREQUENCY AND ORIGIN OF *CYP21A2* ALTERATIONS

CAH constitutes a non-infrequent disease, even in its severe neonatal forms. This seems to be the result of the prolific molecular mechanisms previously mentioned, although a founder effect has also been proposed (33–37). Regarding this latter, some studies have documented a lower mortality in *CYP21A2* carriers mainly due to a decreased number of infections in these individuals (38).

Considering that *de novo* variants in *CYP21A2* are infrequent (27, 29, 30) and that alterations are maintained through generations once originated (33, 39), it is not uncommon that the presence of new/rare pathological variants be the result of the dissemination of single original alleles.

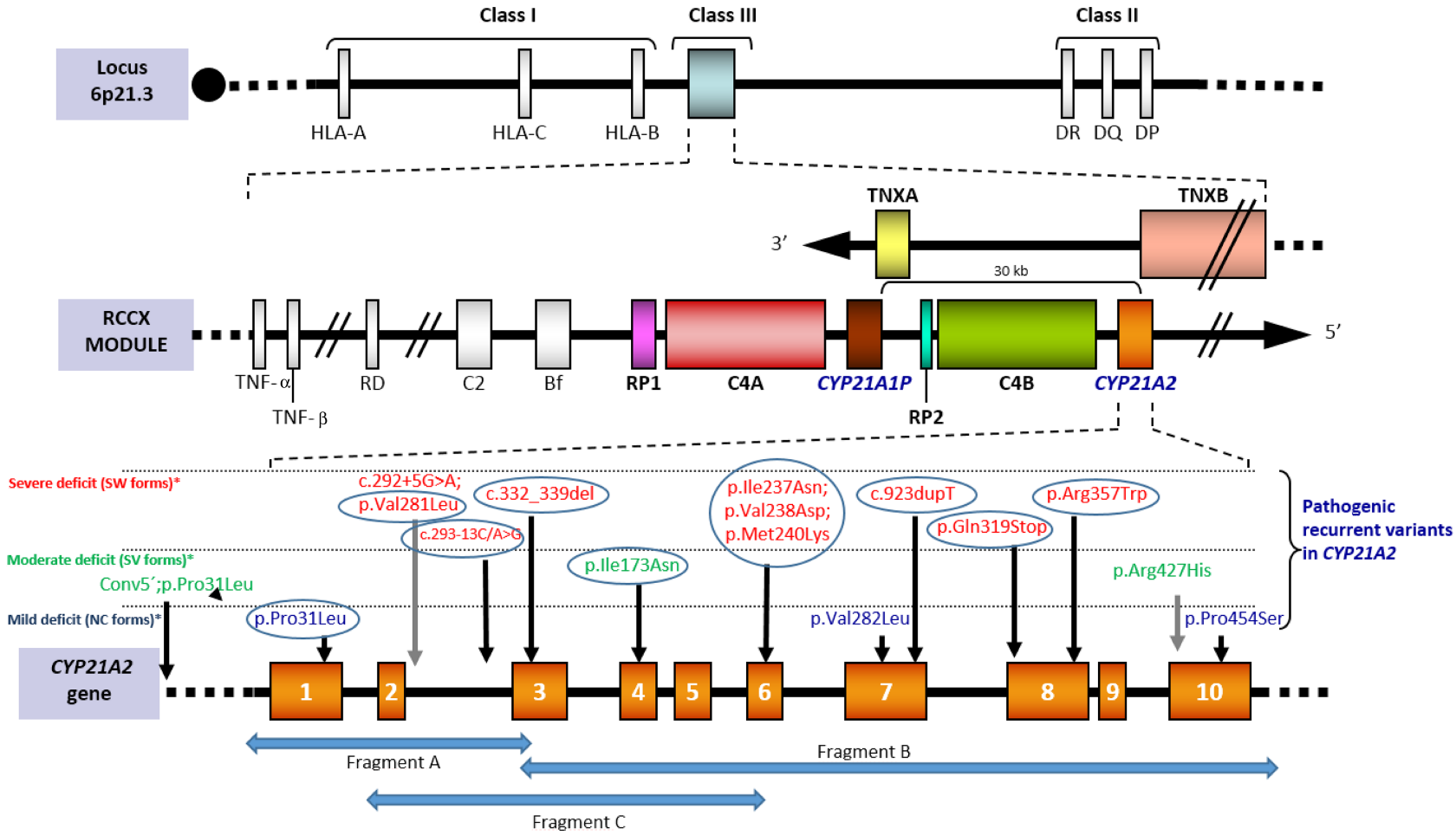

FIGURE 1 | [Adapted from Santomé et al., (21)]. Scheme of the RCCX module located on the short arm of chromosome 6 within the HLA class III region. Tandem duplication affects *CYP21* and C4 genes. In humans only *CYP21A2* gives rise to the functional protein, whereas *CYP21P* is a homologous pseudogene that includes several inactivating point variants that can be transferred to the active gene by small gene conversion events. Both C4A and C4B are functional. Tenascin, also duplicated, is encoded in the complementary chain. The bottom of the image shows the recurrent variants grouped according to how they affect the enzimatic functionality: severely (red), moderately severe (green) or mildly (blue). Recurrent variants in all populations are circled. The complete nomenclature of each variant including the cDNA position (NM_000500.9) would be: c.92C>T [p.Pro31Leu], c.292+5C>A, c.293-13C>G, c.332-339del, c.518T>A [p.Ile173Asn], c.(710T>A; 713T>A; 719T>A) p.[Ile237Asn; Val238Glu; Met240Lys], c.844G>T [p.Val282Leu], c.923dupT, c.955C>T [p.Gln319*], c.1069C>T [p.Arg357Trp], c.1280G>A [p.Arg427His] and c.1360C>T [p.Pro454Ser]). The arrows "Fragment A", "Fragment B" and "Fragment C" represents specific amplicons for *CYP21A2* amplification.

4 GENETIC DIAGNOSIS OF CAH

Since CAH due to 21-OHD accounts for 95% of all CAH cases, *CYP21A2* should be the first gene to investigate in males and virilized girls with adrenal insufficiency. The remaining genes causing CAH (1, 2, 12, 40) as well as other involved in adrenal insufficiency (41) should be investigated using high-throughput approaches (massive sequencing gene panels) (40–42). On regard *CYP11B1*, it is important to highlight its high homology with *CYP11B2* and the consequent existence of hybrid genes (43–45).

5 *CYP21A2* GENOTYPING

Traditional approaches for *CYP21A2* genotyping usually include methods such as capillary sequencing, allele-specific oligonucleotide hybridization, SNaPshot minisequencing and MLPA, which are labor intensive and have limited multiplexing capability, but which keep being used given their proven clinical usefulness and the difficulty of optimizing the current massive sequencing technologies to this complex locus. Conventional massive platforms are poorly equipped to characterize gene-pseudogene pairs and have the limitation of being based on PCR-amplifications and uniquely aligning short reads (that may not include *CYP21A2* gene-specific regions). As a consequence, they are not still the first-choice option for *CYP21A2* genotyping although some promising results have been obtained (31, 46–49). Third-generation platforms based on direct sequencing of long DNA strands without previous amplification seem promising tools (50–52).

5.1 Detection of Point Pathological Variants: Gene-Specific PCR

Current strategies for the specific amplification of *CYP21A2* rely on regions that are known to be different from those of the pseudogene, either as targets for restriction sites prior to PCR or PCR-specific primers. Since one of these latter regions is located on exon 3 (where the variant c.332-339del is located in *CYP21A2P*), an extensively used scheme for the specific amplification of *CYP21A2* is obtaining two fragments (one from 5'UTR to exon 3 and another from exon 3 to 3'UTR). However, alleles carrying the variant c.332-339del as a single microconversion would not be detected in this way (neither chimeras, conversions or gene duplications including it), so a third fragment in which the 3'-end is located on the specific site on exon 6 (where the cluster of three variants is harbored in *CYP21A2P*) can be incorporated (34, 39, 53–56) (**Figure 1**). This last overlapping fragment allows the PCR-detection of pseudogene-gene chimeras with the breaking point before exon 6.

Recurrent variants (**Figure 1**) may be investigated in a first screening performed by allele-specific oligonucleotide hybridization or SNaPshot minisequencing, although they and other point variants are detected with Sanger sequencing on these gene-specific fragments. Whole gene sequencing must guarantee an accurate interpretation based on well-documented alterations due to the lack of complete knowledge regarding the impact of every variant in this small but polymorphic gene. *In vitro* analyses (57–59) and/or models investigation (60–62) should support the involvement of new variants, but only clinical validation in different populations and genotypes will confirm their causality.

Segregation of alterations in parental samples is an important issue since gene chimeras and large or double micro-conversions include several alterations within the same allele (carrier status), a very different situation from that in which alterations are located in different alleles (affected patient). Approximately 5-7% of affected alleles carrying two or more point alterations (63). Patients carrying gene chimeras/conversions that include the specific regions used in PCR protocols result in hemizygosity and directly stablish the segregation, although not the carrier status of progenitors (see *Family Studies*).

5.2 Analysis of Gene Chimeras: MLPA

MLPA allows to identify gene deletions/conversions avoiding the inconvenients linked to Southern blotting. Nevertheless, since it also has unavoidable limitations [reduction of signal when alterations/polymorphisms exist in a probe-binding region (64), inability to detect the *cis*/*trans* disposition of the alterations, or lack of probes addressed to some frequent variants], must be always complemented with other analyses. Unfortunately, a comprehensive revision defining every MLPA pattern and its deduced genotype is still lacking, although some studies are contributing to a better definition of this issue (65). MLPA should also be applied in the complementary characterization of some complex alleles (see *Avoidable Pitfalls Upon Complementary Characterization of Alleles*).

5.3 Avoidable Pitfalls Upon Complementary Characterization of Alleles

Some of the seemingly lacks of genotype-phenotype relationship in several frequent point variants are not further sustained when alleles are better characterized. An efficient multistep approach (64) allows a comprehensive mutation analysis. Apparently mild alleles which are not really such are those carrying the variant c.92C>T [p.Pro31Leu] with a *cis* pseudogene-conversion in 5' (26, 56, 66, 67), and those carrying the variant c.844G>T [p.Val282Leu] in *cis* with the intronic change c.292+5G>A, an alteration observed in SW from Mediterranean populations (15, 68) (**Figure 1** and **Supplementary Table 1**).

Examples of "severe" alleles that are not really such are those with the variant c.955C>T [p.Gln319*] and two copies of the gene, present in several populations (6, 30, 69) (**Supplementary Table 1**). Fortunately, since the whole gene is involved in these alleles, MLPA allows its detection in spite of the salsa MLPA Probemix P050-C1 CAH (MRC Holland) no longer includes exon 8 probes. It is important to mention that some of these alleles carry additional alterations [e.g. c.518T>A (p.Ile173Asn) or the combination of c.293-13 C>G and c.332_339del (6, 70)] and are severe. Some gene conversions involving exons 4 to 8 are not such deficient-alleles. Pseudogenes including the gene-specific region in exon 3, although infrequent (71), result in an identical pattern upon PCR amplification, so these conversions should be investigated with a complementary MLPA analysis.

Discrimination of homo/hemizygosity of mild variants is crucial in NCF (72) as hemizygosity requires genetic counseling. Also essential is to guarantee the efficient amplification of both alleles in order to avoid incorrect interpretations such as false homozygotes due to allele dropout of the normal allele (73). A complementary indirect analysis also provides useful information preventing serious mistakes in prenatal samples (see *Usefulness of an Indirect Analysis*).

6 USEFULNESS OF AN INDIRECT ANALYSIS

Indirect analyses performed by either microsatellite typing or SNPs (6, 74–76) are a useful tool (see *Contribution of CYP21A2 Genotyping*) since informative polymorphic markers on both sides of the gene in each family configure distinct haplotypes in normal and affected chromosomes. They are helpful with prenatal samples, in preimplantation studies and in allele segregation, being able to reveal/discard consanguinity in patients carrying rare variants in homozygosity [useful as a complement of a basic/first study in patients with borderline/false positive results in the neonatal screening (see *Neonatal Screening*)]. Also in epidemiology, since the same haplotype for a new variant in unrelated patients suggests the variant dissemination and the potential interest of its inclusion in the basic screening of that population. Some informative microsatellite loci flanking the *CYP21A2* gene are D6S2792-D6S273 and D6S1014- D6S439 together with two intronic ones in genes *TNF* and *TAP1* (34, 63, 74, 77, 78).

7 CONTRIBUTION OF *CYP21A2* GENOTYPING

7.1 Neonatal Clinical Suspicion

Although clinical manifestations such as adrenal insufficiency or virilization in girls perform the suspicion in the neonatal period, there are unspecific signs (e.g. hypoglycemia, clitoromegaly or genital hyperpigmentation) frequent in combination with 17-OHP elevations (4, 79). Genotyping of *CYP21A2* allows to confirm/discard the disease in both scenarios (80) especially when analytical interferences in the direct immunoassay exist (81, 82). Comprehensive *CYP21A2* genotyping should be guaranteed paying special attention to variants with a significance still poorly established. Failure to detect well-stablished pathogenic variants in *CYP21A2* must prompt further studies.

7.2 Neonatal Screening

Clinical guidelines recommend a second-tier analysis by liquid chromatography–tandem mass spectrometry to improve the positive predictive value of CAH screening (4). Neonates with borderline/high levels of 17-OHP in these programs can take benefit from *CYP21A2* analyses (80, 83–86). Not only CLF, but also neonatal cryptic forms (NCF and SV in males) are detected at this stage, being molecular studies able to correctly classify them (11, 80, 86, 87) by a firstly analysis focused on the identification of recurrent variants (in order to eliminate uncertainty) followed by Sanger sequencing when just one deficient-allele or microsatellite-homozygosity is detected (80).

7.3 Non-Classical Forms

The high recurrence of c.844G>T [p.Val282Leu] in some populations (88, 89) helps to "unmask" severe alleles through the clinical expressiveness of NCF [70% carrying severe alleles (4); 41% in paediatric patients, **Supplementary Table 1**]. *CYP21A2* should be always considered in NCF to allow a proper genetic counseling.

Levels of 17-OHP, either basal or post-ACTH, constitute the most sensitive parameter to define a NCF since *CYP21A2* mild alterations are not fully characterized. A proper threshold for 17-OHP values is difficult to define since some carriers are prone to present a hyperandrogenism similar to that shown in NCF (56, 85, 90–92). Genotyped carriers inside fully characterized segregated families are useful to achieve this goal (56, 91). Compound heterozygosity with severe alleles in NCF may be suspected based on 17-OHP levels (**Figure 2A** and **Supplementary Table 3**) (56, 63) conversely to what happen with carriers of severe *vs.* mild variants.

Monogenic and polygenic models in paediatric hyperandrogenism due to 21-OHD have been detected (91) (**Figure 2B**), being carriers (monoallelic) with hyperandrogenism the counterpart of cryptic forms (biallelic alterations) without clinical expression. Considering the important contribution of the "back door" pathway to circulating levels of the potent androgen 11-ketotestosterone in CAH (93, 94), investigation of gene variants coding for the enzymes involved seems interesting.

7.4 Carrier Detection

The biochemical marker 21-deoxicortisol detects carriers (10, 40, 95), although only molecular analyses are able to discriminate carriers of severe alleles. Individuals with hyperandrogenism and moderately elevated post-ACTH 17-OHP levels (not reaching the NCF threshold) may take benefit from *CYP21A2* analyses since alterations are more frequent in these patients (91) (**Supplementary Table 2**).

7.5 Genetic Counseling

The high carrier frequency of severe variants in general population (about 1:60) (15, 96, 97) (**Supplementary Table 1**, false severe alleles) makes reasonable to refine the risk of having an affected child by genotyping *CYP21A2* in couples where one member is affected/carrier. Individuals with CLF present a risk of 1:120 of having a newborn affected with a CLF. The theoretical risk is lower in NCF [1:250 (4)] although some studies have documented to be higher (1.5-2.5%) (98).

7.6 Family Studies

Family studies are necessary to ascertain parental genotype and segregation of the pathological alleles among the offspring. They are initially addressed to detect/discard alterations documented in the index case, but the high carrier frequency justifies the

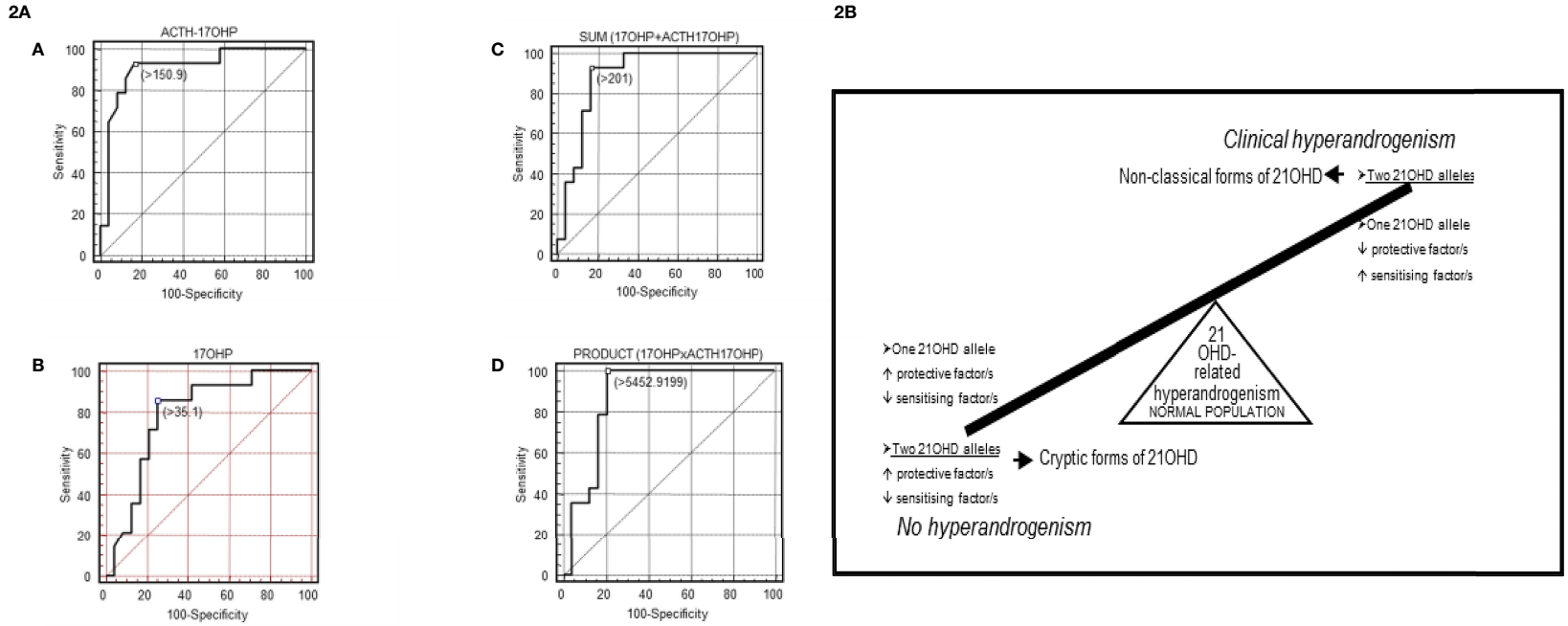

FIGURE 2 | **(2A)** [Taken from Ezquieta et al., (56)]: Receiver operating characteristic (ROC) curves analyses in fully genotyped children affected with NCF of 21-OHD (mild/mild *vs* severe/mild genotype) for: **(A)** adrenocorticotropic hormone (ACTH)-stimulated 17-OHP, **(B)** basal 17-OHP, or **(C, D)** the combination of both parameters [**(C)**: sum; **(D)**: product]. Areas under the curves (SE): ACTH-stimulated 17-OHP, 0.908 (0.057); basal 17-OHP, 0.790 (0.081); sum 0.866 (0.068); product 0.884 (0.064). Cut-off values, nmol/L **(A–C)** and $nmol^2/L^2$ **(D)**. The cut-offs for maximum predictive values are represented by small, empty squares in the Figures. **(2B)** [From Ezquieta et al., (91)] Diagram of a hypothetical interaction between protective and sensitizing factors modulating the clinical expressivity of 21-OHD-related hyperandrogenism. CAPN10-UCSNP44C and TNFR2-R196 are proposed in this study to be sensitizing and protective factors, respectively.

subsequent screening of frequent pathological variants to discard its coexistence in the family. Progenitors must not be considered obligate carriers since *de novo* variants are detected in 1-2% of deficient alleles (27, 29, 30).

7.7 Assisted Reproductive Techniques and Genetic Counseling

CAH due to 21-OHD should be considered in reproductive assistance and genetic counseling (7, 99, 100) due to the associated infertility (7–9, 40) and the high frequency of carriers in general population. The strong genotype-phenotype relationship (13, 101) facilitates counseling in couples even in absence of an index case, but it should not be forgotten that expressivity vary particularly in moderately-severe forms (13).

7.8 Prenatal Diagnosis

Prenatal studies are normally performed inside CLF-families. It is still accomplished on samples from corionic villus through direct analysis addressed to investigate those alterations detected in the index case. An additional indirect analysis (6, 15, 74) provides the possibility of detecting maternal contamination and avoids eventual allele dropout artefacts.

Prenatal treatment prevents virilization in girls affected with CAH but is still considered experimental (4). Prenatal diagnosis establishes treatment withdraw in non-affected foetus (carriers and non-carriers). Protocols must include screening for Y-chromosomal DNA in maternal blood (4) to minimize (40) treatment in males. Since prenatal treatment is only effective if established at 6^{th}-7^{th} weeks (4, 102), it is unfeasible totally avoid treatment in males since cfDNA analyses must be performed in samples with a foetal fraction about 3.5-4% (9^{th}-10^{th} week).

CYP21A2 genotyping from cfDNA in maternal blood is a promising approach not suitable in clinical settings yet (4, 102). For its application, massive sequencing based on an indirect analyses conducted by SNP-haplotypes previously defined in parents and index case is necessary due to the coexistence of foetal and maternal DNA in the same sample (76).

7.9 Preimplantation Genetic Diagnosis

This particular approach enables to study the embryo before the transference to the uterus. These tests are mentioned in the last Clinical Practice Guidelines from the Endocrine Society although subjected to their own risk and ethical controversies (4).

Microsatellite typing is the most appropriate approach, since the paucity of sample hampers a direct gene analysis and haplotypes detected in the directly genotyped index case provide the information.

7.10 Other Prenatal Scenarios

Suspicion of CAH due to anomalies detected by fetal ultrasound or genetic counseling for a couple at risk (not previously genotyped) with an ongoing pregnancy are prenatal situations in which *CYP21A2* genotyping are requested. When the index case is unknown, the only suitable approach is the direct analysis. Only well-documented pathogenic alterations should be considered.

8 CONCLUSIONS

CYP21A2 genotyping favorably contributes to confirm/discard CLF after neonatal or prenatal suspicion. The high frequency of carriers in general population and the infertility associated to the disease turn molecular diagnosis into an irreplaceable tool to detect/discriminate severe alleles in family members and in genetic counseling, as well as in specific areas as assisted reproduction and preimplantational diagnosis. The high complexity of the locus makes essential the performance of *CYP21A2* genotyping under supervision of expert personnel in the field. There is no doubt that molecular diagnosis of 21-OHD definitely contributes to a better management of the disease in every step of the clinical course.

AUTHOR CONTRIBUTIONS

Conception and design: MA and BE. Manuscript writing: MA and BE. Manuscript revision: MA and BE. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.834549/full#supplementary-material

REFERENCES

1. El-Maouche D, Arlt W, Merke DP. Congenital Adrenal Hyperplasia. *Lancet* (2017) 390:2194–210. doi: 10.1016/S0140-6736(17)31431-9
2. Miller WL. Mechanisms in Endocrinology: Rare Defects in Adrenal Steroidogenesis. *Rev Eur J Endocrinol* (2018) 179(3):R125–41. doi: 10.1530/EJE-18-0279
3. White PC, Speiser PW. Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency. *Endocr Rev* (2000) 21:245–91. doi: 10.1210/edrv.21.3.0398
4. Speiser PW, Arlt W, Auchus RJ, Baskin LS, Conway GS, Merke DP, et al. Congenital Adrenal Hyperplasia Due to Steroid 21-Hydroxylase Deficiency: An Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab* (2018) 103:4043–88. doi: 10.1210/jc.2018-01865
5. Baumgartner-Parzer SM, Nowotny P, Heinze G, Waldhäusl W, Vierhapper H. Carrier Frequency of Congenital Adrenal Hyperplasia (21-Hydroxylase Deficiency) in a Middle European Population. *J Clin Endocrinol Metab* (2005) 90:775–8. doi: 10.1210/jc.2004-1728
6. Ezquieta B, Beneyto M, Muñoz-Pacheco R, Barrio R, Oyarzabal M, Lechuga JL, et al. Gene Duplications in 21-Hydroxylase Deficiency: The Importance of Accurate Molecular Diagnosis in Carrier Detection and Prenatal Diagnosis. *Prenat Diagn* (2006) 26(12):1172–8. doi: 10.1002/pd.1584
7. Ezquieta B, Alonso M, Álvarez E, Arnao DR, Rodríguez A, Siguero JP, et al. Should 21-Hydroxylase Deficiency Genotyping be Considered in Assisted Reproductive Technology Programs? *Fertil Steril* (2007) 88(5):1437.e5–11. doi: 10.1016/j.fertnstert.2007.01.030
8. Reichman DE, White PC, New MI, Rosenwaks Z. Fertility in Patients With Congenital Adrenal Hyperplasia. *Fertil Steril* (2014) 101(2):301–9.

doi: 10.1016/j.fertnstert.2013.11.002. Fertility in patients with congenital adrenal hyperplasia.
9. Carmina E, Dewailly D, Escobar-Morreale HF, Kelestimur F, Moran C, Oberfield S, et al. Non-Classic Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency Revisited: An Update With a Special Focus on Adolescent and Adult Women. *Hum Reprod Update* (2017) 23(5):580–99. doi: 10.1093/humupd/dmx014
10. Forest MG, Tardy V, Nicolino M, David M, Morel Y. 21-Hydroxylase Deficiency: An Exemplary Model of the Contribution of Molecular Biology in the Understanding and Management of the Disease. *Ann Endocrinol (Paris)* (2005) 66(3):225–32. doi: 10.1016/s0003-4266(05)81754-8
11. Lajic S, Karlsson L, Zetterström RH, Falhammar H, Nordenström A. The Success of a Screening Program Is Largely Dependent on Close Collaboration Between the Laboratory and the Clinical Follow-Up of the Patients. *Int J Neonatal Screen* (2020) 6(3):68. doi: 10.3390/ijns6030068
12. Krone N, Arlt W. Genetics of Congenital Adrenal Hyperplasia. *Best Pract Res Clin Endocrinol Metab* (2009) 23(2):181–92. doi: 10.1016/j.beem.2008.10.014
13. New MI, Abraham M, Gonzalez B, Dumic M, Razzaghy-Azar M, Chitayat D, et al. Genotype-Phenotype Correlation in 1,507 Families With Congenital Adrenal Hyperplasia Owing to 21-Hydroxylase Deficiency. *Proc Natl Acad Sci USA* (2013) 110(7):2611–6. doi: 10.1073/pnas.1300057110
14. Speiser PW, Azziz R, Baskin LS, Ghizzoni L, Hensle TW, Merke DP, et al. Congenital Adrenal Hyperplasia Due to Steroid 21-Hydroxylase Deficiency: An Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab* (2010) 95:4133–60. doi: 10.1210/jc.2009-2631
15. Ezquieta B, Santomé L, Barrio R, Barrionuevo JL, López-Siguero JP, Oliver A, et al. Carrier Detection and Prenatal Diagnosis of Congenital Adrenal Hyperplasia Must Identify 'Apparently Mild' CYP21A2 Alleles Which Associate Neonatal Salt-Wasting Disease. *Prenat Diagn* (2010) 30(8):758–63. doi: 10.1002/pd.2537
16. Concolino P, Costella A. Congenital Adrenal Hyperplasia (CAH) Due to 21-Hydroxylase Deficiency: A Comprehensive Focus on 233 Pathogenic Variants of CYP21A2 Gene. *Rev Mol Diagn Ther* (2018) 22(3):261–80. doi: 10.1007/s40291-018-0319-y
17. Gomes LG, Huang N, Agrawal V, Mendonça BB, Bachega TA, Miller WL. Extraadrenal 21-Hydroxylation by CYP2C19 and CYP3A4: Effect on 21-Hydroxylase Deficiency. *J Clin Endocrinol Metab* (2009) 94(1):89–95. doi: 10.1210/jc.2008-1174
18. Yang Z, Mendoza AR, Welch TR, Zipf WB, Yu CY. Modular Variations of HLA Class III Genes for Serine/Threonine Kinase RP, Complement C4, Steroid 21-Hydroxylase CYP21 and Tenascin TNX (RCCX). A Mechanism for Gene Deletions and Disease Associations. *J Biol Chem* (1999) 274:12147–56. doi: 10.1074/jbc.274.17.12147
19. Chung EK, Yang Y, Rennebohm RM, Lokki ML, Higgins GC, Jones KN, et al. Genetic Sophistication of Human Complement Components C4A and C4B and RP-C4-CYP21-TNX (RCCX) Modules in the Major Histocompatibility Complex. *Am J Hum Genet* (2002) 71(4):823–37. doi: 10.1086/342777
20. Sweeten TL, Odell DW, Odell JD, Torres AR. C4B Null Alleles are Not Associated With Genetic Polymorphisms in the Adjacent Gene CYP21A2 in Autism. *BMC Med Genet* (2008) 9:1. doi: 10.1186/1471-2350-9-1
21. Santomé JL, Cirujano A, Ferreiro B, Casado C, Muñoz-Pacheco R, Ezquieta B. Simple Virilizing Forms of Congenital Adrenal Hyperplasia: Adaptation and Prospective Validation of the Molecular Screening [Article in Spanish]. *Med Clin (Barc)* (2010) 135(5):195–201. doi: 10.1016/j.medcli.2009.11.039
22. Concolino P, Mello E, Minucci A, Giardina E, Zuppi C, Toscano V, et al. A New CYP21A1P/CYP21A2 Chimeric Gene Identified in an Italian Woman Suffering From Classical Congenital Adrenal Hyperplasia Form. *BMC Med Genet* (2009) 10:72. doi: 10.1186/1471-2350-10-72
23. Vrzalová Z, Hrubá Z, Hrabincová ES, Vrábelová S, Votava F, Kolouśková S, et al. Chimeric CYP21A1P/CYP21A2 Genes Identified in Czech Patients With Congenital Adrenal Hyperplasia. *Eur J Med Genet* (2011) 54(2):112–7. doi: 10.1016/j.ejmg.2010.10.005
24. Miller WL, Merke DP. Tenascin-X, Congenital Adrenal Hyperplasia, and the CAH-X Syndrome. *Horm Res Paediatr* (2018) 89(5):352–61. doi: 10.1159/000481911
25. Lao Q, Merke DP. Molecular Genetic Testing of Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency Should Include CAH-X Chimeras. *Eur J Hum Genet* (2021) 29(7):1047–8. doi: 10.1038/s41431-021-00870-5
26. Chen W, Xu Z, Sullivan A, Finkielstain GP, Van Ryzin C, Merke DP, et al. Junction Site Analysis of Chimeric CYP21A1P/CYP21A2 Genes in 21-Hydroxylase Deficiency. *Clin Chem* (2012) 58(2):421–30. doi: 10.1373/clinchem.2011.174037
27. Koppens PF, Hoogenboezem T, Degenhart HJ. Duplication of the CYP21A2 Gene Complicates Mutation Analysis of Steroid 21-Hydroxylase Deficiency: Characteristics of Three Unusual Haplotypes. *Hum Genet* (2002) 111(4-5):405–10. doi: 10.1007/s00439-002-0810-7
28. Díez I, Rodríguez A, González E, Martínez M, Rodríguez B, Ezquieta B. Virilizing Congenital Adrenogenital Syndrome With a *De Novo* I172N Mutation: Study of a New Case. *Case Rep Pediatr (Barc)* (2010) 72(1):72–8. doi: 10.1016/j.anpedi.2009.08.006
29. Lopes da Silva-Grecco R, de Paula D, Rodrigues C, Pontes P, da Cunha HM, Palandi-de-Mello M, et al. A *De Novo* Mutation in CYP21A2 Gene in a Case of *In Vitro* Fertilization. *Mol Genet Metab Rep* (2015) 5:98–102. doi: 10.1016/j.ymgmr.2015.10.011
30. 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(10):1341–67. doi: 10.1038/s41431-020-0653-5
31. Simonetti L, Bruque CD, Fernández CS, Benavides-Mori B, Delea M, Kolomenski JE, et al. CYP21A2 Mutation Update: Comprehensive Analysis of Databases and Published Genetic Variants. *Hum Mutat* (2018) 39(1):5–22. doi: 10.1002/humu.23351
32. Arriba M, Oriola J, Ezquieta B. A New Synonymous Variant Involving an mRNA Splicing Site in CYP21A2 Detected in 12 Unrelated Patients With Deficiency of 21-Hydroxylase. *Clin Genet* (2021) 100(5):634–6. doi: 10.1111/cge.14035
33. Levo A, Jääskeläinen J, Sistonen P, Sirén MK, Voutilainen R, Partanen J, et al. Tracing Past Population Migrations: Genealogy of Steroid 21-Hydroxylase (CYP21) Gene Mutations in Finland. *Eur J Hum Genet* (1999) 7(2):188–96. doi: 10.1038/sj.ejhg.5200262
34. Ezquieta B, Cueva E, Oyarzabal M, Oliver A, Varela JM, Jariego C, et al. Gene Conversion (655 Splicing Mutation) and the Founder Effect (Q318X) Contribute to the Most Frequent Severe Point Mutations in Congenital Adrenal Hyperplasia in the Spanish Population. *Clin Genet* (2002) 62:181–8. doi: 10.1034/j.1399-0004.2002.620213.x
35. Billerbeck AE, Mendonca BB, Pinto EM, Madureira G, Arnhold IJ, Bachega TA, et al. Three Novel Mutations in CYP21 Gene in Brazilian Patients With the Classical Form of 21-Hydroxylase Deficiency Due to a Founder Effect. *J Clin Endocrinol Metab* (2002) 87(9):4314–7. doi: 10.1210/jc.2001-011939
36. Kleinle S, Lang R, Fischer GF, Vierhapper H, Waldhauser F, Födinger M, et al. Duplications of the Functional CYP21A2 Gene Are Primarily Restricted to Q318X Alleles: Evidence for a Founder Effect. *J Clin Endocrinol Metab* (2009) 94(10):3954–8. doi: 10.1210/jc.2009-0487
37. Silveira EL, Elnecave RH, dos Santos EP, Moura V, Pinto EM, van der Linden I, et al. Molecular Analysis of CYP21A2 Can Optimize the Follow-Up of Positive Results in Newborn Screening for Congenital Adrenal Hyperplasia. *Clin Genet* (2009) 76(6):503–10. doi: 10.1111/j.1399-0004.2009.01274.x
38. Nordenström A, Svensson J, Lajic S, Frisén L, Nordenskjöld A, Norrby C, et al. Carriers of a Classic CYP21A2 Mutation Have Reduced Mortality: A Population-Based National Cohort Study. *J Clin Endocrinol Metab* (2019) 104:6148–54. doi: 10.1210/jc.2019-01199
39. Ezquieta B, Oyarzábal M, Jariego CM, Varela JM, Chueca M. A Novel Frameshift in the first Exon of the 21-OH Gene Found Inhomozygosity in an Apparently Nonconsanguineous Family. *Horm Res* (1999) 51:135–41. doi: 10.1159/000023346
40. Buonocore F, Maharaj A, Qamar Y, Koehler K, Suntharalingham JP, Chan LF, et al. Genetic Analysis of Pediatric Primary Adrenal Insufficiency of Unknown Etiology: 25 Years' Experience in the UK. *J Endocr Soc* (2021) 5(8):bvab086. doi: 10.1210/jendso/bvab086
41. Flück CE. MECHANISMS IN ENDOCRINOLOGY: Update on Pathogenesis of Primary Adrenal Insufficiency: Beyond Steroid Enzyme

Deficiency and Autoimmune Adrenal Destruction. *Eur J Endocrinol* (2017) 177(3):R99–111. doi: 10.1530/EJE-17-0128
42. Roucher-Boulez F, Mallet-Motak D, Tardy-Guidollet V, Menassa R, Goursaud C, Plotton I, et al. News About the Genetics of Congenital Primary Adrenal Insufficiency. *Ann Endocrinol (Paris)* (2018) 79(3):174–81. doi: 10.1016/j.ando.2018.03.016
43. Hampf M, Dao NT, Hoan NT. Bernhardt R.Unequal Crossing-Over Between Aldosterone Synthase and 11beta-Hydroxylase Genes Causes Congenital Adrenal Hyperplasia. *J Clin Endocrinol Metab* (2001) 86 (9):4445–52. doi: 10.1210/jcem.86.9.7820
44. Portrat S, Mulatero P, Curnow KM, Chaussain JL, Morel Y, Pascoe L. Deletion Hybrid Genes, Due to Unequal Crossing Over Between CYP11B1 (11beta-Hydroxylase) and CYP11B2(aldosterone Synthase) Cause Steroid 11beta-Hydroxylase Deficiency and Congenital Adrenal Hyperplasia. *J Clin Endocrinol Metab* (2001) 86(7):3197–201. doi: 10.1210/jcem.86.7.7671
45. Ezquieta B, Luzuriaga C. Neonatal Salt-Wasting and 11 Beta-Hydroxylase Deficiency in a Child Carrying a Homozygous Deletion Hybrid CYP11B2 (Aldosterone Synthase)-CYP11B1 (11 Beta-Hydroxylase). *Clin Genet* (2004) 66(3):229–35. doi: 10.1111/j.1399-0004.2004.00291.x
46. Turan I, Tastan M, Boga DD, Gurbuz F, Kotan LD, Tuli A, et al. 21-Hydroxylase Deficiency: Mutational Spectrum and Genotype-Phenotype Relations Analyses by Next-Generation Sequencing and Multiplex Ligation-Dependent Probe Amplification. *Eur J Med Genet* (2020) 63 (4):103782. doi: 10.1016/j.ejmg.2019.103782
47. Gangodkar P, Khadilkar V, Raghupathy P, Kumar R, Dayal AA, Dayal D, et al. Clinical Application of a Novel Next Generation Sequencing Assay for CYP21A2 Gene in 310 Cases of 21-Hydroxylase Congenital Adrenal Hyperplasia From India. *Endocrine* (2021) 71(1):189–98. doi: 10.1007/s12020-020-02494-z
48. Stephens Z, Milosevic D, Kipp B, Grebe S, Iyer RK, Kocher JP, et al. PB-Motif-A Method for Identifying Gene/Pseudogene Rearrangements With Long Reads: An Application to CYP21A2 Genotyping. *Front Genet* (2021) 12:716586. doi: 10.3389/fgene.2021.716586
49. Wang W, Han R, Yang Z, Zheng S, Li H, Wan Z, et al. Targeted Gene Panel Sequencing for Molecular Diagnosis of Congenital Adrenal Hyperplasia. *J Steroid Biochem Mol Biol* (2021) 211:105899. doi: 10.1016/j.jsbmb.2021.105899
50. Mohammadi MM, Bavi O. DNA Sequencing: An Overview of Solid-State and Biological Nanopore-Based Methods. *Biophys Rev* (2021) 14:99–110. doi: 10.1007/s12551-021-00857-y
51. Goto Y, Akahori R, Yanagi I, Takeda KI. Solid-State Nanopores Towards Single-Molecule DNA Sequencing. *J Hum Genet* (2020) 65(1):69–77. doi: 10.1038/s10038-019-0655-8
52. Girgis H, DuPai CD, Lund J, Reeder J, Guillory J, Durinck S, et al. Single-Molecule Nanopore Sequencing Reveals Extreme Target Copy Number Heterogeneity in Arylomycin-Resistant Mutants. *Proc Natl Acad Sci USA* (2021) 118(1):e2021958118. doi: 10.1073/pnas.2021958118
53. Owerbach D, Crawford YM, Draznin MB. Direct Analysis of CYP21B Genes in 21-Hydroxylase Deficiency Using Polymerase Chain Reaction Amplification. *Mol Endocrinol* (1990) 4(1):125–31. doi: 10.1210/mend-4-1-125
54. Ezquieta B, Oliver A, Gracia R, Gancedo PG. Analysis Ofsteroid 21-Hydroxylase Mutations in the Spanish Population. *HumGenet* (1995) 96:198–204. doi: 10.1007/BF00207379
55. Ezquieta B, Varela JM, Jariego C, Oliver A, Gracia R. Nonisotopic Detection of Point Mutations in CYP21B Gene in Steroid21-Hydroxylase Deficiency. *Clin Chem* (1996) 42:1108–10. doi: 10.1093/clinchem/42.7.1108
56. Ezquieta B, Cueva E, Varela J, Oliver A, Fernández J, Jariego C. Nonclassical 21-Hydroxylase Deficiency in Children: Association of ACTH-Stimulated 17OH Progesterone With Risk for Compound Heterozygosity for Severe Mutations. *Acta Paediatr* (2002) 91:892–8. doi: 10.1080/080352502760148595
57. Concolino P, Mello E, Patrosso MC, Penco S, Zuppi C, Capoluongo E, et al. P.H282N and P.Y191H: 2 Novel CYP21A2 Mutations in Italian Congenital Adrenal Hyperplasia Patients. *Metabolism* (2012) 61(4):519–24. doi: 10.1016/j.metabol.2011.08.008
58. Brønstad I, Breivik L, Methlie P, Wolff AS, Bratland E, Nermoen I, et al. Functional Studies of Novel CYP21A2 Mutations Detected in Norwegian Patients With Congenital Adrenal Hyperplasia. *Endocr Connect* (2014) 3 (2):67–74. doi: 10.1530/EC-14-0032
59. Karlsson L, de Paula D, Gori AL, D'Almeida C, Östberg LJ, Persson B, et al. Novel Non-Classic CYP21A2 Variants, Including Combined Alleles, Identified in Patients With Congenital Adrenal Hyperplasia. *Clin Biochem* (2019) 73:50–6. doi: 10.1016/j.clinbiochem.2019.07.009
60. Robins T, Carlsson J, Sunnerhagen M, Wedell A, Persson B. Molecular Model of Human CYP21 Based on Mammalian CYP2C5: Structural Features Correlate With Clinical Severity of Mutations Causing Congenital Adrenal Hyperplasia. *Mol Endocrinol* (2006) 20(11):2946–64. doi: 10.1210/me.2006-0172
61. Haider S, Islam B, D'Atri V, Sgobba M, Poojari C, Sun L, et al. Structure-Phenotype Correlations of Human CYP21A2 Mutations in Congenital Adrenal Hyperplasia. *Proc Natl Acad Sci USA* (2013) 110(7):2605–10. doi: 10.1073/pnas.1221133110
62. Pallan PS, Lei L, Wang C, Waterman WR, Guengerich FP, Egli M, et al. Research Resource: Correlating Human Cytochrome P450 21a2 Crystal Structure and Phenotypes of Mutations in Congenital Adrenal Hyperplasia. *Mol Endocrinol* (2015) 29(9):1375–84. doi: 10.1210/ME.2015-1127
63. de Carvalho DF, Miranda MC, Gomes LG, Madureira G, Marcondes JA, Billerbeck AE, et al. Molecular CYP21A2 Diagnosis in 480 Brazilian Patients With Congenital Adrenal Hyperplasia Before Newborn Screening Introduction. *Eur J Endocrinol* (2016) 175(2):107–16. doi: 10.1530/EJE-16-0171
64. Xu Z, Chen W, Merke DP, McDonnell NB. Comprehensive Mutation Analysis of the CYP21A2 Gene. An Efficient Multistep Approach to the Molecular Diagnosis of Congenital Adrenal Hyperplasia. *J Mol Diagn* (2013) 15(6):745–53. doi: 10.1016/j.jmoldx.2013.06.001
65. Concolino P. Issues With the Detection of Large Genomic Rearrangements in Molecular Diagnosis of 21-Hydroxylase Deficiency. *Mol Diagn Ther* (2019) 23(5):563–7. doi: 10.1007/s40291-019-00415-z
66. Tardy V, Menassa R, Sulmont V, Lienhardt-Roussie A, Lecointre C, Brauner R, et al. Phenotype-Genotype Correlations of 13 Rare CYP21A2 Mutations Detected in 46 Patients Affected With 21-Hydroxylase Deficiency and in One Carrier. *J Clin Endocrinol Metab* (2010) 95(3):1288–300. doi: 10.1210/jc.2009-1202
67. Araujo RS, Billerbeck AE, Madureira G, Mendonca BB, Bachega TA. Substitutions in the CYP21A2 Promoter Explain the Simple-Virilizing Form of 21-Hydroxylase Deficiency in Patients Harbouring a P30L Mutation. *Case Rep Clin Endocrinol (Oxf)* (2005) 62(2):132–6. doi: 10.1111/j.1365-2265.2005.02184.x
68. Friães A, Rêgo AT, Aragüés JM, Moura LF, Mirante A, Mascarenhas MR, et al. CYP21A2 Mutations in Portuguese Patients With Congenital Adrenal Hyperplasia: Identification of Two Novel Mutations and Characterization of Four Different Partial Gene Conversions. *Mol Genet Metab* (2006) 88(1):58–65. doi: 10.1016/j.ymgme.2005.11.015
69. Parajes S, Quinteiro C, Domínguez F, Loidi L. High Frequency of Copy Number Variations and Sequence Variants at CYP21A2 Locus: Implication for the Genetic Diagnosis of 21-Hydroxylase Deficiency. *PloS One* (2008) 3 (5):e2138. doi: 10.1371/journal.pone.0002138
70. Wedell A, Stengler B, Luthman H. Characterization of Mutations on the Rare Duplicated C4/CYP21 Haplotype in Steroid 21-Hydroxylase Deficiency. *Hum Genet* (1994) 94(1):50–4. doi: 10.1007/BF02272841
71. Cantürk C, Baade U, Salazar R, Storm N, Pörtner R, Höppner W. Sequence Analysis of CYP21A1P in a German Population to Aid in the Molecular Biological Diagnosis of Congenital Adrenal Hyperplasia. *Clin Chem* (2011) 57(3):511–7. doi: 10.1373/clinchem.2010.156893
72. Ezquieta B, Muñoz-Pacheco R, Santomé L, Ferreiro B, García D, Casado C, et al. Pitfalls in the Molecular Diagnosis of 21OH Deficiency Due to Point Mutations Identification Without Further Characterizations of Gene Deletions and Duplications. *Horm Res* (2008) 71(Suppl 1):45. 47th Annual Meeting ESPE. Horm Res. 2008. 70, pp. 45 - 45. 01/01/2008. ISSN. doi: 10.1159/000157521
73. Schulze E, Bettendorf M, Maser-Gluth C, Decker M, Schwabe U. Allele-Dropout Using PCR-Based Diagnosis for the Splicing Mutation in Intron-2 of the CYP21B-Gene: Successful Amplification With a Taq/Pwo-Polymerase Mixture. *Endocr Res* (1998) 24(3-4):637–41. doi: 10.3109/07435809809032662
74. Ezquieta B, Jariego C, Varela JM, Oliver A, Gracia R. Microsatellite Markers in the Indirect Analysis of the Steroid 21-Hydroxylase Gene. *Prenat Diagn*

(1997) 17:429–34. doi: 10.1002/(sici)1097-0223(199705)17:5<429::aid-pd77>3.0.co;2-9
75. Coates BS, Sumerford DV, Miller NJ, Kim KS, Sappington TW, Siegfried BD, et al. Comparative Performance of Single Nucleotide Polymorphism and Microsatellite Markers for Population Genetic Analysis. *J Hered* (2009) 100 (5):556–64. doi: 10.1093/jhered/esp028
76. New MI, Tong YK, Yuen T, Jiang P, Pina C, Chan KC, et al. Noninvasive Prenatal Diagnosis of Congenital Adrenal Hyperplasia Using Cell-Free Fetal DNA in Maternal Plasma. *J Clin Endocrinol Metab* (2014) 99(6):E1022–30. doi: 10.1210/jc.2014-1118
77. Fitness J, Dixit N, Webster D, Torresani T, Pergolizzi R, Speiser PW, et al. Genotyping of CYP21, Linked Chromosome 6p Markers, and a Sex-Specific Gene in Neonatal Screening for Congenital Adrenal Hyperplasia. *J Clin Endocrinol Metab* (1999) 84(3):960–6. doi: 10.1210/jcem.84.3.5550
78. Karell K, Klinger N, Holopainen P, Levo A, Partanen J. Major Histocompatibility Complex (MHC)-Linked Microsatellite Markers in a Founder Population. *Tissue Antigens* (2000) 56(1):45–51. doi: 10.1034/j.1399-0039.2000.560106.x
79. Soriano L, Velázquez M, Ezquieta B. Usefulness of Molecular Analysis in the Differential Diagnosis of Congenital 21-Hidroxylase Deficiency Detected in Neonatal Screening. *Med Clin (Barc)* (2011) 136(7):313–4. doi: 10.1016/j.medcli.2009.06.008
80. Dulín E, Ezquieta B. Newborn Screening of Congenital Adrenal Hyperplasia. *Endocrinol Diabetes Nutr* (2018) 65:1–4. doi: 10.1016/j.endien.2017.11.015
81. Balcells C, Gili T, Pérez J, Corripio R. Pseudohypoaldosteronism Without Nephropathy Masking Salt-Wasting Congenital Adrenal Hyperplasia Genetically Confirmed. *BMJ Case Rep* (2013) 2013:bcr2012008281. doi: 10.1136/bcr-2012-008281
82. Tuhan HU, Catli G, Anik A, Onay H, Dundar B, Bober E, et al. Cross-Reactivity of Adrenal Steroids With Aldosterone may Prevent the Accurate Diagnosis of Congenital Adrenal Hyperplasia. *J Pediatr Endocrinol Metab* (2015) 28(5-6):701–4. doi: 10.1515/jpem-2014-0170
83. Nordenström A, Thilén A, Hagenfeldt L, Larsson A, Wedell A. Genotyping is a Valuable Diagnostic Complement to Neonatal Screening for Congenital Adrenal Hyperplasia Due to Steroid 21-Hydroxylase Deficiency. *J Clin Endocrinol Metab* (1999) 84(5):1505–9. doi: 10.1210/jcem.84.5.5651
84. Gidlöf S, Falhammar H, Thilén A, von Döbeln U, Ritzén M, Wedell A, et al. One Hundred Years of Congenital Adrenal Hyperplasia in Sweden: A Retrospective, Population-Based Cohort Study. *Lancet Diabetes Endocrinol* (2013) 1(1):35–42. doi: 10.1016/S2213-8587(13)70007-X
85. Falhammar H, Wedell A, Nordenström A. Biochemical and Genetic Diagnosis of 21-Hydroxylase Deficiency. *Endocrine* (2015) 50(2):306–14. doi: 10.1007/s12020-015-0731-6
86. Marino S, Perez N, Ramírez P, Pujana M, Dratler G, Belgorosky A, et al. Molecular Analysis of the CYP21A2 Gene in Dried Blood Spot Samples. *Medicina (B Aires)* (2020) 80(3):197–202.
87. Castro PS, Rassi TO, Araujo RF, Pezzuti IL, Rodrigues AS, Bachega TA, et al. High Frequency of non-Classical Congenital Adrenal Hyperplasia Form Among Children With Persistently Elevated Levels of 17-Hydroxyprogesterone After Newborn Screening. *J Pediatr Endocrinol Metab* (2019) 32(5):499–504. doi: 10.1515/jpem-2018-0398
88. New M. Inborn Errors of Adrenal Steroidogenesis. *Mol Cell Endocrinol* (2003) 211(1-2):75–83. doi: 10.1016/j.mce.2003.09.013
89. Ezquieta B, Fernandez ML, Dulín E, Rodriguez D, Rodríguez A. Prevalence of Frequent Recessive Diseases in the Spanish Population Through DNA Analyses on Samples From the Neonatal Screening. *Med Clin (Barc)* (2005) 125(13):493–5. doi: 10.1157/13080213
90. Admoni O, Israel S, Lavi I, Gur M, Tenenbaum-Rakover Y. Hyperandrogenism in Carriers of CYP21 Mutations: The Role of Genotype. *Clin Endocrinol (Oxf)* (2006) 64(6):645–51. doi: 10.1111/j.1365-2265.2006.02521.x
91. Ezquieta B, Oyarzabal M, Barrio R, Luzuriaga C, Hermoso F, Lechuga JL, et al. Monogenic and Polygenic Models Detected in Steroid 21-Hydroxylase Deficiency-Related Paediatric Hyperandrogenism. *Horm Res* (2009) 71 (1):28–37. doi: 10.1159/000173739
92. Guarnotta V, Niceta M, Bono M, Marchese S, Fabiano C, Indelicato S, et al. Clinical and Hormonal Characteristics in Heterozygote Carriers of Congenital Adrenal Hyperplasia. *J Steroid Biochem Mol Biol* (2020) 198:105554. doi: 10.1016/j.jsbmb.2019.105554
93. Turcu AF, Rege J, Auchus RJ, Rainey WE. 11-Oxygenated Androgens in Health and Disease. *Nat Rev Endocrinol* (2020) 16(5):284–96. doi: 10.1038/s41574-020-0336-x
94. Sumińska M, Bogusz-Górna K, Wegner D, Fichna M. Non-Classic Disorder of Adrenal Steroidogenesis and Clinical Dilemmas in 21-Hydroxylase Deficiency Combined With Backdoor Androgen Pathway. *Mini-Review Case Rep Int J Mol Sci* (2020) 21(13):4622. doi: 10.3390/ijms21134622
95. Costa-Barbosa FA, Carvalho VM, Oliveira KC, Vieira JG, Kater CE. Reassessment of Predictive Values of ACTH-Stimulated Serum 21-Deoxycortisol and 17-Hydroxyprogesterone to Identify CYP21A2 Heterozygote Carriers and Nonclassic Subjects. *Clin Endocrinol (Oxf)* (2021) 95(4):677–85. doi: 10.1111/cen.14550
96. Speiser PW, Dupont B, Rubinstein P, Piazza A, Kastelan A, New MI. High Frequency of Nonclassical Steroid 21-Hydroxylase Deficiency. *Am J Hum Genet* (1985) 37:650–67.
97. Pignatelli D, Carvalho BL, Palmeiro A, Barros A, Guerreiro SG, Macut D, et al. The Complexities in Genotyping of Congenital Adrenal Hyperplasia: 21-Hydroxylase Deficiency. *Front Endocrinol (Lausanne)* (2019) 10:432. doi: 10.3389/fendo.2019.00432
98. Livadas S, Bothou C. Management of the Female With Non-Classical Congenital Adrenal Hyperplasia (NCCAH): A Patient-Oriented Approach. *Front Endocrinol (Lausanne)* (2019) 10:366. doi: 10.3389/fendo.2019.00366
99. Trakakis E, Loghis C, Kassanos D. Congenital Adrenal Hyperplasia Because of 21-Hydroxylase Deficiency. A Genetic Disorder of Interest to Obstetricians and Gynecologists. *Obstet Gynecol Surv* (2009) 64(3):177–89. doi: 10.1097/OGX.0b013e318193301b
100. Chatziaggelou A, Sakkas EG, Votino R, Papagianni M, Mastorakos G. Assisted Reproduction in Congenital Adrenal Hyperplasia. *Front Endocrinol (Lausanne)* (2019) 10:723. doi: 10.3389/fendo.2019.00723
101. Narasimhan ML, Khattab A. Genetics of Congenital Adrenal Hyperplasia and Genotype-Phenotype Correlation. *Fertil Steril* (2019) 111(1):24–9. doi: 10.1016/j.fertnstert.2018.11.007
102. Claahsen-van HL, Speiser PW, Ahmed SF, Arlt W, Auchus RJ, Falhammar H, et al. Congenital Adrenal Hyperplasia - Current Insights in Pathophysiology, Diagnostics and Management. *Endocr Rev* (2022) 43 (1):91–159. doi: 10.1210/endrev/bnab016

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Effect of Recombinant Gonadotropin on Testicular Function and Testicular Sperm Extraction in Five Cases of *NR0B1* (*DAX1*) Pathogenic Variants

Jordan Teoli[1,2,3], *Vincent Mezzarobba*[4], *Lucie Renault*[5], *Delphine Mallet*[1], *Hervé Lejeune*[2,3,5], *Pierre Chatelain*[2,6], *Frédérique Tixier*[6], *Marc Nicolino*[2,6], *Noël Peretti*[2,7], *Sandrine Giscard D'estaing*[2,3,5], *Béatrice Cuzin*[8], *Frédérique Dijoud*[2,3,9], *Florence Roucher-Boulez*[1,2] *and Ingrid Plotton*[1,2,3,5]*

[1] Service de Biochimie et Biologie Moléculaire, UM Pathologies Endocriniennes, CR DEV-GEN, Centre de Biologie et Pathologie Est, Hospices Civils de Lyon, Bron, France, [2] Université Claude Bernard Lyon 1, Lyon, France, [3] Institut Cellule Souche et Cerveau (SBRI), Unité INSERM, Centre de Recherche INSERM, Bron, France, [4] Fédération d'Endocrinologie, Hôpital Louis Pradel, Hospices Civils de Lyon, Bron, France, [5] Service de Médecine de la Reproduction, Hôpital Femme-Mère-Enfant, Hospices Civils de Lyon, Bron, France, [6] Service d'Endocrinologie Pédiatrique, Hôpital Femme Mère Enfant, Hospices Civils de Lyon, Bron, France, [7] Service de Gastroentérologie, Hépatologie et Nutrition Pédiatriques, Hôpital Femme Mère Enfant, Hospices Civils de Lyon, Bron, France, [8] Chirugie Urologique, Centre Lyonnais d'Urologie Bellecour, Lyon, France, [9] Service d'Anatomie Pathologique, Centre de Biologie et de Pathologie Est, Hospices Civils de Lyon, Bron, France

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Maria Fragoso,
Institute of Cancer of Sao Paulo, Brazil

Reviewed by:
David William Cooke,
Johns Hopkins Medicine, United States
Sasha R. Howard,
Queen Mary University of London, United Kingdom

***Correspondence:**
Ingrid Plotton
ingrid.plotton@chu-lyon.fr

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Background: *NR0B1* pathogenic variants can cause congenital adrenal hypoplasia or primary adrenal insufficiency in early childhood usually associated with hypogonadotropic hypogonadism. *NR0B1* is necessary for organogenesis of the adrenal cortex and to maintain normal spermatogenesis. In humans, restoration of fertility in patients carrying *NR0B1* pathogenic variants is challenging.

Objective: The aim of the study was to investigate the clinical, hormonal, histological, spermiological, and molecular genetic characteristics of a cohort of patients with *NR0B1* pathogenic variants, monitored for fertility preservation.

Patients: We included five patients, including four teenagers, with *NR0B1* pathogenic or likely pathogenic variants. They all had primary adrenal insufficiency and were receiving replacement therapy with glucocorticoids and mineralocorticoids. Patients received recombinant follicle-stimulating hormone and recombinant human chorionic gonadotropin in order to induce spermatogenesis. Combined gonadotropin treatment was initiated between 13 years and 15 years and 6 months for the four teenagers and at 31 years and 2 months for the only adult. Physical and hormonal assessments were performed just before starting gonadotropin treatment. After 12 months of gonadotropin treatment, physical examination and hormonal assessments were repeated, and semen analyses were performed. If no sperm cells were observed in at least 2 semen collections at 3-month interval, testicular biopsy for testicular sperm extraction was proposed.

Results: Bilateral testicular volume increased from 8 ml (interquartile range, 6–9) to 12 ml (10–16) after gonadotropin treatment. Inhibin B levels were relatively stable: 110 ng/L (46–139) before and 91 ng/L (20–120) at the end of gonadotropin treatment. Azoospermia

was observed in all semen analyses for all cases during gonadotropin treatment. Three patients agreed to testicular biopsy; no mature sperm cells could be retrieved in any.

Conclusion: We characterized a cohort of patients with *NR0B1* pathogenic or likely pathogenic variants for fertility preservation by recombinant gonadotropin treatment, which began either at puberty or in adulthood. No sperm cells could be retrieved in semen samples or testicular biopsy even after gonadotropin treatment, indicating that gonadotropin treatment, even when started at puberty, is ineffective for restoring fertility.

Keywords: testicular biopsy, adrenal hypoplasia, hypogonadotrophic hypogonadism, spermatogenesis, gonadotropin, congenital, male infertility, adrenal insufficiency

1 INTRODUCTION

X-linked adrenal hypoplasia congenita (X-AHC) is a pathology characterized by primary adrenal insufficiency (Addison's disease), with onset most often at birth or in early childhood, and frequently associated with hypogonadotropic hypogonadism and spermatogenesis failure detected after puberty (1–6). Adrenal insufficiency can be treated effectively by glucocorticoid and mineralocorticoid replacement therapy. However, and contrary to other forms of hypogonadotropic hypogonadism (7), restoration of spermatogenesis remains challenging in X-AHC patients with azoospermia because of an added primary testicular injury in X-AHC (5, 6). Azoospermia in X-AHC patients classically does not respond to gonadotropin treatment, and no sperm cells were obtained in semen or even in testicular biopsies after this therapy (5, 6, 8–12) except in one study (13).

X-AHC is related to *NR0B1* (or *DAX1)* gene alterations. *NR0B1* is a gene with only two exons, carried by the X chromosome, belonging to the nuclear hormone receptor superfamily (1). It encodes a 470-amino acid (AA) protein with a suspected ligand-binding domain in the carboxyl-terminal portion and a 3.5-fold repeated motif responsible for protein–protein interactions in the amino-terminal portion (1, 4). This protein appears essential for organogenesis of the adrenal cortex, gonadal sex determination, development of the hypothalamic-pituitary-gonadotropic axis, and spermatogenesis (2, 3). Pathogenic variants in the *NR0B1* gene occur in 1:70,000 to 1:600,000 boys (14). More than one hundred pathogenic variants in the *NR0B1* gene have been described (4, 14, 15). Duplication of *NR0B1* is related to 46,XY sex reversal whereas deletion, indel or frameshift, splice sites, and nonsense or missense pathogenic variants are responsible for X-AHC in humans (2, 4, 14).

Here, we describe the impacts of pathogenic or likely pathogenic *NR0B1* variants on clinical, hormonal, histological, and spermiological aspects and on gonadotropin treatment response in five male patients, including four teenagers. Three of the five variants explored here have never been reported, whether in the literature or in databases.

2 MATERIALS AND METHODS

2.1 Patients

This retrospective study included male patients monitored in the reproductive medicine department of Lyon University Hospital for fertility preservation. Patients were included if they had received a gonadotropin treatment after 2010 and if a hemizygous *NR0B1* pathogenic or likely pathogenic variant was identified in the molecular endocrinology unit of the laboratory using Sanger sequencing on DNA extracted from whole blood. Variants were described using reference NP_000466.2 for DAX1 protein and NM_000475.5 for *NR0B1* transcript on GRCh37/hg19 human genome assembly. Pathogenic or likely pathogenic classification was based on the American College of Medical Genetics and Association of Medical Pathologists (ACMG) consensus recommendation (16) with the help of the Gnomad_v2 (https://gnomad.broadinstitute.org/), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), and dbSNP databases (https://www.ncbi.nlm.nih.gov/snp/) and *in silico* prediction tools listed by Mobidetails (an online DNA variant interpretation tool) (17).

Patients and, as appropriate, their parents signed an informed written consent form for genetic study. In accordance with French legislation, review board submission was not required, owing to the observational nature of the study. The study was conducted in accordance with the principles of the Declaration of Helsinki.

2.2 Protocol

During the fertility preservation procedure, history, psychological evaluation, and clinical data were recorded systematically, including physical examination at each visit: before treatment and the day of the semen analysis and/or blood sampling for hormonal analysis.

Abbreviations: AA, amino acid; ACMG, American College of Medical Genetics and Genomics; X-AHC, X-linked adrenal hypoplasia congenita; AMH, anti-Müllerian hormone; BTV, bilateral testicular volume; CVb, interassay coefficient of variation; FSH, follicle-stimulating hormone; LBD, ligand-binding domain; LH, luteinizing hormone; LOQ, limit of quantification; rFSH, recombinant follicle-stimulating hormone; rhCG, recombinant human chorionic gonadotropin; TESE, testicular sperm extraction.

At the time of inclusion, bilateral testicular volume (BTV: sum of right and left testis volume, considered normal if ≥30 ml) was assessed using a Prader orchidometer. Blood samples were taken for follicle-stimulating hormone (FSH), luteinizing hormone (LH), total testosterone, anti-Müllerian hormone (AMH), and inhibin B assay (pretherapeutic assessment).

Patients included in the fertility preservation protocol received a combination of recombinant FSH (rFSH) and recombinant human chorionic gonadotropin (rhCG). The gonadotropin treatment was increased gradually to reach a subcutaneous injection dose of 150 IU rFSH three times per week and 1,500 IU rhCG twice a week. During follow-up visits, rFSH dosage was adapted to inhibin B level and rhCG dosage to testosterone level.

After 12- to 42-month gonadotropin treatment, BTV was reassessed and blood samples were repeated for hormonal measurement (total testosterone, AMH, inhibin B). Semen was collected under gonadotropin treatment for laboratory analysis. Testicular biopsy for testicular sperm extraction (TESE) was proposed if azoospermia was observed on two semen analyses 3 months apart (**Figure 1**).

2.3 Laboratory Assays

2.3.1 Hormonal Measurements

Plasma FSH and LH were assessed by an automated chemiluminescence immunometric assay on Architect i2000SR (Abbott, Chicago, IL, USA). The interassay coefficient of variation (CVb) was ≤3.9% at 6, 20, and 40 IU/L and ≤4.2% at 4, 14, and 44 IU/L for FSH and LH, respectively. The limit of quantification (LOQ) was 0.05 IU/L for FSH and LH. In men with normal testicular function, normal ranges extended from 1.1 to 7.2 IU/L and 1.3 to 5.8 IU/L for FSH and LH, respectively.

Plasma total testosterone was assessed on in-house liquid chromatography coupled with tandem mass spectrometry after supported liquid–liquid extraction using diatomaceous earth. CVb was ≤7.8% at 1.96, 7.50, 8.20, and 23.46 nmol/L. LOQ was 0.13 nmol/L. Normal ranges were 10.40 to 26.00 nmol/L in young men and 0.28 ± 0.01 nmol/L (mean ± standard deviation) in prepubescent boys.

Serum AMH was assessed by automated electrochemiluminescence assay on Cobas e601 (Roche Diagnostics, Basel, Switzerland). CVb was ≤2.8% at 5.5 and 30 pmol/L. LOQ was 0.21 pmol/L. Normal ranges extended from 15 to 60 pmol/L in men with normal spermatogenesis (18).

Serum inhibin B was assessed by enzyme immunoassay using the Inhibin B Gen II ELISA kit (Beckman Coulter, Brea, CA, USA). CVb was ≤13.7% at 115 and 420 ng/L. LOQ was 5 ng/L. Normal lab ranges for men with normal testicular function were taken from the normozoospermic cohort of Pierik et al.: 55 to 309 ng/L (19) and, for boys aged 12–17 years were taken from Crofton et al.: 74 to 470 ng/L (20).

2.3.2 Semen Analysis

Semen collection was carried out by masturbation in the reproduction laboratory after 3–5 days of sexual abstinence. Analysis was performed according to the 2010 World Health Organization criteria (21).

2.3.3 Testicular Biopsy and Conventional TESE

The multiple bilateral testicular biopsies and conventional TESE procedure was as previously described (22). TESE was performed in the reproduction unit of the laboratory.

2.3.4 Histological Analysis

In parallel, some biopsy fragments were sent to the pathology unit of the laboratory, fixed in alcohol, formalin, and acetic acid (AFA) and paraffin embedded. Three-micrometer slices were stained by hematoxylin–phloxin–saffron.

Slide evaluation was performed on a Leica DM2500 microscope. All tubules within five image fields were evaluated. The presence of a lumen and the most advanced germ cell were noted. Germ cells were identified on the basis on their morphology (size and shape) and location (23). The number of Leydig cells was estimated on three fields at ×40 magnification.

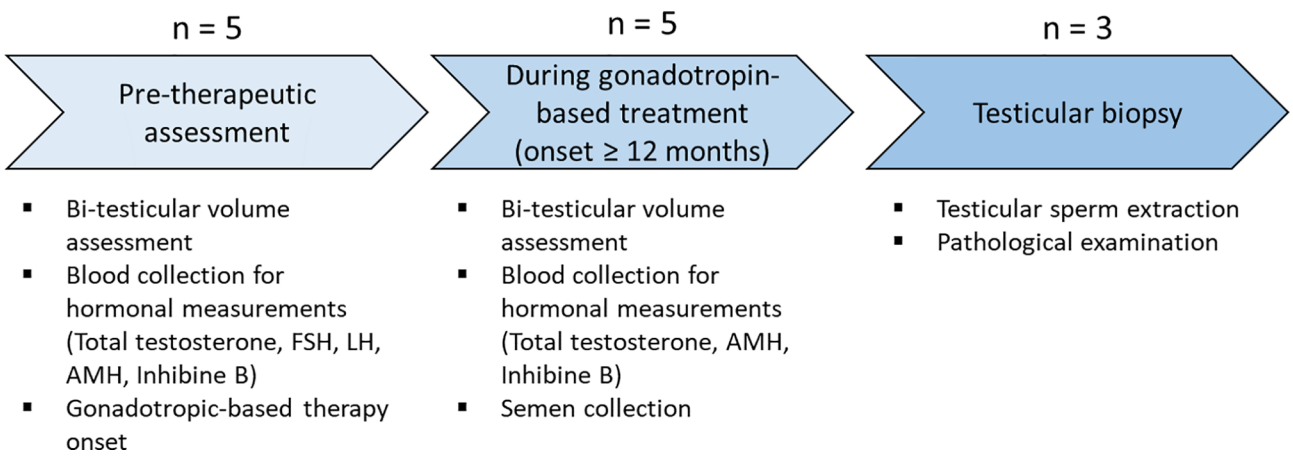

FIGURE 1 | Protocol design. Physical examination and biological samples at each step of the protocol. The number of patients undergoing each step is indicated. Each step of the protocol is represented by an arrow. AMH, anti-Müllerian hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone.

2.4 Statistical Analysis

Quantitative data were expressed as median (interquartile range). Values below LOQ were considered equal to the LOQ. Analysis used R software v3.6.3 (R Foundation for Statistical Computing, Vienna, Austria).

3 RESULTS

Five patients from unrelated families were included in the study. A primary adrenal insufficiency crisis occurred at birth in three patients (patients 1, 2, and 4) and in childhood up to 10 years of age in the other two (patients 3 and 5). Patients started glucocorticoid and mineralocorticoid replacement therapy. Genetic analysis found *NR0B1* variants that confirmed X-AHC diagnosis.

Locations of patients' *NR0B1* variants are reported in **Table 1** and displayed in **Figure 2**. Based on the ACMG criteria, all the variants were considered likely pathogenic or pathogenic (**Table 1**). Two of the five variants were previously reported elsewhere. The variant NM_000475.5: c.1411T>C p.(*471Glnext*18) (patient 3) was recently reported in the literature and considered likely pathogenic in ClinVar. The variant NM_000475.5: c.919G>T p.(Glu307*) (patient 4) was reported in dbSNP and considered pathogenic in ClinVar. The other three variants had not been reported in the literature, ClinVar, or dbSNP. None of the variants were reported in Gnomad_v2. All variants were clustered in the putative ligand-binding protein domain (**Figure 2**).

Patient 1 carried the novel variant NM_000475.5: c.857_862dup p.(Leu286_Val287dup), which adds two additional AA in an alpha helix of the putative ligand-binding domain of nuclear hormone receptor of *NR0B1*. His mother was heterozygous for the variant. His healthy brother did not carry the variant. He had one infertile maternal uncle and one maternal uncle who died at the age of 1 month of life.

Patient 2 was hemizygous for the novel substitution NM_000475.5: c.896T>C p.(Leu299Pro), inherited from his heterozygous mother. His healthy brother did not carry the variant.

Patient 3 had a stop-loss variant NM_000475.5: c.1411T>C p.(*471Glnext*18), which extended the C-terminal portion of the protein by 18 additional AA. Genetic analysis was not performed on his mother, but he had two healthy brothers who did not carry the variant. He had one maternal uncle with adrenal insufficiency.

Patient 4 had a stop gain variant NM_000475.5: c.919G>T p.(Glu307*), leading to loss of part of the putative ligand-binding domain of the nuclear hormone receptor of *NR0B1*. His mother was heterozygous for the variant. His healthy brother did not carry the variant.

Patient 5 carried a new frameshift variant NM_000475.5: c.950_966del p.(Leu317Hisfs*66) caused by a 17-bp deletion. Genetic analysis was not performed on his mother, and family history was not available.

These patients were enrolled in a fertility preservation protocol based on gonadotropin treatment after 2010: four at puberty (patients 1 to 4) and one in adulthood (patient 5). FSH, LH, and plasma total testosterone levels assessed in four of the five patients (patients 1 to 4) immediately before starting gonadotropin treatment showed low testosterone levels (0.58nmol/L [0.15–1.61]) compared with FSH (2.00 IU/L [1.53–2.85]) and LH concentrations (0.60 IU/L [0.32–0.83]), which were not elevated. BTV was low (8 ml [6–9]). AMH levels were high in three patients. Inhibin B levels were low or near the lower limit of normal (110 ng/L [46–139]) (**Table 1**).

Patients then received combined gonadotropin treatment. Patient 5 had received testosterone therapy for 15 years before starting the gonadotropin treatment, and patient 3 received only rFSH during the first 6 months (priming rFSH) of gonadotropin treatment.

After at least 12 months under gonadotropin treatment, physical and hormonal assessments were repeated. BTV rose from 8 ml (6–9) before to 12 ml (10–16) after gonadotropin treatment, and values remained very low. Total testosterone increased from 0.58 nmol/L (0.15–1.61) to normal adult values at 15.51 nmol/L (14.07–16.17). AMH levels decreased from 192.9 pmol/L (58.6–249.5) before to 41 pmol/L (36.9–60.4) after gonadotropin treatment. AMH levels approached usual values after gonadotropin treatment. Inhibin B levels stayed quite stable: 110 ng/L (46–139) at the beginning to 91 ng/L (20–120) at the end of gonadotropin treatment and remained quite low (**Table 1** and **Figure 3**).

Semen analysis was performed after at least 12 months of gonadotropin treatment. No sperm cells were retrieved in any of the five patients. Four patients (patients 1, 2, 4, and 5) were eligible for TESE, since azoospermia was observed on two semen analyses 3 months apart, whereas only one semen analysis was performed in the other patient (patient 3). Only three patients (patients 1, 4, and 5) agreed to testicular biopsy, and the TESE procedure was negative for all: no spermatozoa could be extracted and cryopreserved (**Table 1**).

Histological analysis of testicular biopsies showed pubescent testicular parenchyma with severe hypospermatogenesis lesions and maturation arrest. In all three patients, interstitial tissue was edematous and there was no dysplasia.

Histological examination in patient 1 (16y7m) showed severe hypospermatogenesis with maturation arrest (histological mosaicism profile) associated with Leydig cell hyperplasia. Most of the seminiferous tubules showed Sertoli cell-only syndrome. Some tubules showed incomplete spermatogenesis with a few spermatocytes and round spermatids but no mature germ cells. Rare prepubertal tubules were observed, without a central lumen and with very rare spermatogonia (**Figures 4A, B**).

Patient 4 (16y7m) showed pubescent testis with Sertoli cells only. The stroma was edematous, with some Leydig cells but without hyperplasia (**Figures 4C, D**).

Patient 5 (34y8m) showed severe hypospermatogenesis with numerous tubules with Sertoli cells only and some tubules with maturation arrest at spermatocyte level. Nodular Leydig cell hyperplasia was also observed. The tubules were surrounded by thickened lamina propria and some were totally atrophic (**Figures 4E, F**).

TABLE 1 | Patients' characteristics.

	Patient	1	2	3	4	5
Diagnosis	Age at diagnosis	At birth	At birth	10 years	At birth	6 years
	Diagnostic context	Acute primary adrenal insufficiency	Acute primary adrenal insufficiency	Acute primary adrenal insufficiency	Acute primary adrenal insufficiency	Acute primary adrenal insufficiency
Pretherapeutic assessment (at age of gonadotropin initiation)	Bilateral testicular volume (ml)	10	9	6	8	6
	FSH/LH (IU/L)	1.8/0.4	4.8/0.8	2.2/0.91	0.7/0.07	NA
	Total testosterone (nmol/L)	3.44	1	0.16	<0.13	NA
	AMH (pmol/L)	414.1	58.6	249.5	192.9	46.4
	Inhibin B (ng/L)	139	46	187	110	42
Gonadotropin treatment	Age of gonadotropin treatment initiation	13y11m	15y6m	13y	14y	31y2m
	Age of gonadotropin treatment termination	16y7m	18y8m	15y11m	17y	34y8m
	Gonadotropin treatment	rFSH + rhCG	rFSH + rhCG	Priming rFSH for 6 months then rFSH + rhCG	rFSH + rhCG	rFSH + rhCG after 15 years of testosterone supplementation
	Total duration of gonadotropin treatment	32 months	38 months	35 months	36 months	42 months
Assessment during gonadotropin treatment or the earliest assessment after termination	Age at assessment	16y7m	20y11m	15y8m	15y	34y8m
	Bilateral testicular volume (ml)	10	12	20	16	7
	Total testosterone (nmol/L)	16.82	NA	12.60	15.51	NA
	AMH (pmol/L)	60.4	15.2	41.0	105.4	36.9
	Inhibin B (ng/L)	91	<5	214	120	20
Semen collection	Result	Azoospermia On 4 samples	Azoospermia On 2 samples	Azoospermia On 1 sample	Azoospermia On 3 samples	Azoospermia On 3 samples
	Time of sampling since start of therapy	Between 12 and 32 months	At 35 and 38 months	At 35 months	Between 12 and 31 months	Between 16 and 42 months
TESE	Result	No sperm cells retrieved	Not done	Not done	No sperm cells retrieved	No sperm cells retrieved
	Time of testicular biopsy since start of therapy	32 months	NA	NA	31 months	42 months
	Age at biopsy	16y7m	NA	NA	16y7m	34y8m
NR0B1 variant (NP_000466.2, NM_000475.5, GRCh37/hg19)	Location	p.(Leu286_Val287dup) c.857_862dup	p.(Leu299Pro) c.896T>C	p.(*471Glnext*18) c.1411T>C	p.(Glu307*) c.919G>T	p.(Leu317Hisfs*66) c.950_966del
	ACMG class (ACMG criteria)		Likely pathogenic			Pathogenic (PVS1, PM1, PM2, PP4)

(Continued)

TABLE 1 | Continued

Patient	1	2	3	4	5
	Likely pathogenic (PM1, PM2, PM4, PP3, PP4)	(PM1+PM2 +PP3 PP4)	Likely pathogenic (PM2, PM4, PP3, PP4, PP5)	Pathogenic (PVS1, PM1, PM2, PP3, PP4, PP5)	

AA, amino acid; ACMG, American College of Medical Genetics and Genomics; AMH, anti-Müllerian hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; NA, not applicable; rFSH, recombinant follicle-stimulating hormone; rhCG, recombinant human chorionic gonadotropin; TESE, testicular sperm extraction.
ACMG criteria: pathogenic moderate (PM); pathogenic supporting (PP); pathogenic strong (PS); pathogenic very strong (PVS).
"PVS1: null variant (nonsense, frameshift, canonical +/−1 or 2 splice sites, initiation codon, single or multiexon deletion) in a gene where loss of function is a known mechanism of disease."
"PM1: located in a mutational hotspot and/or critical and well-established functional domain (e.g., active site of an enzyme) without benign variation."
"PM2: absent from controls (or at extremely low frequency if recessive) in Exome Sequencing Project, 1,000 Genomes or ExAC."
"PM4: protein length changes due to in-frame deletions/insertions in a nonrepeat region or stop-loss variants."
"PP3: multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc.)."
"PP4: patient's phenotype or family history is highly specific for a disease with a single genetic etiology."
"PP5: reputable source recently reports variant as pathogenic but the evidence is not available to the laboratory to perform an independent evaluation."

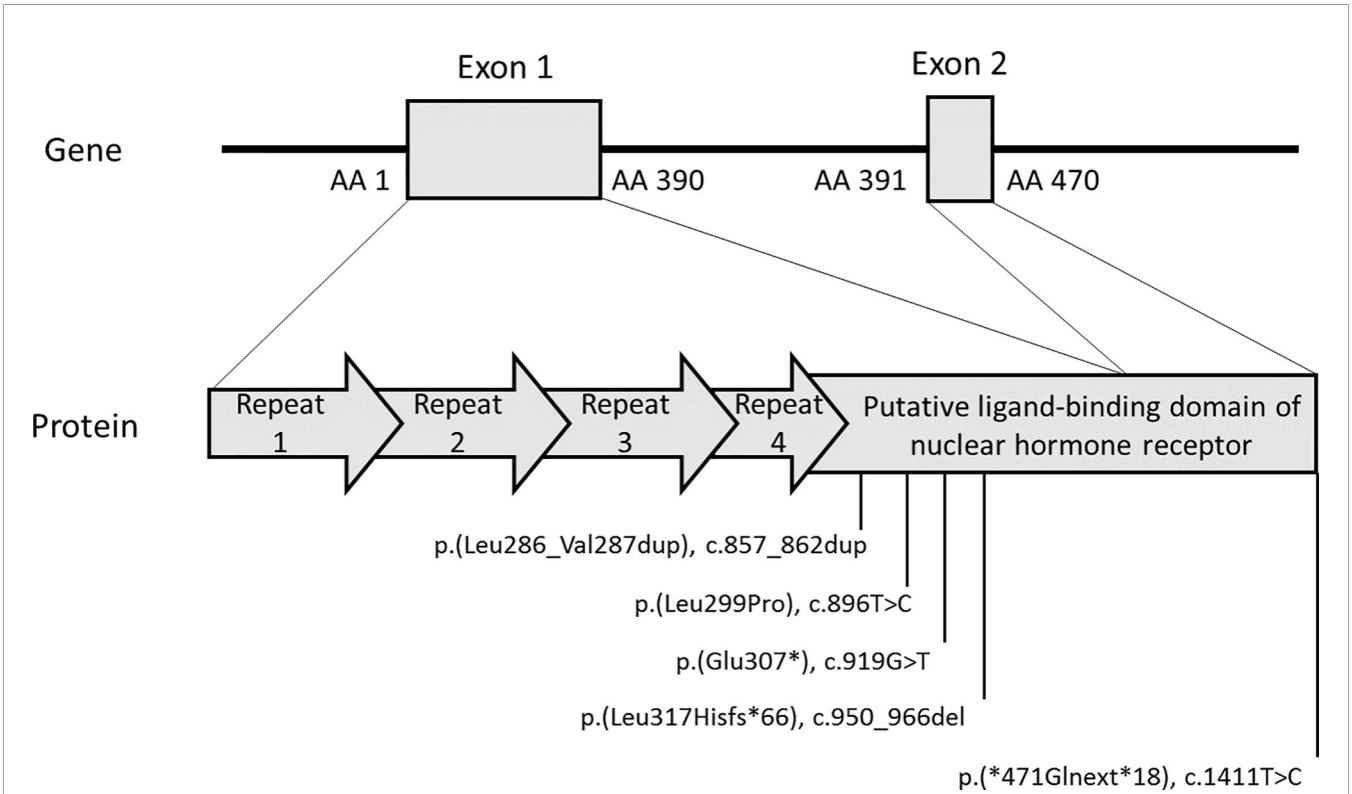

FIGURE 2 | *NR0B1* variants identified in patients. The figure depicts the two exons of the *NR0B1* gene confronted with the 470 AA DAX1 protein. The DAX1 protein diagram resumes the 3.5-fold repeated motif in the amino-terminal portion (arrows) and the putative ligand-binding domain of nuclear hormone receptor in the carboxyl-terminal portion. The five variants identified in the five patients are positioned on the protein diagram. All the reported variants cluster in the putative ligand-binding domain of nuclear hormone receptor. Variants were described using reference NP_000466.2 for DAX1 protein and NM_000475.5 for *NR0B1* transcript on GRCh37/hg19 human genome assembly. AA, amino acid.

4 DISCUSSION

We characterized a cohort of patients with pathogenic or likely pathogenic *NR0B1* variants, for fertility preservation associated to gonadotropin treatment, at onset of puberty in four cases and in adulthood for the other. To our knowledge, this is the first time that the impact of gonadotropin treatment has been reported in literature in adolescents.

Here, X-AHC was diagnosed during acute primary adrenal insufficiency at birth or in childhood up to 10 years of age. Variants were classified as pathogenic or likely pathogenic; all were located in a hotspot: the putative ligand-binding domain of nuclear hormone receptor. All variants modified the length of the protein if one was produced, except the missense variant NM_000475.5: c.896T>C p.(Leu299Pro). Leu299 is included in the same highly LLxLxLx-conserved domain as Leu295 and

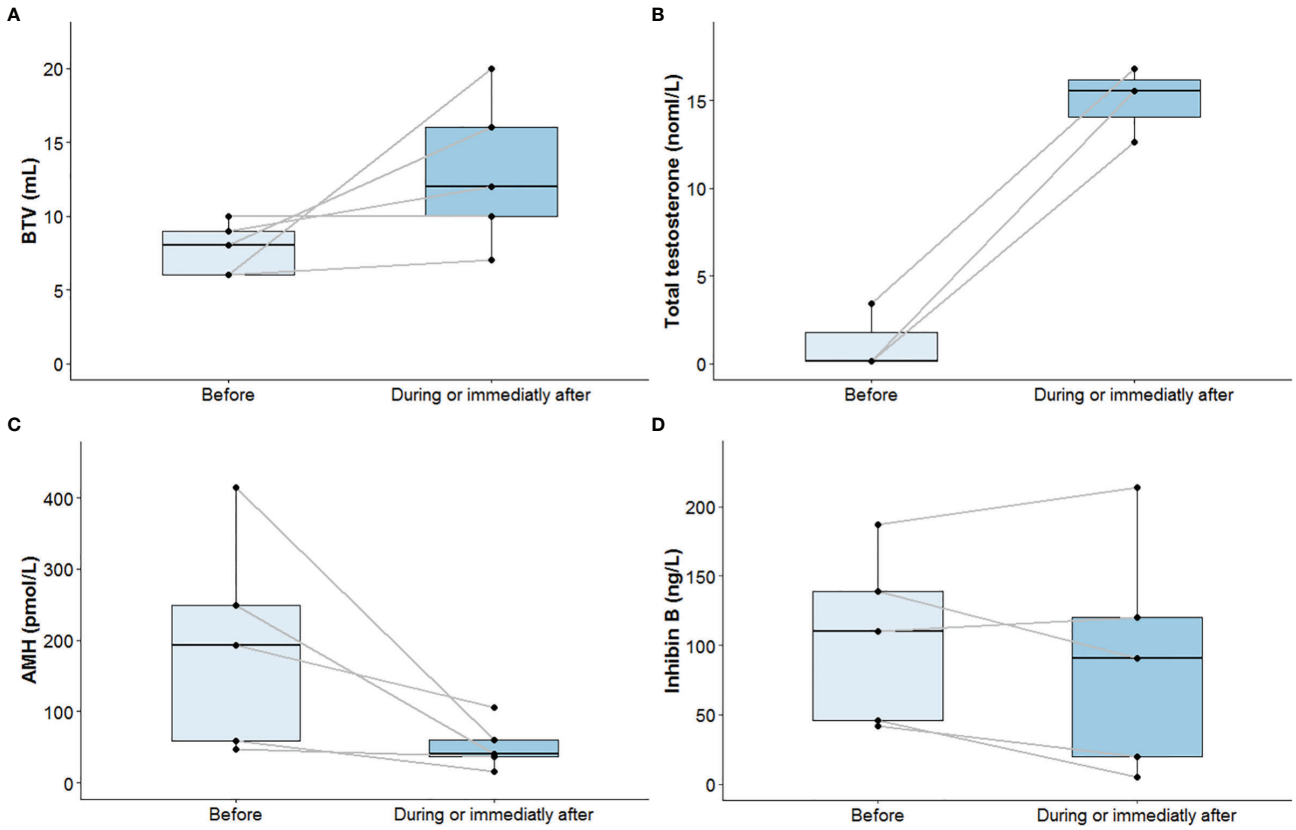

FIGURE 3 | Bi-testicular volume **(A)**, plasma total testosterone **(B)**, serum AMH **(C)**, and serum inhibin B **(D)** progression before and during or immediately after termination of gonadotropin treatment. Median values and interquartile ranges are presented in two boxplots for each parameter: one for values measured before initiation of gonadotropin treatment and one for values measured during or immediately after termination. Each black dot corresponds to one patient. Black dots obtained from the same patient before and during or immediately after termination of gonadotropin treatment are connected by solid gray lines. For plasma total testosterone, only patients with values before and during or immediately after termination of gonadotropin treatment were considered. An increase in BTV and total testosterone levels and a decrease in AMH levels after at least 12 months under gonadotropin treatment is observed. Variation in inhibin B levels is not as clear. AMH, anti-Müllerian hormone; BTV, bi-testicular volume.

Leu297, substitution of which in Proline was reported in X-AHC patients (9, 24). This highlighted the potential deleterious effect of p.(Leu299Pro) for DAX1 protein function. The variant NM_000475.5: c.1411T>C p.(*471Glnext*18) was reported in an X-AHC boy with precocious puberty (25). Protein–protein docking showed the addition of 18 additional AA after the stop codon decreased interaction between DAX1 protein and SF1 protein. The boy was followed up from 11 to 15.1 years of age; we do not know whether he would develop hypogonadotropic hypogonadism later. No semen collection was performed, but testicular volume was 3 ml bilaterally and inhibin B levels were low, suggesting a spermatogenesis defect (25). In contrast, the patient carrying NM_000475.5: c.1411T>C p.(*471Glnext*18) (patient 3) did not present precocious puberty and had subnormal BTV of 20 ml and normal inhibin B level at 15 years and 8 months.

Patients with frameshift variant or stop-gain variant might have been expected to have earlier revelation of the pathology than those with missense variant or in-frame insertion. However, hormonal levels, BTV, age at diagnosis, and gonadotropin treatment response showed no correlations with the molecular variant. This confirms the absence of any clear genotype–phenotype relation in X-AHC patients and the heterogeneity of the pathology reported elsewhere (4, 10, 12, 26, 27).

Low levels of total testosterone in parallel to the defect of increased FSH and LH levels indicated that patients presented hypogonadotropic hypogonadism. Inhibin B is secreted by Sertoli cells, and BTV and inhibin B are markers of spermatogenic potential (28–30). Therefore, reduced BTV and low or low-normal inhibin B values for age suggest spermatogenesis failure which can be integrated in the hypogonadotropic hypogonadism profile and in the primary gonadal defect reported in X-AHC patients (5, 9, 10, 13). As observed here, some authors reported testicular volume ranging from 3 to 6 ml bilaterally in most X-AHC cases (6, 9, 11, 25, 26, 31, 32). However, testicular volume may be normal in mild forms of X-AHC (10). Likewise, inhibin B was reported to be low or in the lowest range of normal at puberty or after (6, 10, 25, 26, 31, 33).

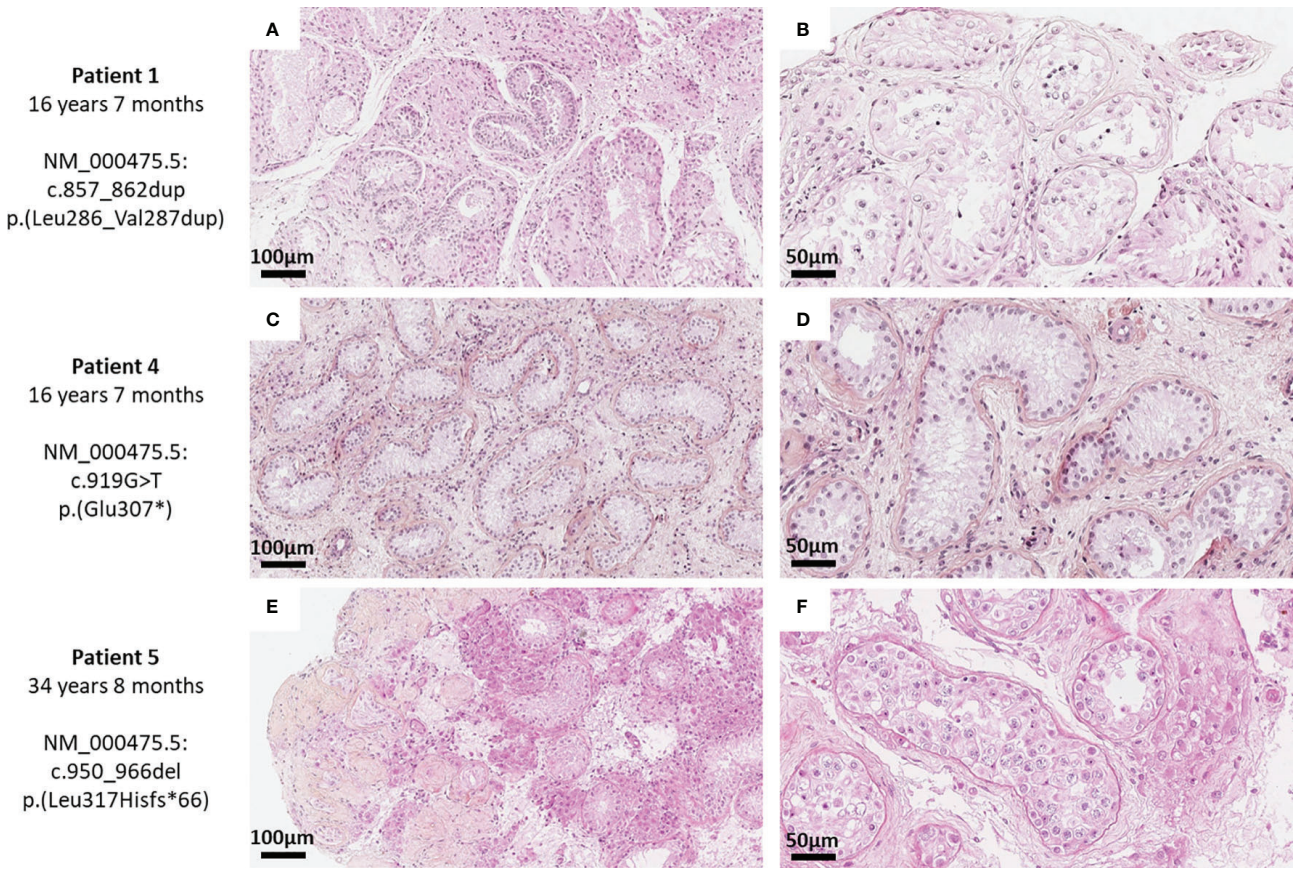

FIGURE 4 | Histological sections of testicular biopsies of patient 1 **(A**, **B)**, patient 4 **(C**, **D)**, and patient 5 **(E, F)** after gonadotropin treatment. Histological sections of testicular biopsies are shown at magnifications ×200 **(A, C, E)** and ×400 **(B, D, F)** after a hematoxylin–phloxin–saffron staining. All biopsies showed severe hypospermatogenesis with either Sertoli cell-only profile **(C**, **D)**, histological mosaicism profile with Leydig cell hyperplasia **(A**, **E)**, or maturation arrest at round spermatid stage **(B)** or spermatocyte stage **(F)**.

After gonadotropin treatment, total testosterone levels increased and AMH levels decreased. This indicates that Leydig cells retain their cellular function and ability to be stimulated by gonadotropins in X-AHC patients. The level of testosterone secretion stimulation by hCG in X-AHC patients varies from case to case (5, 9, 11–13, 34). It is already known physiologically that testosterone induces maturation of Sertoli cells, which express the androgen receptor, manifested by a stop in their multiplication and a sharp decrease of their AMH secretion (35, 36). Consequently, the decrease in AMH observed in our patients could suggest the presence of mature Sertoli cells which express the androgen receptor in X-AHC patients.

After gonadotropin treatment, we also showed an overall increase in BTV, although it remained below the normal range, suggesting a modest increase in germ cells. BTV did not respond to treatment in the adolescent patient where it was highest before the start of the gonadotropin treatment (patient 1) or in the patient treated in adulthood (patient 5). Interestingly, the greatest increase in BTV was in the patient who received rFSH alone (priming rFSH) during the first 6 months (patient 3). As reported by Dwyer et al. in congenital hypogonadotropic hypogonadism due to GnRH defect, priming rFSH treatment can increase the Sertoli cell population before testosterone secretion (induced by the addition of hCG) stops their multiplication (37). Unfortunately, patient 3 was not eligible for testicular biopsy and TESE to see if priming rFSH could improve his spermatogenesis. Inhibin B level variations were less clear, with a slight increase after gonadotropin treatment in some patients and a decrease in others, indicating a primary defect in Sertoli cell function. Our observations were consistent with other cases reported in literature which showed an increase in testicular volume (5, 13, 34) but no or only slight increase in inhibin B levels after gonadotropin therapy (9). However, there were some discrepancies. A study in an azoospermic 36-year-old man showed a rise in inhibin B after 5 months of combined gonadotropin treatment, but in a mild form of X-AHC (10), and another study reported no significant increase in testicular volume after gonadotropin treatment in seven adult patients (4.0 ± 2.9 vs. 4.9 ± 3.3 ml) (8). In any case, inhibin B response to

gonadotropin treatment, as BTV response, was generally much lower than in other forms of hypogonadotropic hypogonadism such as Kallmann syndrome (7, 38), which reinforces the idea of a peripheral gonadal defect in X-AHC.

Some authors reported progressive degradation of the hypothalamic-pituitary-gonadal axis with age in X-AHC patients. Galeotti et al. reported a cohort of eight X-AHC patients with normal minipuberty (27). Others described infants with normal or increased testicular volume for age and a physiologic minipuberty whereas the maternal uncle, bearing the same *NR0B1* variant, failed to enter puberty (32, 39). In terms of fertility, a mutated *NR0B1* murine model suggested that spermatogenesis may deteriorate gradually with age (40). This was supported in humans in a mild form of X-AHC in which inhibin B decreased from 148 ng/L at 35 years to 38 ng/L at 43 years (normal range, 80–270 ng/L), sperm count decreased from 4 million at 23 years to 0.05 million at 37 years, and testicular volume decreased from 20 ml bilaterally at 32 years to 15 ml at 47 years (10). Data from Galeotti et al. were also consistent with progressive degradation of spermatogenesis, with normal inhibin B values in the first year of life, decreasing in adolescence and adulthood according to the age-related normal ranges (27). Previous studies, using several combined gonadotropin drugs, doses, and treatment duration ranging from 5 months to 3 years, failed to restore spermatogenesis in X-AHC patients (5, 6, 9–12). None of these studies used priming rFSH. What these studies had in common was also that gonadotropin treatment was initiated in adulthood (5, 6, 8–12); it may therefore be advantageous to start gonadotropin treatment earlier in life. As inhibin B levels were not below the limit of quantification for any of our patients and in some cases BTV responded to gonadotropin therapy, we might expect to retrieve sperm cells in semen or testicular biopsy after TESE, but in fact failed to do so, whether gonadotropin treatment was started either in adulthood or at age of puberty. The gonadotropin treatment protocol we used may be contested, but a similar one allowed sperm cells to be retrieved from semen in almost the entire cohort of adults with hypogonadotropic hypogonadism (including 11 patients with Kallmann syndrome) after around 12 months of treatment on average (7). Inhibin B and BTV appear to be poorer biomarkers of spermatogenesis in X-AHC patients than in other forms of nonobstructive azoospermia, where elevation after gonadotropin treatment correlated with the presence of sperm cells in semen collection (7, 38).

Interestingly, some studies held out hope for X-AHC patients to be able to father children. Some cases of spontaneous paternity were reported, free of any drugs, but in mild forms of X-AHC. Vargas et al. reported a kindred with late-onset X-AHC where a man and his uncle had children at respectively 32 and 39 years of age, before diagnosis of primary adrenal insufficiency (26). The uncle was then totally azoospermic at 64 years of age. Raffin-Sanson et al. reported a man who had two sons, one at 35 years of age by *in vitro* fertilization and one naturally at 37 years of age. He was diagnosed with adrenal insufficiency at 19 years of age, and his sperm count decreased drastically with age (10). Otherwise, using a gonadotropin supplementation protocol almost identical to ours (administration of menotropin consisting of 150 IU FSH and 150 IU LH three times per week, combined with administration of 1,500 IU hCG two times per week for 20 months), Frapsauce et al. succeeded in retrieving sperm cells from a 25-year-old man with a classic form of X-AHC (adrenal crisis at 3 weeks of life) by testicular sperm extraction, with intracytoplasmic sperm injection resulting in the birth of a heathy child (13).

In the present study, testicular biopsies showed severe hypospermatogenesis with the absence of mature germ cells, although biopsy was realized during adolescence for two patients and a combined gonadotropin treatment was used. Spermatogonia and hyperplastic Leydig cells were seen in two of the three patients with testicular biopsy (one with biopsy during adolescence and the other during adulthood). Sertoli cell injury was found in all patients. These observations are consistent with the absence of sperm cells in semen or TESE after gonadotropin therapy, and with decreased inhibin B levels and reduced testicular volume unresponsive or poorly responsive to gonadotropin treatment. Histological examination in a murine model with mutated *NR0B1* highlighted a progressive degeneration of seminiferous tubules with hyperplastic Leydig cells and failure to maintain germ cells (40). In humans, Seminara et al. showed a Sertoli cell-only syndrome with scarce spermatogonia not maturing into sperm cells in a 27-year-old man treated with hCG for 7 years (5). In a 20-year-old man with X-AHC, testicular biopsy found a disorganized structure of seminiferous tubules with moderate hyperplastic Leydig cells and proliferative interstitial tissue after 6 months of gonadotrophin treatment (6), in line with the abnormalities found in the present patients. Interestingly, postmortem histological testicular examination of a newborn baby who had a mutated *NR0B1* and died of adrenal crisis at 23 days showed physiologic testicular histology for age with numerous Sertoli cells and numerous spermatogonia (9). Normal testicular histology was also described in a 9-year-old boy. The structure of his seminiferous tubes was conserved, and they contained spermatogonia, while Leydig cells were not hyperplastic (41). In the 25-year-old patient with a classic form of X-AHC and who fathered a child, reported by Frapsauce et al., biopsy showed mostly incomplete spermatogenesis up to spermatocyte stage but very rare focal spermatogenesis leading to mature sperm cells for TESE-intracytoplasmic sperm injection (13). The discrepancy between the present TESE results and those of Frapsauce et al. may be due to the fact that their patient did not carry the same *NR0B1* variant as ours, and it is important to bear in mind the heterogeneous spectrum of X-AHC even in patients carrying the same *NR0B1* variant (4, 10, 26, 27). However, it cannot be excluded that focal spermatogenesis existed elsewhere in our patients' testes but simply not in the multiple bilateral biopsied fragments.

5 PERSPECTIVES

Testicular biopsy failed to retrieve mature sperm cells although it was performed during adolescence in two patients under

gonadotropin treatment, suggesting that testicular biopsy may be performed as early as possible (at the age of usual spermatogenesis onset) after diagnosis of X-AHC. If spermatogonia and functional seminiferous tubules can be retrieved from the testicular biopsy, X-AHC may be an indication for the emerging *in vitro* spermatogenesis technology (42), on the hypothesis that spermatogenesis defect in X-AHC patients is due to impaired Sertoli cell function. In 2016, Perrard et al. managed for the first time to perform complete spermatogenesis from culture of adult human seminiferous tubule segments, using a bioreactor in a specific culture medium (42). Earlier gonadotropin treatment, at onset of puberty (around 11 years of age) or even before the rise in intratesticular testosterone secretion, with priming rFSH to optimize Sertoli cell function, should also be investigated.

6 CONCLUSION

The present data extend our understanding of X-AHC, reporting three new *NR0B1* variants. These variants were associated with classic forms of X-AHC with azoospermia not responding to combined gonadotropin treatment. No sperm cells could be retrieved from semen collection or testicular biopsy even when gonadotropin treatment was started at the age of puberty. However, spermatogonia were seen in testicular biopsies of two out of three patients, holding out hope for X-AHC patients to father children using *in vitro* spermatogenesis technique currently in development.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

VM, LR and JT wrote the manuscript. IP, DM, FD, and FR supervised the laboratory procedures. JT and VM performed the statistical analysis. JT, VM, FD, FR, and IP interpreted the data. Patient care was performed by HL, PC, FT, MN, NP, SGD, BC, and IP. All authors read, revised, and approved the final version of the manuscript.

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REFERENCES

1. Zanaria E, Muscatelli F, Bardoni B, Strom TM, Guioli S, Guo W, et al. An Unusual Member of the Nuclear Hormone Receptor Superfamily Responsible for X-Linked Adrenal Hypoplasia Congenita. *Nature* (1994) 372:635–41. doi: 10.1038/372635a0
2. Muscatelli F, Strom TM, Walker AP, Zanaria E, Récan D, Meindl A, et al. Mutations in the DAX-1 Gene Give Rise to Both X-Linked Adrenal Hypoplasia Congenita and Hypogonadotropic Hypogonadism. *Nature* (1994) 372:672–6. doi: 10.1038/372672a0
3. Mou L, Xie N, Yang L, Liu Y, Diao R, Cai Z, et al. A Novel Mutation of DAX-1 Associated With Secretory Azoospermia. *PloS One* (2015) 10:e0133997. doi: 10.1371/journal.pone.0133997
4. Jadhav U, Harris RM, Jameson JL. Hypogonadotropic Hypogonadism in Subjects With DAX1 Mutations. *Mol Cell Endocrinol* (2011) 346:65–73. doi: 10.1016/j.mce.2011.04.017
5. Seminara SB, Achermann JC, Genel M, Jameson JL, Crowley WF. X-Linked Adrenal Hypoplasia Congenita: A Mutation in DAX1 Expands the Phenotypic Spectrum in Males and Females. *J Clin Endocrinol Metab* (1999) 84:4501–9. doi: 10.1210/jcem.84.12.6172
6. Ozisik G, Mantovani G, Achermann JC, Persani L, Spada A, Weiss J, et al. An Alternate Translation Initiation Site Circumvents an Amino-Terminal DAX1 Nonsense Mutation Leading to a Mild Form of X-Linked Adrenal Hypoplasia Congenita. *J Clin Endocrinol Metab* (2003) 88:417–23. doi: 10.1210/jc.2002-021034
7. Rohayem J, Sinthofen N, Nieschlag E, Kliesch S, Zitzmann M. Causes of Hypogonadotropic Hypogonadism Predict Response to Gonadotropin Substitution in Adults. *Andrology* (2016) 4:87–94. doi: 10.1111/andr.12128
8. Zheng JJ, Wu XY, Nie M, Liu ZX, Wang X, Huang BK, et al. Dysfunction of Hypothalamic-Pituitary-Testicular Axis in Patients With Adrenal Hypoplasia Congenita Due to DAX-1 Gene Mutation. *Zhonghua Yi Xue Za Zhi* (2016) 96:1183–7. doi: 10.3760/cma.j.issn.0376-2491.2016.15.008
9. Brown P, Scobie GA, Townsend J, Bayne RAL, Seckl JR, Saunders PTK, et al. Identification of a Novel Missense Mutation That Is as Damaging to DAX-1 Repressor Function as a Nonsense Mutation. *J Clin Endocrinol Metab* (2003) 88:1341–9. doi: 10.1210/jc.2002-021560
10. Raffin-Sanson M-L, Oudet B, Salenave S, Brailly-Tabard S, Pehuet M, Christin-Maitre S, et al. A Man With a DAX1/NR0B1 Mutation, Normal Puberty, and an Intact Hypothalamic–Pituitary–Gonadal Axis But Deteriorating Oligospermia During Long-Term Follow-Up. *Eur J Endocrinol* (2013) 168:K45–50. doi: 10.1530/EJE-12-1055
11. Mantovani G, Ozisik G, Achermann JC, Romoli R, Borretta G, Persani L, et al. Hypogonadotropic Hypogonadism as a Presenting Feature of Late-Onset X-Linked Adrenal Hypoplasia Congenita. *J Clin Endocrinol Metab* (2002) 87:44–8. doi: 10.1210/jcem.87.1.8163
12. Mantovani G, De Menis E, Borretta G, Radetti G, Bondioni S, Spada A, et al. DAX1 and X-Linked Adrenal Hypoplasia Congenita: Clinical and Molecular Analysis in Five Patients. *Eur J Endocrinol* (2006) 154:685–9. doi: 10.1530/eje.1.02132
13. Frapsauce C, Ravel C, Legendre M, Sibony M, Mandelbaum J, Donadille B, et al. Birth After TESE-ICSI in a Man With Hypogonadotropic Hypogonadism and Congenital Adrenal Hypoplasia Linked to a DAX-1 (NR0B1) Mutation. *Hum Reprod* (2011) 26:724–8. doi: 10.1093/humrep/deq372
14. Lin L, Gu W-X, Ozisik G, To WS, Owen CJ, Jameson JL, et al. Analysis of DAX1 (NR0B1) and Steroidogenic Factor-1 (NR5A1) in Children and Adults With Primary Adrenal Failure: Ten Years' Experience. *J Clin Endocrinol Metab* (2006) 91:3048–54. doi: 10.1210/jc.2006-0603
15. Suntharalingham JP, Buonocore F, Duncan AJ, Achermann JC. DAX-1 (NR0B1) and Steroidogenic Factor-1 (SF-1, NR5A1) in Human Disease. *Best Pract Res Clin Endocrinol Metab* (2015) 29:607–19. doi: 10.1016/j.beem.2015.07.004
16. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and Guidelines for the Interpretation of Sequence Variants: A Joint Consensus Recommendation of the American College of Medical Genetics and Genomics

and the Association for Molecular Pathology. *Genet Med* (2015) 17:405–24. doi: 10.1038/gim.2015.30
17. Baux D, Van Goethem C, Ardouin O, Guignard T, Bergougnoux A, Koenig M, et al. MobiDetails: Online DNA Variants Interpretation. *Eur J Hum Genet* (2021) 29:356–60. doi: 10.1038/s41431-020-00755-z
18. Plotton I, Garby L, Morel Y, Lejeune H. Decrease of Anti-Mullerian Hormone in Genetic Spermatogenic Failure. *Andrologia* (2012) 44:349–54. doi: 10.1111/j.1439-0272.2010.01092.x
19. Pierik FH, Vreeburg JTM, Stijnen T, De Jong FH, Weber RFA. Serum Inhibin B as a Marker of Spermatogenesis. *J Clin Endocrinol Metab* (1998) 83:3110–4. doi: 10.1210/jcem.83.9.5121
20. Crofton PM, Evans AEM, Groome NP, Taylor MRH, Holland CV, Kelnar CJH. Inhibin B in Boys From Birth to Adulthood: Relationship With Age, Pubertal Stage, FSH and Testosterone. *Clin Endocrinol (Oxf)* (2002) 56:215–21. doi: 10.1046/j.0300-0664.2001.01448.x
21. World Health Organization. *WHO Laboratory Manual for the Examination and Processing of Human Semen* (2010). Available at: https://www.who.int/docs/default-source/reproductive-health/srhr-documents/infertility/examination-and-processing-of-human-semen-5ed-eng.pdf (Accessed December 16, 2021).
22. Plotton I, Giscard d'Estaing S, Cuzin B, Brosse A, Benchaib M, Lornage J, et al. Preliminary Results of a Prospective Study of Testicular Sperm Extraction in Young Versus Adult Patients With Nonmosaic 47,XXY Klinefelter Syndrome. *J Clin Endocrinol Metab* (2015) 100:961–7. doi: 10.1210/jc.2014-3083
23. Nistal M, Riestra ML, Galmés-Belmonte I, Paniagua R. Testicular Biopsy in Patients With Obstructive Azoospermia. *Am J Surg Pathol* (1999) 23:1546–54. doi: 10.1097/00000478-199912000-00013
24. Zhang Y-H, Huang B-L, Anyane-Yeboa K, Carvalho JAR, Clemons RD, Cole T, et al. Nine Novel Mutations in NR0B1 (DAX1) Causing Adrenal Hypoplasia Congenita. *Hum Mutat* (2001) 18:547. doi: 10.1002/humu.1236
25. Yang H, Wei H, Shen L, Kumar CS, Chen Q, Chen Y, et al. A Novel Stop-Loss DAX1 Variant Affecting its Protein-Interaction With SF1 Precedes the Adrenal Hypoplasia Congenital With Rare Spontaneous Precocious Puberty and Elevated Hypothalamic-Pituitary-Gonadal/Adrenal Axis Responses. *Eur J Med Genet* (2021) 64:104192. doi: 10.1016/j.ejmg.2021.104192
26. Vargas MCC, Moura FS, Elias CP, Carvalho SR, Rassi N, Kunii IS, et al. Spontaneous Fertility and Variable Spectrum of Reproductive Phenotype in a Family With Adult-Onset X-Linked Adrenal Insufficiency Harboring a Novel DAX-1/NR0B1 Mutation. *BMC Endocr Disord* (2020) 20:21. doi: 10.1186/s12902-020-0500-2
27. Galeotti C, Lahlou Z, Goullon D, Sarda-Thibault H, Cahen-Varsaux J, Bignon-Topalovic J, et al. Longitudinal Evaluation of the Hypothalamic-Pituitary-Testicular Function in 8 Boys With Adrenal Hypoplasia Congenita (AHC) Due to NR0B1 Mutations. *PloS One* (2012) 7:e39828. doi: 10.1371/journal.pone.0039828
28. Jensen TK, Andersson A-M, Hjollund NHI, Scheike T, Kolstad H, Giwercman A, et al. Inhibin B as a Serum Marker of Spermatogenesis: Correlation to Differences in Sperm Concentration and Follicle-Stimulating Hormone Levels. A Study of 349 Danish Men. *J Clin Endocrinol Metab* (1997) 82:4059–63. doi: 10.1210/jcem.82.12.4456
29. Kumanov P, Nandipati K, Tomova A, Agarwal A. Inhibin B is a Better Marker of Spermatogenesis Than Other Hormones in the Evaluation of Male Factor Infertility. *Fertil Steril* (2006) 86:332–8. doi: 10.1016/j.fertnstert.2006.01.022
30. Rey R. Regulation of Spermatogenesis. *Endocr Dev* (2003) 5:38–55. doi: 10.1159/000069300
31. Tabarin A, Achermann JC, Recan D, Bex V, Bertagna X, Christin-Maitre S, et al. A Novel Mutation in DAX1 Causes Delayed-Onset Adrenal Insufficiency and Incomplete Hypogonadotropic Hypogonadism. *J Clin Invest* (2000) 105:321–8. doi: 10.1172/JCI7212
32. Takahashi I, Takahashi T, Shoji Y, Takada G. Prolonged Activation of the Hypothalamus- Pituitary-Gonadal Axis in a Child With X-Linked Adrenal Hypoplasia Congenita: Adrenal Hypoplasia Congenita. *Clin Endocrinol* (2000) 53:127–9. doi: 10.1046/j.1365-2265.2000.01037.x
33. Bergadá I, Andreone L, Bedecarrás P, Ropelato MG, Copelli S, Laissue P, et al. Seminiferous Tubule Function in Delayed-Onset X-Linked Adrenal Hypoplasia Congenita Associated With Incomplete Hypogonadotrophic Hypogonadism. *Clin Endocrinol* (2007) 68:240–6. doi: 10.1111/j.1365-2265.2007.03026.x
34. Caron P, Imbeaud S, Bennet A, Plantavid M, Camerino G, Rochiccioli P. Combined Hypothalamic-Pituitary-Gonadal Defect in a Hypogonadic Man With a Novel Mutation in the DAX-1 Gene. *J Clin Endocrinol Metab* (1999) 84:3563–9. doi: 10.1210/jcem.84.10.6030
35. Rey R, Lordereau-Richard I, Carel J-C, Barbet P, Cate RL, Roger M, et al. Anti-Müllerian Hormone and Testosterone Serum Levels are Inversely During Normal and Precocious Pubertal Development. *J Clin Endocrinol Metab* (1993) 77:1220–6. doi: 10.1210/jcem.77.5.8077315
36. Rey R. Recent Advancement in the Treatment of Boys and Adolescents With Hypogonadism. *Ther Adv Endocrinol Metab* (2022) 13:1–17. doi: 10.1177/20420188211065660
37. Dwyer AA, Sykiotis GP, Hayes FJ, Boepple PA, Lee H, Loughlin KR, et al. Trial of Recombinant Follicle-Stimulating Hormone Pretreatment for GnRH-Induced Fertility in Patients With Congenital Hypogonadotropic Hypogonadism. *J Clin Endocrinol Metab* (2013) 98:E1790–5. doi: 10.1210/jc.2013-2518
38. Rohayem J, Hauffa BP, Zacharin M, Kliesch S, Zitzmann Mthe "German Adolescent Hypogonadotropic Hypogonadism Study Group". Testicular Growth and Spermatogenesis: New Goals for Pubertal Hormone Replacement in Boys With Hypogonadotropic Hypogonadism? -a Multicentre Prospective Study of hCG/rFSH Treatment Outcomes During Adolescence-. *Clin Endocrinol* (2017) 86:75–87. doi: 10.1111/cen.13164
39. Kaiserman KB, Nakamoto JM, Geffner ME, McCabe ERB. Minipuberty of Infancy and Adolescent Pubertal Function in Adrenal Hypoplasia Congenita. *J Pediatr* (1998) 133:300–2. doi: 10.1016/s0022-3476(98)70242-2
40. Yu RN, Ito M, Saunders TL, Camper SA, Jameson JL. Role of Ahch in Gonadal Development and Gametogenesis. *Nat Genet* (1998) 20:353–7. doi: 10.1038/3822
41. Morii M, Takahashi T, Takahashi I, Komatsu K, Sagishima M, Nanjo H, et al. X-Linked Adrenal Hypoplasia Congenita: Testicular Histology Before Puberty. *Pediatr Int* (2007) 49:526–9. doi: 10.1111/j.1442-200X.2007.02416.x
42. Perrard M-H, Sereni N, Schluth-Bolard C, Blondet A, d'Estaing SG, Plotton I, et al. Complete Human and Rat Ex Vivo Spermatogenesis From Fresh or Frozen Testicular Tissue. *Biol Reprod* (2016) 95:89–9. doi: 10.1095/biolreprod.116.142802

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Genotype, Mortality, Morbidity, and Outcomes of 3β-Hydroxysteroid Dehydrogenase Deficiency in Algeria

Asmahane Ladjouze[1], Malcolm Donaldson[2], Ingrid Plotton[3], Nacima Djenane[4], Kahina Mohammedi[1], Véronique Tardy-Guidollet[3], Delphine Mallet[3], Kamélia Boulesnane[1], Zair Bouzerar[1], Yves Morel[3] and Florence Roucher-Boulez[3]*

[1] Department of Paediatrics, Centre Hospitalo-Universitaire Bab El Oued, Algiers, Algeria, [2] Section of Child Health, School of Medicine, Queen Elizabeth University Hospital, Glasgow, United Kingdom, [3] Molecular Endocrinology and Rare Diseases, Hospices Civils de Lyon, Lyon University Hospital, Bron-Lyon, France, [4] Department of Pathological Anatomy, Centre Hospitalo-Universitaire Bab El Oued, Algiers, Algeria

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***Correspondence:**
Asmahane Ladjouze
a.ladjouze@univ-alger.dz

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Background: 3β-hydroxysteroid dehydrogenase 2 (3βHSD2) deficiency is a rare form of congenital adrenal hyperplasia (CAH), with fewer than 200 cases reported in the world literature and few data on outcomes.

Patients and Methods: We report a mixed longitudinal and cross-sectional study from a single Algerian center between 2007 and 2021. Virilization and under-masculinization were assessed using Prader staging and the external masculinization score (EMS), pubertal development staged according to the system of Tanner. Adrenal steroids were measured using mass spectrophotometry (LC-MS/MS). A genetic analysis of *HSD3B2* was performed using Sanger sequencing.

Results: A 3βHSD2 defect was confirmed in 6 males and 8 females from 10 families (8 consanguineous), with p.Pro222Gln mutation in all but two siblings with a novel deletion: c.453_464del or p.(Thr152_Pro155del). Probable 3βHSD2 deficiency was diagnosed retrospectively in a further 6 siblings who died, and in two patients from two other centers. In the genetically confirmed patients, the median (range) age at presentation was 20 (0–390) days, with salt-wasting (n = 14) and genital anomaly (n = 10). The Prader stage for female patients was 2 (1–2) with no posterior fusion of the labia. The EMS for males was 6 (3–9). Median (range) values at diagnosis for 17-hydroxyprogesterone (17-OHP), dehydroepiandrosterone sulfate (DHEA-S), and 17-hydroxypregnenolone (17OHPreg) were elevated: 73.7 (0.37–164.3) nmol/L; 501.2(9.4–5441.3) nmol/L, and 139.7 (10.9–1500) nmol/l (NB >90 nmol/L diagnostic of 3βHSD2 defect). Premature pubarche was observed in four patients (3F:1M). Six patients (5F:1M) entered puberty spontaneously, aged 11 (5–13) years in 5 girls and 11.5 years in one boy. Testicular adrenal rest tumors were found in three boys. Four girls reached menarche at 14.3 (11–14.5) years, with three developing adrenal masses (surgically excised in two) and polycystic ovary syndrome (PCOS), with radiological evidence of ovarian adrenal rest tumor in one. The median IQ was 90 (43–105), >100 in only two patients and <70 in three.

Conclusions: The prevalence of 3βHSD2 deficiency in Algeria appears high, with p.Pro222Gln being the most frequent mutation. Mortality is also high, with significant morbidity from adrenal tumors and PCOS in adolescence and an increased risk of learning disability. The finding of adrenal tumors in older patients with 3βHSD2 indicates under-replacement, requiring effective hydrocortisone and fludrocortisone treatment rather than surgical removal.

Keywords: 3-β hydroxysteroid dehydrogenase deficiency, 3βHSD2, HSD3B2, congenital adrenal hyperplasia, newborn screening, adrenal rest tumors, polycystic ovary syndrome

INTRODUCTION

3β-hydroxysteroid dehydrogenase type 2 (3βHSD2) deficiency is a rare cause of congenital adrenal hyperplasia (CAH) with an estimated birth prevalence of less than 1/1,000,000 (1) and with fewer than 200 families reported in the world literature (2). The condition is transmitted in an autosomal recessive pattern and results from mutations in the *HSD3B2* gene, which encodes the type II 3βHSD isoenzyme (3). With a severe *HSD3B2* gene defect, biosynthesis of all steroids—mineralocorticoid, glucocorticoid, and sex hormones—is impaired, resulting in varying degrees of salt-wasting (SW), and under-masculinisation in 46, XY individuals. The phenotype of 3βHSD deficiency is linked to the type of *HSD3B2* mutation and to the residual activity of the 3βHSD enzyme. Thus, as in 21-hydroxylase deficiency (21OHD), classical salt-wasting forms have been described in 3βHSD deficiency as well as classical non-salt-wasting forms presenting with isolated under-masculinisation in 46,XY individuals (4). In affected women, virilization is usually absent or limited to clitoral enlargement. No *HSD3B2* mutation has been found in presumed non-classical forms with milder hyperandrogenism (5). While testicular adrenal rest tumor is well-recognized in 21OHD and has also been described in the ovaries of female patients with this variety of CAH (6–9), there have been only rare reports of adrenal rests in 46, XY patients with 3βHSD2 deficiency. Moreover, there are no confirmed cases of ovarian adrenal rest tumor, with adrenal rest nodules having been found in the broad ligament and near the ovarian hilus in a 35-year-old woman with 3βHSD deficiency, but not in the ovaries themselves (10).

To date, only two series of 3βHSD deficiency with more than 10 subjects have been reported (11, 12), and there is no large series describing the characteristics of patients with the p.Pro222Gln mutation which is the most frequent mutation encountered in Algeria, being found in all families but one in our series, and is also found in Latin American countries such as Colombia and Brazil (13–15). The hormonal criteria of a high 17OHPreg [basal or ACTH stimulated >90 nmol/l (16)] is preferred to the Δ5/Δ4 ratio [17 OH-Pregnenolone/Cortisol>103 or 181 nmol/l (16, 17)]. With liquid chromatography coupled to the tandem mass spectrometry (LC-MS/MS) method, these cut-offs have yet to be established. Moreover, genetic testing (when available) is recommended to confirm the diagnosis.

In Algeria, a country with a high birth rate of 22.2 births/1,000 population (18) and high levels of consanguinity (38%), we have accumulated a series of 14 patients from 10 families with confirmed 3βHSD2 deficiency (3BHSD2). We have been struck by the relative frequency of the disorder compared with other causes of CAH, how frequently it is misdiagnosed as 21-OHD, and the high rate of sibling deaths in the families. The purpose of this study, therefore, is to detail the presentation and outcome of 3βHSD deficiency in our Algerian families, make an estimate of its prevalence among other forms of CAH, and draw attention to some long-term problems and complications. These include developmental delay, ovarian adrenal rest tumor, and polycystic ovary syndrome (PCOS).

PATIENTS AND METHODS

Clinical and hormonal data were collected from the medical records of patients attending a single center, the Pediatric Department of the Centre Hospitalo-Universitaire (CHU) of Bab El Oued, Algiers, Algeria over a fourteen-year period (2007–2021). Although patients from all over Algeria attend CHU Bab El Oued, at least ten other units (pediatric and adult) also receive endocrine referrals. In the absence of a national registry of CAH or rare diseases, and in an attempt to ascertain the exact number of patients followed for 3βHSD2 deficiency during the study period, we contacted all pediatric endocrinologists in Algeria, asking if they had seen one or more confirmed cases. Also, to estimate the prevalence of 3βHSD2 deficiency among other forms of CAH, we compared the number of patients with 3βHSD2 deficiency to the number of patients with other forms of CAH in our department.

Data Retrieval

Details from the case notes of the patients studied were recorded using an electronic form (Epi-info7) and included the following: date and year of birth, sex, birth weight and gestation, mode of delivery, age at presentation, start of medical treatment, and definitive diagnosis of 3βHSD deficiency. Details of the presence and degree of consanguinity; and a history of sibling deaths from a) salt-wasting (indicative of 3βHSD2 deficiency); and b) unclassified illness during infancy, were recorded. Examination findings including Prader stage (19) and the External Masculinisation Score (EMS) described by Ahmed and colleagues (20) were also recorded. Finally, biochemical and radiological data, and details of surgical and medical treatment were collated.

Clinical Review

In April 2019, and again in March 2021, all patients were invited to attend CHU Bab El Oued for clinical assessment, which included auxology, expressed according to the 2007 WHO References and standards (21, 22), blood pressure measurement, pubertal staging, Prader and EMS scoring, and clarification (where necessary) concerning consanguinity and sibling health. An IQ test was also performed using the Wechsler scale [Wechsler Preschool and Primary Scales of Intelligence (WPPSI) (23)] and the Khos block-design test (24) for preschool children. Further biochemistry and radiology assessments were also carried out at this time. When patients were fully assessed in both 2019 and 2021, the most recent clinical and biochemical data are given in the *Results* section.

Biochemistry Assays

Blood samples were normally collected between 8 and 10 a.m. Cortisol, 17-hydroxyprogesterone (17-OHP), serum dehydroepiandrosterone sulfate (DHEA-S), delta4-androstenedione (Δ4A) and testosterone were measured in the laboratory of the department of nuclear medicine in CHU Bab El Oued using radioimmunoassay (RIA). Renin levels were measured in the laboratory of the Centre Pierre Marie Curie Hospital, Algiers, using RIA (Cisbio Bioassays).

Since 17-hydroxypregnenolone (17OHPreg) assay is not available in Algeria, blood samples were sent to Laboratoire Cerba, France and measured using liquid chromatography coupled to tandem Mass Spectrometry LC MS/MS method. Some steroids were reassessed in 2019 and 2021 by LC MS/MS at Lyon University Hospital, France (17OHP, DHEA, 17OHPregnenolone).

Age-appropriate reference ranges are given in the *Results* section and are taken from values established in the laboratory of Lyon, France, supplemented in the case of DHEA by data from Kushnir et al. (25) (please see **Supplementary Table S1**). Normative data from Lyon were determined from plasma samples, drawn at 8 a.m. in subjects beyond early childhood, using the LC MS/MS technique.

Genetic Analysis

Genetic analysis, after informed consent, was performed at the Department of Molecular Endocrinology and Rare Diseases, Lyon University Hospital, France, as previously described by Sanger sequencing (26) and *in vitro* functional studies (14).

Ethical Approval

Written informed consent was obtained from all families for genetic testing. The local ethics committee was informed and approved the study as a clinical audit.

Statistical Analysis

Anthropometric data were expressed as standard deviation score (SDS) using the World Health Organization 2007 data (WHO 2007, Anthro plus software) (21, 22). Data analysis was carried out using the software Epi Info 7 (7.2.2.6). A Student t-test was used to compare the age at diagnosis and treatment in male and female patients.

RESULTS

At the end of the study period, 273 patients from 227 families had been diagnosed with classic CAH in our clinic at CHU Bab El Oued. Of these, 3βHSD2 deficiency was diagnosed and confirmed by molecular studies in 14 patients from 10 families, and their pedigrees are shown in **Figure 1**. After 21-hydroxylase deficiency, with 243 patients from 207 families, 3βHSD2 deficiency was the next most common form of CAH, accounting for 5% of cases, and was more frequent than 11-βhydroxylase deficiency (13 patients from 8 families) and StAR protein deficiency (6 patients from 4 families).

The fourteen patients (eight females) were from ten families, with consanguinity (parents first cousins) in eight. Four patients from three families (F, G, and I) were from the same region in the north-center of Algeria, the province of Boumerdès (**Figure 1**).

Four children (3 boys) from family E, a family with poor socioeconomic circumstances, suffered from a separate severe congenital motor disability syndrome. Two of these children, E II-4 and II-5, died at the ages of 8 and 12 years with severe malnutrition.

Six siblings from 4 families died in infancy, of whom three (Family E II-1, II-2, and II-3) had a clear history of salt-wasting, while three (Family F II-2, G II-2, and J II-1) died with adrenal insufficiency while on hydrocortisone treatment. The median (range) age at death for these six siblings was 19.4 (0.5–48) months.

Table 1 shows the clinical features of the 14 patients with confirmed 3βHSD2 deficiency. Four patients had been diagnosed originally as having 21-OH deficiency (B II-1; B II-2; E II-8; and G II-1) and were treated with hydrocortisone only; two patients (A II-3 and B II-2) were diagnosed soon after birth by screening since one sibling was already being managed for CAH (A II-2 and B II-1). Patient B II-2 was screened at birth, but with suspected 21OHD.

Prevalence of 3βHSD Deficiency

Apart from the 14 confirmed and six unconfirmed but probable patients mentioned, we are aware of only two other patients with 3βHSD, one diagnosed biochemically in our center, in whom genetic studies are pending, and the other being followed by a colleague in France. However, since children are also sometimes followed by adult endocrinologists and other children have probably died in infancy, this number is almost certainly an underestimate.

Presentation of the 14 Confirmed Patients

(See **Table 1**) In the absence of any systematic newborn screening program in our country, all but two patients (A.II.3 and B II-2), who were diagnosed by neonatal family screening, presented with severe salt-wasting (SW) during infancy, mean ± SD (range) age 2.2 ± 3.3 (0.1–13) months. SW syndrome was associated with a disorder of sex development (DSD) in all male patients, but was not the principal cause of referral. Two patients presented with SW in the early neonatal period (3–10 days), 7 aged 11–28 days, and 5 after 28 days.

The median (range) age at presentation with either SW, DSD or both was 2.4 weeks (3 days–13 months). There was no male predominance in our patients, despite the absence of ambiguous

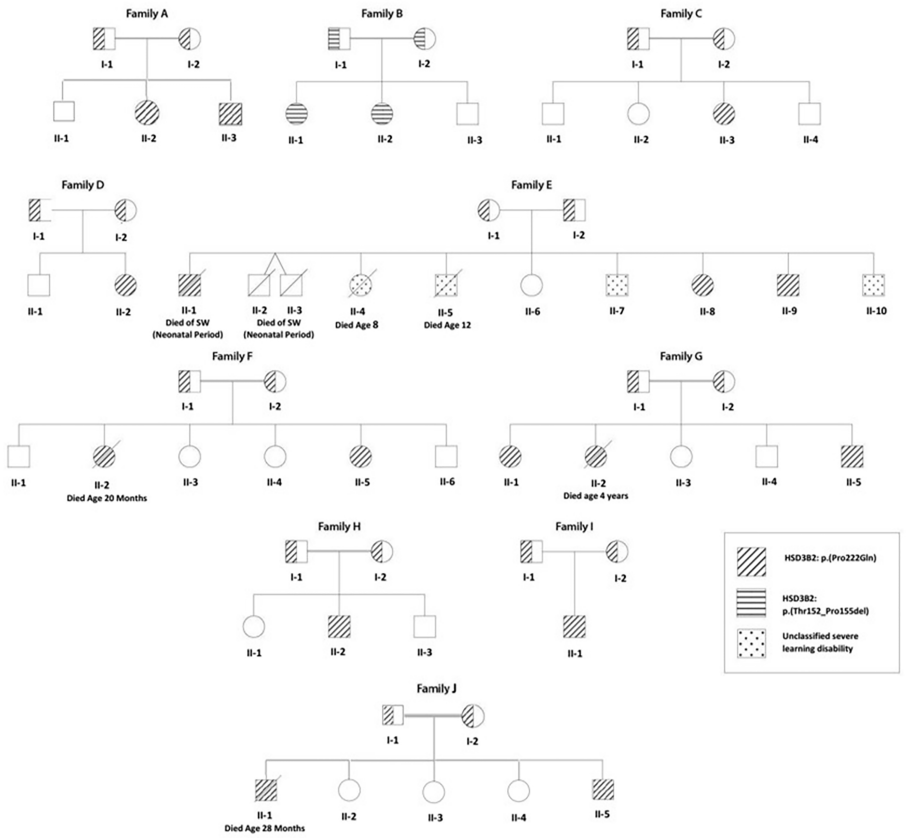

FIGURE 1 | Family tree of 10 Algerian families (8 consanguineous) with a) 3βHSD2 deficiency (individuals shown as hatched circles or squares); and b) an unclassified severe learning disability syndrome (affected individuals shown as speckled circles or squares). A HSD3B2: p.Pro222Gln mutation was found in 9 families (diagonal hatching), while Family B shows a novel HSD3B2: p.Thr152_Pro155del mutation (horizontal hatching). Of 42 children born to the 10 families, 3βHSD2 deficiency was genetically proven in 18 (2 deaths) and suspected on clinical grounds in twins from Family E with neonatal death from salt-wasting.

genitalia in females. Mean ± SD age at clinical/biochemical diagnosis was 1.3 ± 1.5 months in males and 2.4 ± 4.3 months in females ($p = 0.5$).

The median (range) age at the start of treatment with hydrocortisone was 1.25 (0.1–13) months. Since fludrocortisone is not widely available in Algeria, mineralocorticoid treatment was not always possible and was often not administered regularly.

The median (range) age of the patients at the time of referral to our department at CHU Bab El Oued for further investigations was 50.5 months (3 days–16.5 years). Ten were seen within the first year of life, while 4 females (B II-1, B II-2, E II-8, and G II-1) were referred after the age of 10 years (10.4–16.5 years). These four patients were already receiving steroid treatment and had been misdiagnosed as having 21 OHD.

Presentation, DSD Status and Definitive Diagnosis in Females

The eight females presented with salt-wasting only (5), salt-wasting with clitoromegaly (2), and after being screened at birth (1). Virilization in girls was mild, with two patients not significantly virilized, two at Prader stage 1 (clitoromegaly only), and 4 at Prader stage 2 (clitoromegaly with narrowing of the distal vagina) (see **Figure 2A**). None had labial fusion. Clitoromegaly was more severe (4 cm) in patient E II-8, in whom the diagnosis was made well after the neonatal period at 3 months (**Figure 2A**). At presentation at 13 months, one girl (C.II.3) had Prader-stage P2 pubic hair.

Due to non-availability of fludrocortisone, the four older female patients who had been initially misdiagnosed as 21-OH deficiency and had been treated with hydrocortisone alone. In these patients, adjustments to hydrocortisone dosing had been made in relation to 17OHP levels and not to 17OHPreg levels, leading to inadequate treatment.

Presentation, DSD Status and DSD Management in Males

The six males presented following family screening (1), with genital anomaly (1), salt-wasting (1), and both genital anomalies and salt-wasting (3). Two males were severely under-masculinized with EMS scores of 3 and 3.5/12, including patient E II-9 (**Figures 2B, C**) and two mildly under-masculinized (EMS scores of 6 and 9/12), including patient G II-5. All six patients received testosterone enanthate (50 mg/

TABLE 1 | Clinical data and features at first examination for 14 Algerian patients from 10 families with confirmed 3β-hydroxysteroid dehydrogenase (3βHSD) deficiency.

Patient No.	Pedigree	Parental consanguinity	Sex	BW (kg)	GA (w)	Mode of presentation	Genital status at diagnosis		SW	Age at presentation	Age at start of treatment	Age at definitive diagnosis of 3βHSD	Genetic mutation
							Females Prader stage (Clitoral length in cm)	Males EMS (Penile length in cm)					
1	A II-2	1st cousin	F	3.35	37	SW	1 (ND)	–	+	3w	7w	7w	p.(Pro222Gln)
2	A II-3	1st cousin	M	3.25	39	DSD + SCR	–	3.5 (2)	+	3 d	3 d**	3 d	p.(Pro222Gln)
3	B II-1	1st cousin	F	4	41	SW + DSD	2 (4)	–	+	3m	4m*	6w	p.(Thr152_Pro155del)
4	B II-2	1st cousin	F	2.7	41	SCR	1 (0.5)	–	+	4w	1m *	5w	p.(Thr152_Pro155del)
5	C II-3	2nd cousin	F	3.2	41	SW + DSD	2 (1.5)	–	+	13m	13m	16m	p.(Pro222Gln)
6	D II-1	No	F	2.6	41	SW	1 (ND)	–	+	14d	6w	6w	p.(Pro222Gln)
7	E II-8	2nd cousin	F	ND	41	SW	2 (1.5)	–	+	14d	4w*	5w	p.(Pro222Gln)
8	E II-9	2nd cousin	M	3	40	SW + DSD	–	6 (2)	+	19d	3w	5.3m	p.(Pro222Gln)
9	F II-1	1st cousin	F	ND	41	SW	2 (1)	–	+	17d	3m	7.3m	p.(Pro222Gln)
10	G II-5	2nd cousin	M	5	41.5	SW + DSD	–	9 (3)	+	3d	3d	3d	p.(Pro222Gln)
11	G II-1	2nd cousin	F	3.4	41	SW + DSD	2 (ND)	–	+	14d	1m*	15y	p.(Pro222Gln)
12	H II-2	1st cousin	M	4	41	SW	–	6 (2)	+	2m	2m	3.8m	p.(Pro222Gln)
13	I II-1	No	M	3.3	40	DSD		6 (2)	+	3w	3w	3w	p.(Pro222Gln)
14	J II-5	2nd cousin	M	3.7	40	DSD + SW		3 (2.8)	+	4m	4m	16m	p.(Pro222Gln)

*Age at presentation, start of treatment and definitive diagnosis of 3βHSD deficiency is given in days (d), weeks (w), months (m) or years (y). BW, birth weight; GA, gestational age; SW, salt-wasting; F, female; M, male; EMS, External Masculinization score (maximum 12); SCR, screening; ND, not documented; DSD, disorder of sex development. *Initially diagnosed as 21-OH deficiency. **Treatment was started at birth, the patient presented with SW subsequently.*

month for 3 months) during the first months of life, and four underwent uncomplicated surgical correction of hypospadias. So far, one patient (E II-9) has developed spontaneous puberty without any need for testosterone supplementation.

Biochemical Data

Table 2 shows the initial and current biochemical status of the 14 patients with confirmed 3βHSD deficiency. The sensitivity of the hormones measured in showing values above the reference range was 100% for 17 OH-pregnenolone and DHEA-S except in patients in whom the measurements were obtained while on treatment.

Initial 17OH-Progesterone (17OHP) was mildly elevated at 79.2 (7.7–804) nmol/l) [normal values 0.4–3.3], while 17 OH-Pregnenolone (17OHPreg), DHEA-S and renin were elevated in all patients, respectively—157 (112.2–1500) nmol/l for 17OHPreg [normal values 0.13–13.7]; 687 (53–5442) µg/dl for DHEA-S [30–333]; and 892 (360–16,634) pg/ml for Renin [360–1,040]. Delta4-Androstenedione was only mildly elevated in some patients (2.24 (0.01–6.06) ng/dl [normal values 0.21–3.08]. When reassessed by LC-MS/MS (patients off treatment for one day), 17OH-Preg was high in most patients at 89.13 (1.06–132) nmol/l, while 17 OHP [2.7 (0.08–7.3) nmol/l] and DHEA-S [4.82 (2.88–20.14) nmol/l], were normal or only slightly elevated in all patients.

Genetic Analysis

(See **Figures 1**, **3** and **Table 1**) All but two of the 14 patients were homozygous for the null mutation, p.(Pro222Gln) (c.665C >A). The two sisters of Family B were homozygous for a novel 12bp deletion (c.453_464del) deleting 4 amino acids p.(Thr152_Pro155del). As these amino acids are located within the characteristic catalytic Y-X-X-X-K site, this mutation should be a null mutation, hence the good genotype/phenotype correlation observed (**Figure 3**).

Clinical Outcomes

Table 3 shows the status of the 14 patients at the last review in 2019 or 2021. All patients were treated with hydrocortisone at a mean (± SD) dose of 15.2 ± 0.8 mg/m²/day. Owing to problems with fludrocortisone availability, three patients were not receiving this at the time of their last evaluation, and the remaining patients were on a dose of 54 ± 25 µg/day. Of note, fludrocortisone treatment is either imported from Spain twice a year in bulk by compassionate health professionals or provided at cost or for free to the patients at the discretion of the pediatric endocrinologist or shipped directly by family members living abroad (28).

At the most recent visit, the median age was 8.7 (1.7–21.7) years, height 0.24 (−1.96 to +1.45) SDS, with 5 patients <−1 SDS; BMI +1.06 (−1.36 to +6.3) SDS, with 7 patients >+1 SDS and 3 patients >+2 SDS.

Seven girls reached Tanner B2 and P2 during the study period, at 9 (8–13) and 10 (1.25–10) years old. Only one boy (E II-9) had entered puberty at G2 aged 11.5 years. In the absence of adequate treatment, this patient had already presented with premature pubarche aged 7 years. Another boy (H II-2) presented with premature pubarche at the age of 8 years.

Complications of 3βHSD Deficiency

Six of the 14 patients experienced one or more acute illnesses with SW crises after diagnosis, but there were no deaths.

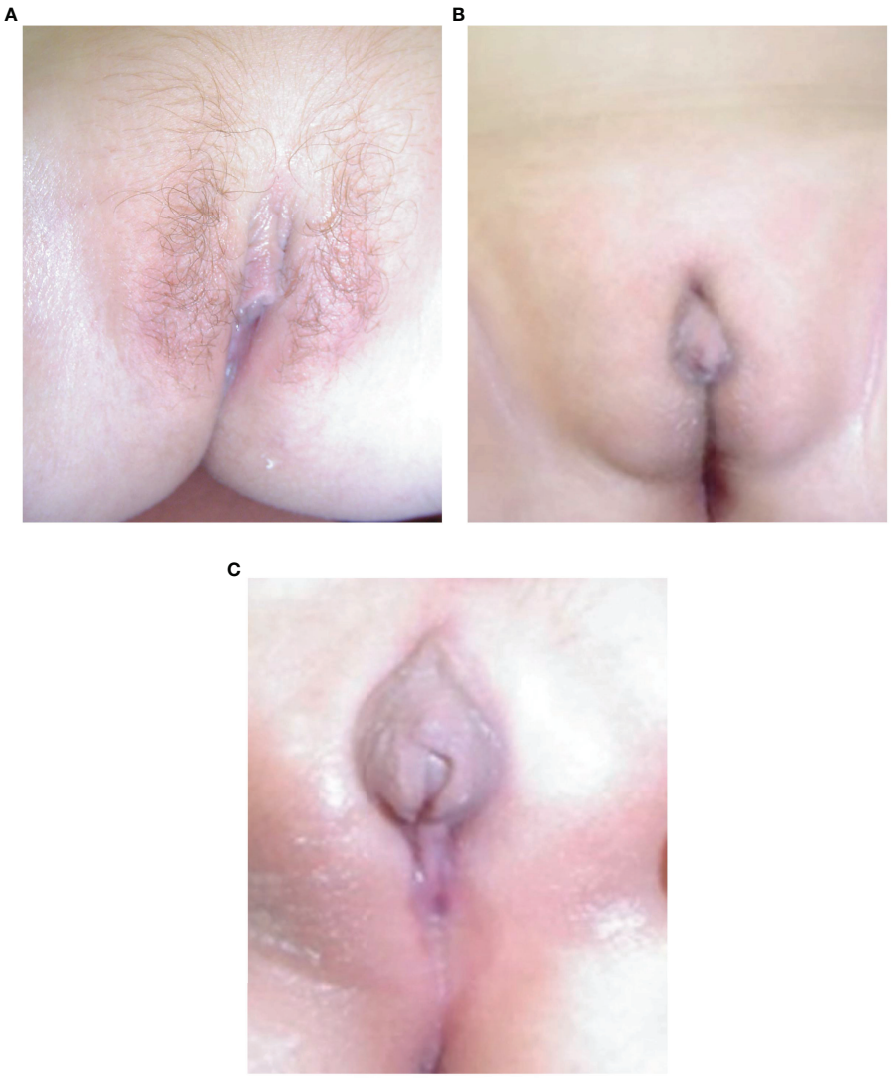

FIGURE 2 | **(A–C)** Appearance of external genitalia in two siblings from family E with 3βHSD2 deficiency due to a p.P222Q mutation, showing virilization with clitoromegaly and pubic hair in the sister, E II-8 **(A)** and under-masculinisation in the brother, EII-9 **(B, C)**.

Overweight (BMI >1 SDS) was seen in seven patients. Only three patients were obese (BMI >+2 SDS) even though all subjects were receiving hydrocortisone doses that were above the physiologic replacement level of 8 mg/m^2/day. However, we were unable to demonstrate a direct relationship between obesity and hydrocortisone dose, which was between 13 and 14.8 mg/m^2/day in the three obese patients.

Although the four girls reaching menarche during the study period experienced this within the normal age range (11.5–14.5 years), three of these girls (patients B II-1, B II-2, and G II-1) had oligo-amenorrhea and met the criteria for PCOS (29) with a combination of menstrual irregularity, clinical features of hyperandrogenism (hirsutism and severe acne), and enlarged, cystic ovaries. Ovarian volumes were very large in all three girls: 73 × 47 × 40 mm and 54 × 40 × 30 with cysts up to 68 × 40 mm in B II-1; 54 × 20 × 30 and 63 × 30 × 20 with cysts >25–35 mm in B II-2; and 48 × 42 × 55 and 84 × 55 × 40 mm with cysts >40 mm in G II-1. Patient E II-8 also had large ovaries (29 × 28 × 49 and 33 × 22.5 × 39) with large cysts measuring 38 × 36 mm on the most recent pelvic ultrasound. However, this girl did not have either prolonged amenorrhea or severe hyperandrogenism, and so the diagnosis was one of the polycystic ovaries rather than PCOS.

Stature was normal, although one patient had received growth hormone therapy to offset short stature with bone age advance.

Adrenal Tumor Formation, Testicular Adrenal Rest Tumor and Ovarian Adrenal Rest Tumor

Two male patients (E II-9 and G II-5) were diagnosed with testicular adrenal rest tumor (TART) by systematic testicular

TABLE 2 | Hormonal data in 14 Algerian patients with 3β-HSD deficiency.

Patient No.	First available analysis						Last available analysis (LC-MS/MS)			
	17OHP (nmol/l) RIA	Delta4-A (ng/dl)RIA	17 OH-Preg (nmol/l)(LC MS/MS)	DHEA-S (µg/dl)RIA	ACTH (pg/ml)	Renin (pg/ml)	17OHP LC-MS/MS (nmol/l)	17 OH-Preg LC-MS/MS (nmol/l)	DHEA LC-MS/MS (nmol/l)	DHEA-S LC-MS/MS (nmol/l)
1/A II-2	60	6.06	140.4	464.38		933	4.12	119.69	30.60	4.82
2/A II-3	0.37*			9.4*		2*	<0.3*	1.06*	0.89*	
3/B II-1	164.3	5.73	1297	900	2,135	851	2.14			
4/B II-2	320	2.34	20.8*	1,000	133	960	1.47			
5/C II-3	242	2.24	112.21	538	475	360	4.3	67.57	30.60	3.98
6/D II-1	19.26*	1	159.33	1,105	30.98	1,040	3.5	131.99	77.28	2.88
7/E II-8	7.75*	0.98	127	120		597	1.2	27.65	77.08	
8/E II-9	99	4.95	139	5,441.29		540	7.3	92.63		
9/F II-1	84.85	0.32	295	150			1	43.02	3.46	
10/G II-5	1.83*	2.33*	17.75*	1,080*		16,634	3.5	89.13	27.62	16.63
11/G II-1	804		157	687		10,665	2.14	93	4.37*	20.14
12/H II-2	60	0.01	10.9*	4.32	234.8	802	4.15	127.2		19.35
13/I II-1	73.7		1,500	53			0.08*	1.75*	0.30*	2.9*
14/J II-5	41.6			34.84			3.23*			
Reference Range	[0.4–3.3]	[0.21–3.08]	[0.13–13.7]	[30–333]	[29–38]	[360–1,040]	[0.49–1.87]	[0.13–13.7]	0.5–2 y [0.2–8.7]	1–4 y: [10–530] 5–9 y [80–2,310]

**Analysis performed while on hydrocortisone treatment. 17-OHP, 17 hydroxyprogesterone; 17OH-Preg, 17 hydroxypregnenolone; DHEAS, dehydroepiandrosterone sulfate; ACTH, adrenocorticotrophic hormone; Delta4-A, Delta 4-Androstenedione; LC-MS/MS, Liquid Chromatography coupled to tandem Mass Spectrometry.*
*Please see **Supplementary Table S1** for age-appropriate reference ranges. Hormonal analysis was performed where possible either before starting treatment or within a day of treatment.*

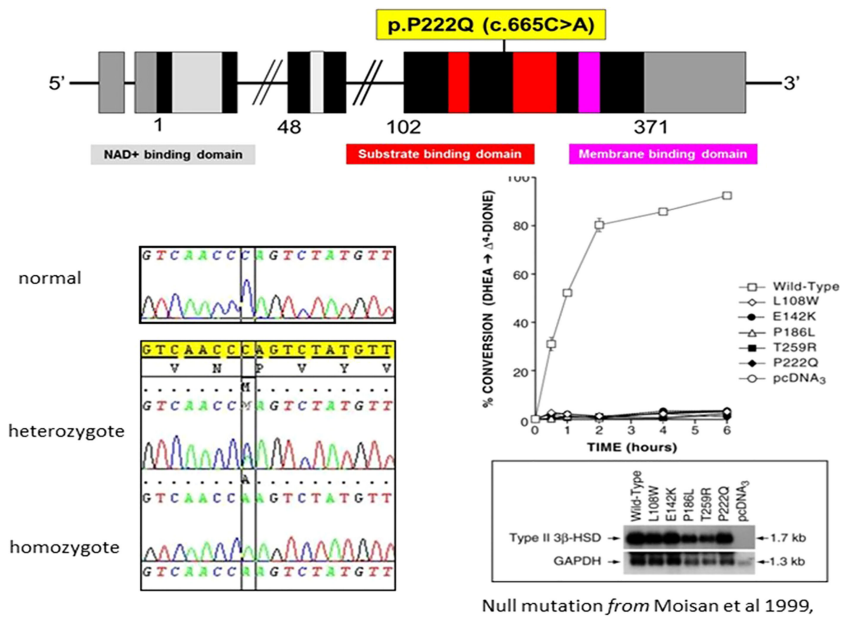

FIGURE 3 | Characteristics of the p.P222Q mutation of the HSD3B2 gene [From Moisan et al, 1999 (27)] —reproduced by kind permission of Oxford University Press).

ultrasonography at 5 and 10 years, testicular examination having revealed no abnormality. One patient (E II-9) had been inadequately treated during infancy and childhood because of fludrocortisone unavailability and poor compliance.

The three older girls (B II-1, B II-2, and G II-1) with PCOS also presented with adrenal masses at 13, 15, and 16 years of age (see **Table 3** and patient B II-1 in **Figure 4**). In patient G II-1, routine pelvic ultrasonography showed a large right adrenal mass, measuring 27 × 30 mm. This mass was of suspect appearance on pelvic computed tomography with heterogeneous enhancement, including necrotic areas in contact with the inferior vena cava, and was therefore surgically removed and analyzed in view of the

TABLE 3 | Status at most recent follow-up in 14 Algerian patients with genetically confirmed 3β-hydroxysteroid dehydrogenase.

Patient No. Pedigree	Sex	Age (yr)	BA (yr)	BP (mmHg)	HC dose (mg/m²/d)	FC dose (µg/d)	Height (cm/SDS)	BMI (kg/m²)/SDS	Tanner Stage	Age at B2/G2	Age at P2	Age at menarche	Complication	IQ/DQ
1/A II-2	F	11.5	12	95/60	16.2	25	150/+0.31	16.4/–0.54	B3P4A3	8.5	5	–	Premature pubarche	78
2/A II-3	M	1.67	ND	80/50	15	50	79/–1.96	18.3/+1.67	G1P1A1	–	–	–	Short stature	ND
3/B II-1	F	18.32	>18	115/75	15	100	157/–0.93	24.7/+0.97	B4P4A3	9	10	11.5	PCOS Adrenal tumor	105
4/B II-2	F	17.75	18	110/75	15	100	151/–1.81	19.7/–0.53	B4P4A3	11	10	14.5	GH treatment for short stature PCOS Adrenal tumor	99
5/C II-3	F	8.75	8.83	90/60	15.9	50	128/–0.53	25/–0.58	B2P3A1	8.5	1.25	–	Premature pubarche	98
6/D II-1	F	8	9	90/60	14.8	50	133/+1.06	37.7/+2.21	B2P3A1	8	5.5	–	Premature pubarche Obesity	87
7/E II-8	F	18.32	17	90/70	14.5	*	151/–1.8	18.4/–1.09	B4P5A3	11	10	14	Learning disability Probable OART	43
8/E II-.9	M	14.32	14	100/60	14.38	*	161/–1.15	16.6/–1.36	G4P5A3	11.5	7	–	TART Learning disability	49
9/F II-1	F	8.22	7.83	90/40	15	50	120.5/–1.25	20/+1.77	B1P1A1	–	–	–	–	80
10/G II-5	M	6.7	7	80/60	15	50	127.5/+1.45	17.2/+1.15	G1P1A1	–	–	–	TART	90
11/G II-1	F	21.7	>18	90/70	16;2	50	165/+0.28	27.5/+1.58	B5P5A3	13	10	14.5	PCOS Adrenal tumor	104
12/H II-2	M	9	12	90/70	14	*	136/+0.549	24.9/+311	G1P2A1	–	8	–	Obesity Learning disability	55
13/I II-1	M	4.37	4	80/60	13.5	25	106/+0.05	25.8/+6.3	G1P1A1	–	–	–	Obesity	98
14/J II-5	M	4.7	4	90/65	16.5	50	110/–0.37	14/–0.95	G1P1A1	–	–	–	–	90

*BA, bone age; BP, Blood Pressure; HC, hydrocortisone; FC, fludrocortisone; BMI, body mass index; PCOS, polycystic ovary syndrome; TART, testicular adrenal rest tumor; OART, ovarian adrenal rest tumor; IQ, Intellectual quotient; DQ, developmental quotient (in children aged <3 years); NA, not available; ND, not done (not appropriate for age); *FC stopped due to lack of availability. IQ could not be done but the child had bad results at school.*

suspicion of malignancy. Initial pathological analysis favored an adrenocortical tumor. After a second analysis, the diagnosis was revised to adrenal cortical hyperplasia secondary to under-suppressed CAH (**Figure 5**). Post-operatively, hyperandrogenism persisted in this patient, and pelvic computed tomography revealed a large solid mass measuring 40 × 42 mm within the left ovary, which was polycystic as described above. This finding was considered highly suggestive of an ovarian adrenal rest tumor (OART).

Systematic pelvic ultrasonography also showed adrenal masses in the two affected sisters of family B. The older sister (B II-1) was 15 when the mass was diagnosed, a large left adrenal mass measuring 63 × 52 × 51 mm (see **Figures 4A, B**). The evaluation showed no clinical, biological, or radiological evidence of pheochromocytoma. The adrenal mass was removed, and the analysis favored adrenal cortical hyperplasia. Her sister (B II-II) had a left adrenal mass measuring 20 × 25 mm which is currently being kept under surveillance.

Intelligence Quotient

(see **Table 3**, Far Right-Hand Column) An intelligence quotient was assessed in all but one patient, who was too young to be tested. The median IQ (range) was 90 (43–109) (n = 13) and the scores were ranked as follows: 100–110, n = 2; 90–99, n = 5; 80–89, n = 2; 70–79, n = 1; <70, n = 3. The three patients with IQ scores <70 were H II-2 (IQ 55), E II-9 (IQ 49), and E II-8 (IQ 43). Of note, siblings E II-8 and 9 are from kinship in which other siblings had an unclassified global neuro-disability disorder featuring severe cerebral palsy, which appears unrelated to 3βHSD2 deficiency. However, both parents of family E and their one unaffected child (E II-6) are of normal intelligence. There was no correlation between IQ and age at the start of treatment (p = 1).

DISCUSSION

Prevalence

Despite the impossibility of establishing the exact prevalence of 3βHSD2 deficiency, given the absence of a national program of neonatal screening and the lack of national registries for rare diseases in the Maghreb countries, we have nevertheless observed that the prevalence of 3βHSD2 deficiency appears higher in Algeria than elsewhere. Indeed, in a large cohort including all patients with defects in steroid biosynthesis investigated in the laboratory of molecular endocrinology and rare diseases of Lyon Hospital, France, 3βHSD2 deficiency is the most rare form of CAH (2). Globally, 3βHSD2 deficiency is estimated to account for less than 5% of all CAH and is extremely rare except in specific populations such as the Old Order Amish in North America (11) and Turkey (12). Even so, the prevalence described in our series is certain to be an underestimate because of patients

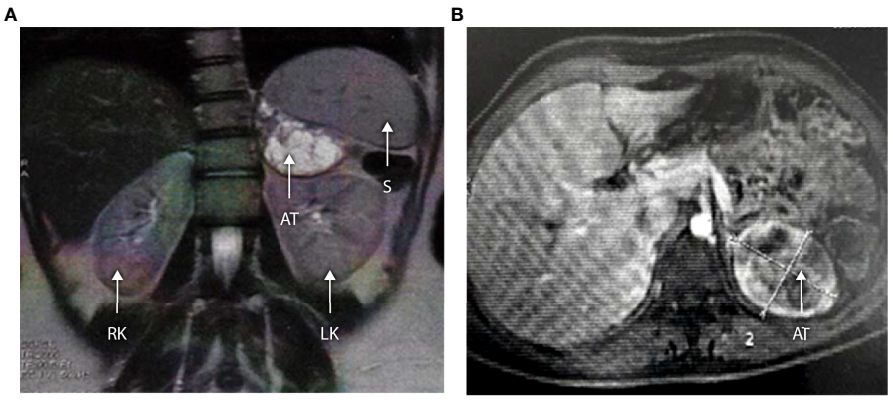

FIGURE 4 | **(A, B)** Abdominal MRI scan in a 16-year-old with 3βHSD2 deficiency (Patient B II-1). Axial and coronal sections demonstrate a large left-sided adrenal tumor measuring 63 × 52 × 51 mm. The lesion shows central cystic degeneration and is pushing the kidney downwards. RK, right kidney; LK, left kidney; S, spleen; AT, adrenal tumor.

dying undiagnosed and the misdiagnosis of 3βHSD as 21-OHD. In countries with neonatal screening programs for 21OHD, patients with 3βHSD2 deficiency may be detected at birth (30). In the absence of such a program, diagnosis depends on clinical awareness, as discussed below.

Diagnosis

Diagnosis in a salt-wasting under-masculinized male is easy, but paradoxically difficult in females who are more likely to die undiagnosed with salt wasting (31). This situation, in which girls die undiagnosed with 3βHSD2 deficiency is to be compared to boys with 21-OHD who die undiagnosed.

The p.Pro222Gln mutation of the HSD3B2 gene is one of the most frequent severe mutations and is predominant in the Algerian population. It has also been found in Colombia and Brazil (13, 15), probably due to a founder effect (2). Although this mutation is described as severe with severe SW forms, one of our patients was diagnosed at 13 months with a delayed SW presentation, clitoromegaly, and premature pubarche. This observation, in contrast with those of patients presenting very early with SW, illustrates the phenotypic variability that may occur with the same genetic defect, although there is usually a good genotype/phenotype correlation. This discrepancy could be explained by the presence of other possible mutations in non-explored genes involved in steroidogenesis in a consanguineous family.

The biochemical diagnosis of 3βHSD2 deficiency is based on the elevation of Δ5-steroids (17 OHPreg, DHEA-S) compared to Δ4 steroids [(17 OHP, Delta4-Androstenedione)]. Because of the conversion of 17OH-pregnenolone to 17 OH-progesterone by the 3βHSD 1 enzyme in peripheral tissues, 17 OHP levels may be increased, leading to the misdiagnosis of 3βHSD2 deficiency as 21OHD (31). We have observed that 17 OHP was mildly elevated in our patients compared to 17 OHPreg. Unfortunately, the 17 OHPreg assay is not widely available in Algeria and is only available in specialist laboratories, which therefore necessitates sending blood samples abroad—a measure that is costly and too expensive for some families.

Therefore, in the absence of available and affordable analysis of 17OH-pregnenolone, and any newborn screening program, clinicians should consider the diagnosis of 3βHSD2 deficiency in all under-masculinized boys and non-virilized or slightly virilized girls who present with mildly elevated 17OHP, elevated ACTH, and SW with elevated renin.

The elevation of 17-OHP on RIA observed in this series is of potential interest regarding newborn screening for CAH. After excluding four patients who were already receiving steroid treatment, the initial 17-OHP values in the remaining 10 patients were all above the French threshold of ≥17 nmol/L for infants ≥36 weeks of gestation (32). By contrast, when using the 17OHP–LC-MS/MS method, all values were well below this cut-off, the difference being attributable to cross-reaction with other steroids when the immunometric assay is used. At present, newborn screening techniques are usually immunological and cross-react with 17-OH pregnenolone, so that 3βHDS2 deficiency would be expected to be detectable. However, if these immunological techniques were to be replaced by LC-MS/MS (which has the advantage of reducing false positive tests and the significant cost they generate), 3βHDS2 deficiency might not be detected. Therefore, if newborn screening for CAH was established in Algeria and other Maghreb countries in the future, an immunological technique combined with current French thresholds would be preferable, to detect both 3βHSD2 and 21-OH deficiency.

The diagnosis of 3βHSD2 deficiency should always be confirmed by 17-OHPregnenolone measurement and by genetic analysis in countries where it is available.

Outcomes

Unlike 21-OH deficiency, very few studies have described the outcomes of patients with 3βHSD2 deficiency (see **Table 4**) and most have focused on male patients.

Most of the male patients with 3βHSD2 deficiency described in the literature have entered puberty spontaneously (1, 11, 12, 27, 33, 35, 39–42), probably because of the peripheral conversion

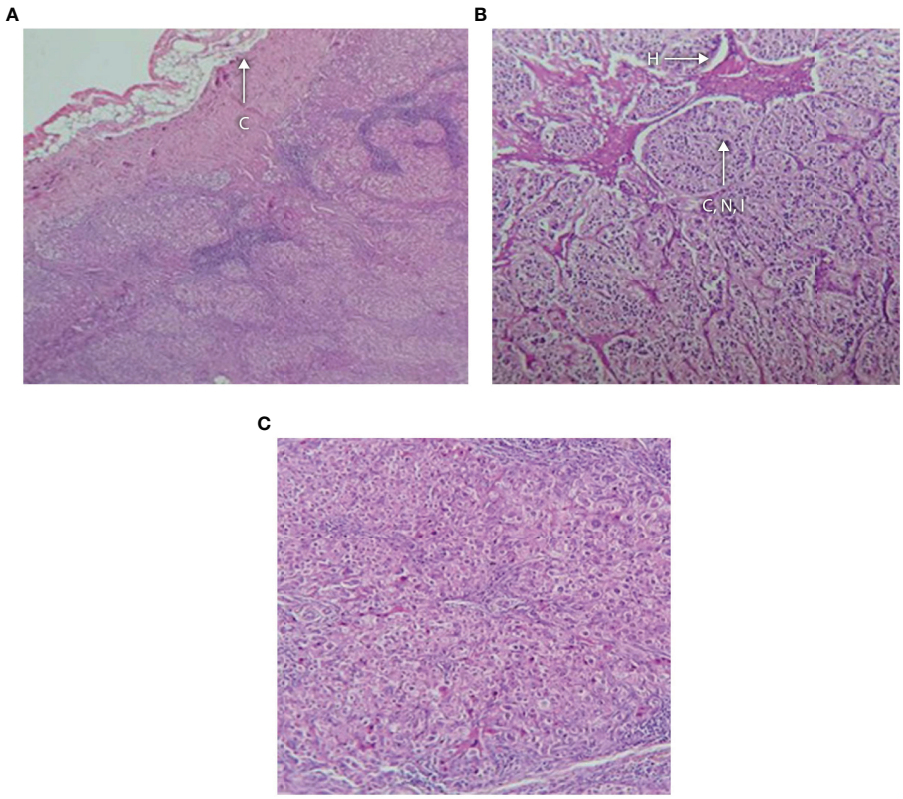

FIGURE 5 | **(A–C)** Histology of adrenal tumor from patient G II-1 following surgical removal showing **(A)** fibrous capsule with an underlying neoplasm containing hemorrhagic foci, no vascular or capsular invasion; **(B)** tumor composed of cells arranged in nests and cords separated by vasculature and lymphoid tissue; and **(C)** higher magnification showing that the cells have distinct boundaries and clear cytoplasm with monomorphic nuclei and foci of oncocytic metaplasia. There is hyperchromasia of the nuclei and apoptosis. C, capsule; H, hemorrhagic focus; CNI, cords, nests and islands of tumor.

of DHEA-S to testosterone (41). At present, only one male patient in our study has reached puberty at a normal age, the others being currently of prepubertal age.

Previous case reports have reported a relative frequency of gynecomastia (27, 33, 34, 38, 41, 45, 46) in boys with 3βHSD2 deficiency, attributed to the conversion of the large number of androgen precursors to androstenedione and testosterone by HSD3B1, with these latter hormones being then converted to estrogens with the help of HSD17B1, HSD17B5, and CYP19A1 (41). However, this problem was not reported in the larger case series (11, 12).

Two of the six males in our patients have developed premature pubic hair. Guran (12) and Benkert (11) have reported a high prevalence of premature pubarche and precocious puberty in their patients, despite hydrocortisone treatment. This may be attributed to the increased expression of 3βHSD1, which increases testosterone and Δ4 steroid concentrations in extra-gonadal and extra-adrenal tissues as children mature (12).

Despite the spontaneous development of puberty in most of the male patients, some needed testosterone treatment. Azoospermia (39, 42, 43) was reported in pubertal or adults patients and testicular anatomy was abnormal in some patients, with immature histology. As with 21 OHD, TARTs were frequently reported in male patients, due to sub-optimal treatment (11, 12).

The association of TARTs, incomplete gonadal maturation, and pathological testicular histology are likely to have a negative impact on the fertility of patients with 3βHSD2 deficiency, although this area is not yet well documented (1, 41). However, some patients have shown normal gonadal development with normal testis histology and, normal sperm count (15, 40). One patient was also reported as having fathered two children, although there was no genetic confirmation of 3βHSD2 deficiency in this case (27).

Few studies have evaluated puberty in female patients with 3βHSD2 deficiency (11, 12, 27, 36, 37, 39, 40). In our study, all female patients at an appropriate age had reached puberty spontaneously and had their menarche at a normal age, consistent with reports in the literature. However, we are struck by the relative frequency of premature pubarche in our patients. Indeed, similar to male patients, and probably for the same reasons, premature pubarche and precocious puberty have been reported in female patients with 3βHSD2 deficiency (11, 12). PCOS was also evident in three girls in our series, with polycystic ovaries but not PCOS in a fourth. PCOS has already been described in female patients with 3βHSD2 deficiency (11, 12, 37) as a probable effect of androgen overproduction.

TABLE 4 | Studies showing outcomes in patients with 3-β hydroxysteroid dehydrogenase 2 deficiency.

First Author/year of publication (Reference)	Country/ Ethnicity	Sex	Mutation	Complication/Puberty/gonadal status
Parks/1971 (33)	USA	M [1]	W171X	Acne 11 years, pubic hair and gynecomastia at 12 years
Jänne/1974 (34)	Finland	M [1]		Premature pubarche, gynecomastia. Testosterone gel started at 9 years Normal testicular histology
Schneider/1975 (35)	USA	M [1]		Onset of puberty at 10 years, gynecomastia aged 11 years with acne, obesity Immature testis, predominantly Sertoli cells, Leydig cell hyperplasia, spermatogenic arrest
Zachman/1979 (36)	Switzerland	F [1]		Severe salt wasting crises during infancy, normal mental development Bone age delay; puberty induced. Adult height 159.5 cm
Martin/1980	Finland	M [1]		Obesity, gynecomastia
Mendonca/1987 (15) Moisan/1999 (14)	Brazil	M [1]	A82T	46, XY individual, initially raised as a girl, virilization during puberty; changed gender at 17 years Gonadectomy and penile surgery at 7 years; Induced puberty; normal testicular histology
Rheaume/1992 (27)	Switzerland	F [1]	W171X	Lack of spontaneous breast development, virilization
	USA	M [1]	W171X/ 186insC-fs	Spontaneous puberty at 13 years, gynecomastia; normal spermatogenesis Fathered two children (but no genetic confirmation)
Chang/1993 (37) Moisan/1999 (14)	USA	F [1] M [1]	G129r/ c6651G>A	Breast development 10 years, menarche at 12 years; adult height 158 cm; irregular menses, hirsutism; bilateral enlarged ovaries, multiple cysts (PCOS) Androgen excess, advanced bone age
Yoshimoto/1997 (38)	Japan	M [1]	R249X	Gynecomastia at 7.5 years, Normal pubertal development; no mature spermatogenesis
Alos/2000 (39)	French	F [1]	A10E	Advanced puberty and bone age at 8 years. Menarche at 10.3 y; enlarged ovaries with multiple cysts
Moisan/1999 (14)	Canadian	M [1]		Pubic hair at 10 years; G2 at 10.5 years; TART; azoospermia
BinAbbas/2004 (40)	Saudi Arabia	M [1] F [1]		Normal puberty; adult height 155 cm; normal sperm count Normal puberty at 14 years, adult height 150 cm; mild hirsutism, menstrual irregularities.
Burckhardt/2015 (41)	Canada/Sri Lanka	M [1]	c.687del27	Cerebral palsy, psychomotor retardation, dyskinetic movement disorder Normal puberty; gynecomastia; spermatogenic arrest (Sertoli cells only)
Lolis/2018 (42)	Sweden	M [1]	Cys-72-Arg	Cryptorchidism. Spontaneous puberty with advanced bone age. Extensive bilateral TARTs from 13 years, mimicking Leydig cell tumor; azoospermia. Adult height 174.5 cm (–2 DS/TH). Cushingoid with obesity and osteoporosis
Falhammar/2012 (43)	Sweden	M [1]		TART, azoospermia
Donadille/2018 (1)	France	M [1]	687 del27	Normal puberty; normal sperm count; adult height 170 cm
Benkert/2015 (11)	USA/Amish	M (2), F (3)	c.35G>A	TART (2 M), PCOS with irregular menses (2 F), obesity (5), early puberty [4] with advanced bone age, hirsutism/acne (5), ischemic encephalopathy (1)
Guran/2020 (12)	Turkey	F [5] M [9]	p.N323D, p.S218P p.W355R	Premature pubarche (F = 5), non-progressive precocious puberty (1 F); central precocious puberty (2F), menarche at 12 years (2F), PCOS (1 F) Premature pubarche (M = 9), non-progressive precocious puberty (2 M), Tanner G5 (3 M) at 14.6, 15.6, and 17 (partial gonadal insufficiency), TART (2 M)
Ladjouze/2022 (44)	Algeria	F [8] M [6]	p.Pro222Gln	Premature pubarche (3 F), menarche at a normal age (4 F), ART (3 F), OART (1 F), PCOS (3 F), Obesity (1 F) Premature pubarche (2 M), spontaneous puberty (1 M). TART (2 M), learning disability (2 M), obesity (2 M)

FH, Final Height; TART, Testicular adrenal rest tumor; ART, Adrenal rest tumor, OART, Ovarian adrenal rest tumor; PCOS, Polycystic ovary syndrome.

Adrenal tumors have been reported in inadequately treated patients with 21OH deficiency, but not to date in patients with 3βHSD2 deficiency. They are a consequence of chronic elevation of ACTH that leads to adrenal cortical hyperplasia in patients with suboptimal hydrocortisone treatment. In our series, we have been surprised by the discovery, on systematic ultrasonography evaluation, of voluminous adrenal tumors in two female patients. Both had been treated since early infancy and were initially misdiagnosed as 21OH deficiency. Because of this misdiagnosis, the treatment was inadequate; the physicians titrating the hydrocortisone dose according to 17OHP and not to 17OHPreg. Both had very large adrenal tumors that led to surgical removal. One of the tumors was large and presented radiologically and histologically as an adrenocortical tumor. Further histological analysis and the benign evolution of the case allowed the correct final diagnosis to be made.

Unlike TART, OART is rarely described in the CAH literature. As mentioned above, only one publication describes adrenal rest tissue in a woman with 3βHSD2 deficiency (10) but in this case the nodules were adjacent to, rather than within, the ovaries. OART was considered highly likely in one girl in our series (G II-1) who had both PCOS and had also undergone removal of adrenal mass. However, in the absence of histological confirmation, the diagnosis of OART in this girl remains unproven.

Growth patterns in our patients were normal, despite the relatively high doses of hydrocortisone used during some periods because of the problems with mineralocorticoid availability. One patient in our series had short stature and was treated with growth hormone therapy. The few patients who reached final height (FH) had a normal height compared to the WHO references. Few studies report final height in patients with 3βHSD2 deficiency. Normal final height was reported in well-treated patients (1), but FH may be compromised when treatment is suboptimal (36, 37, 40, 42).

Median IQ (range) was in the lower half of the normal range in all but two patients in our series, with subnormal IQ (<70) in three patients, two of which were from the same family (E) in which there is an additional neuro-disability disorder. Given that

both the parents and an unaffected sister (E II-6) of this family are of normal intelligence, indicating that putative carriers for the neurological disorder have no cognitive deficit, it is likely the IQ alteration in siblings E II-8 and E II-9 is attributable to 3βHSD deficiency.

Learning difficulties have already been described in patients with 21 OHD CAH patients, probably due to hypoglycemia at presentation (47). We have noticed the same effects on intelligence in children with 21OHD CAH in our patients, with more than 20% of the children having a low IQ (44). This is probably due to the late presentation of our patients, who initially presented with severe hyponatremia and hypoglycemia. The intellectual deficit seen with 3βHSD2 deficiency in this series serves only to strengthen the case for setting up a national screening program for CAH in our country.

CONCLUSIONS

3βHSD2 deficiency appears more prevalent in Algeria than elsewhere, with p.Pro222Gln the most frequent mutation. Mortality is high, with significant morbidity from PCOS and adrenal tumors in adolescence. IQ is usually in the lower half of the population range, with an increased risk of learning disability.

The diagnosis should be considered in all under-masculinized males with SW and healthy female patients with SW. Access to fludrocortisone is an important issue in our country and needs to be redressed urgently. The finding of adrenal masses in older patients with 3βHSD2 deficiency suggests adrenal hyperplasia requiring improved disease control rather than surgical intervention.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, on reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the CHU Bab El Oued Ethical comittee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

AL designed and oversaw the study and wrote the manuscript. MD helped design and structure the manuscript and wrote the paper with AL. IP carried out the LC-MS/MS biochemistry studies and hormonal analyses in Lyon. ND performed the histological analysis and provided the pathology photographs. KM and KB examined the children and collected the data during the visits in 2021. VT oversaw the genetic analyses. DM carried out the genetic analyses. ZB oversaw the visits in 2021. YM carried out the genetic analyses and the LC-MS/MS biochemistry studies. FR-B supervised the hormonal analyses in Lyon, coordinated the genetic studies and helped write the manuscript with AL and MD. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.867073/full#supplementary-material

REFERENCES

1. Donadille B, Houang M, Netchine I, Siffroi JP, Christin-Maitre S. Human 3beta-Hydroxysteroid Dehydrogenase Deficiency Associated With Normal Spermatic Numeration Despite a Severe Enzyme Deficit. *Endocr Connect* (2018) 7:395–402. doi: 10.1530/EC-17-0306
2. Morel Y, Roucher F, Plotton I, Simard J, Coll M. 3β-Hydroxysteroid Dehydrogenase Deficiency. In: *Genetic Steroid Disorders*. Elsevier. p. 99–110. doi: 10.1016/B978-0-12-416006-4.00008-9
3. Simard J, Ricketts ML, Moisan AM, Tardy V, Peter M, Van Vliet G, et al. A New Insight Into the Molecular Basis of 3beta-Hydroxysteroid Dehydrogenase Deficiency(2000) (Accessed February 15, 2015).

4. Russell AJ, Wallace AM, Forest MG, Donaldson MD, Edwards CR, Sutcliffe RG. Mutation in the Human Gene for 3 Beta-Hydroxysteroid Dehydrogenase Type II Leading to Male Pseudohermaphroditism Without Salt Loss. *J Mol Endocrinol* (1994) 12:225–37. doi: 10.1677/jme.0.0120225
5. Zerah M, Rhéaume E, Mani P, Schram P, Simard J, Labrie F, et al. No Evidence of Mutations in the Genes for Type I and Type II 3 Beta-Hydroxysteroid Dehydrogenase (3 Beta HSD) in Nonclassical 3 Beta HSD Deficiency. *J Clin Endocrinol Metab* (1994) 79:1811–7. doi: 10.1210/JCEM.79.6.7989489
6. Thomas TT, Ruscher KR, Mandavilli S, Balarezo F, Finck CM. Ovarian Steroid Cell Tumor, Not Otherwise Specified, Associated With Congenital Adrenal Hyperplasia: Rare Tumors of an Endocrine Disease. *J Pediatr Surg* (2013) 48:e23–7. doi: 10.1016/j.jpedsurg.2013.04.006
7. Tiosano D, Vlodavsky E, Filmar S, Weiner Z, Goldsher D, Bar-Shalom R. Ovarian Adrenal Rest Tumor in a Congenital Adrenal Hyperplasia Patient With Adrenocorticotropin Hypersecretion Following Adrenalectomy. *Horm Res Paediatr* (2010) 74:223–8. doi: 10.1159/000295722
8. Zaarour MG, Atallah DM, Trak-Smayra VE, Halaby GH. Bilateral Ovary Adrenal Rest Tumor in a Congenital Adrenal Hyperplasia Following Adrenalectomy. *Endocr Pract* (2014) 20:e69–74. doi: 10.4158/EP13092.CR
9. Chen Hd, Huang L-E, Zhong Zh, Su Z, Jiang H, Zeng J, et al. Ovarian Adrenal Rest Tumors Undetected by Imaging Studies and Identified at Surgery in Three Females With Congenital Adrenal Hyperplasia Unresponsive to Increased Hormone Therapy Dosage. *Endocr Pathol* (2017) 28:146–51. doi: 10.1007/s12022-016-9461-4
10. Paula FJA, Dick-De-Paula I, Pontes A, Schmitt FCL, Mendonca BB, Foss MC. Hyperandrogenism Due to 3β-Hydroxysteroid Dehydrogenase Deficiency With Accessory Adrenocortical Tissue: A Hormonal and Metabolic Evaluation. *Braz J Med Biol Res* (1994) 27:1149–58.
11. Benkert AR, Young M, Robinson D, Hendrickson C, Lee PA, Strauss KA. Severe Salt-Losing 3β-Hydroxysteroid Dehydrogenase Deficiency: Treatment and Outcomes of HSD3B2 C.35G>A Homozygotes. *J Clin Endocrinol Metab* (2015) 100:E1105–15. doi: 10.1210/jc.2015-2098
12. Guran T, Kara C, Yildiz M, Bitkin EC, Haklar G, Lin J-C, et al. Revisiting Classical 3β-Hydroxysteroid Dehydrogenase 2 Deficiency: Lessons From 31 Pediatric Cases. *J Clin Endocrinol Metab* (2020) 105(4). doi: 10.1210/clinem/dgaa022/5707567
13. Lusa LG, de Lemos-Marini SHV, Soardi FC, Ferraz LFC, Guerra-Júnior G, de Mello MP. Structural Aspects of the P.P222Q Homozygous Mutation of HSD3B2 Gene in a Patient With Congenital Adrenal Hyperplasia. *Arq Bras Endocrinol Metabol* (2010) 54:768–74. doi: 10.1590/s0004-27302010000800018
14. Moisan AM, Tardy V, Ricketts ML, Cabrol S, Raux-demay MC, Forest MG, et al. New Insight Into the Molecular Basis of 3β - Hydroxysteroid Dehydrogenase Deficiency: Identification of Eight Mutations in the HSD3B2 Gene in Eleven Patients From Seven New Families and Comparison of the Functional Properties of Twenty-Five Mutant Enzym. *J Clin Endocrinol Metab* (1999) 84:4410–25. doi: 10.1210/jcem.84.12.6288
15. Marui S, Castro2 M, Latronico AC, Elias2 LLK, Arnhold IJP, Moreira2 AC, et al. Mutations in the Type II 3b-Hydroxysteroid Dehydrogenase (HSD3B2) Gene can Cause Premature pubarche in Girls. *Clin Endocrinol (Oxf)* (2000) 52:67–75. doi: 10.1046/j.1365-2265.2000.00873.x
16. Mermejo LM, Elias LLK, Marui S, Moreira AC, Mendonca BB, De Castro M. Refining Hormonal Diagnosis of Type II 3β-Hydroxysteroid Dehydrogenase Deficiency in Patients With Premature pubarche and Hirsutism Based on HSD3B2 Genotyping. *J Clin Endocrinol Metab* (2005) 90:1287–93. doi: 10.1210/jc.2004-1552
17. Lutfallah C, Wang W, Mason JIAN, Chang YTAI, Haider A, Rich B, et al. Newly Proposed Hormonal Criteria *Via* Genotypic Proof for Type II 3 Beta-Hydroxysteroid Dehydrogenase Deficiency. *J Clin Endocrinol Metab* (2002) 87:2611–22. doi: 10.1210/jcem.87.6.8615
18. Demographie Algérienne 2017. Office of National Statistics, Algeria. *Demographie ALGERIENNE 2017*. (2019). pp. 7–11.
19. PRADER A. Genital Findings in the Female Pseudo-Hermaphroditism of the Congenital Adrenogenital Syndrome; Morphology, Frequency, Development and Heredity of the Different Genital Forms (1954) (Accessed December 22, 2014).
20. Ahmed SF, Khwaja O, Hughes IA. The Role of a Clinical Score in the Assessment of Ambiguous Genitalia (2000) (Accessed December 22, 2014).
21. WHO. WHO. *The WHO Child Growth Standards* (2007). Available at: http://www.who.int/childgrowth/standards/en/ (Accessed May 2, 2015).
22. WHO. WHO. *Development of a WHO Growth Reference for School-Aged Children and Adolescents* (2007). Available at: http://www.who.int/growthref/growthref_who_bull/en/ (Accessed May 2, 2015).
23. Cognet G, Bachelier DChapitre 4. Wppsi-Iv Échelle D'intelligence De Wechsler Pour La Période Préscolaire Et Primaire – Quatrième Version (Accessed January 22, 2022).
24. Kohs SC. *Intelligence Measurement: A Psychological and Statistical Study Based Upon the Block-Design Tests*. New York: Macmillan (1923).
25. Kushnir MM, Blamires T, Rockwood AL, Roberts WL, Yue B, Erdogan E, et al. Liquid Chromatography-Tandem Mass Spectrometry Assay for Androstenedione, Dehydroepiandrosterone, and Testosterone With Pediatric and Adult Reference Intervals. *Clin Chem* (2010) 56:1138–47. doi: 10.1373/clinchem.2010.143222
26. Mébarki F, Sanchez R, Rhéaume E, Laflamme N, Simard J, Forest MG, et al. Nonsalt-Losing Male Pseudohermaphroditism Due to the Novel Homozygous N100S Mutation in the Type II 3 Beta-Hydroxysteroid Dehydrogenase Gene. *J Clin Endocrinol Metab* (1995) 80:2127–34. doi: 10.1210/jcem.80.7.7608265
27. Rhéaume E, Simard J, Morel Y, Mebarki F, Zachmann M, Forest MG, et al. Congenital Adrenal Hyperplasia Due to Point Mutations in the Type II 3 Beta-Hydroxysteroid Dehydrogenase Gene. *Nat Genet* (1992) 1:239–45. doi: 10.1038/ng0792-239
28. Rowlands A, Deeb A, Ladjouze A, Hamza RT, Musa SA, Raza J, et al. Access to Fludrocortisone and to Hydrocortisone in Children With Congenital Adrenal Hyperplasia in the WHO Eastern Mediterranean Region: It Takes a Village. *BMJ Glob Heal* (2021) 6:e007195. doi: 10.1136/BMJGH-2021-007195
29. Ibáñez L, Oberfield SE, Witchel S, Auchus RJ, Chang RJ, Codner E, et al. An International Consortium Update: Pathophysiology, Diagnosis, and Treatment of Polycystic Ovarian Syndrome in Adolescence. *Horm Res Paediatr* (2017) 88:371–95. doi: 10.1159/000479371
30. Coulm B, Coste J, Tardy V, Ecosse E, Roussey M, Morel Y, et al. Efficiency of Neonatal Screening for Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency in Children Born in Mainland France Between 1996 and 2003. *Arch Pediatr Adolesc Med* (2012) 166:113–20. doi: 10.1001/archpediatrics.2011.774
31. Nordenström A, Forest MG, Wedell A. A Case of 3β-Hydroxysteroid Dehydrogenase Type II (HSD3B2) Deficiency Picked Up by Neonatal Screening for 21-Hydroxylase Deficiency: Difficulties and Delay in Etiologic Diagnosis. *Horm Res* (2007) 68:204–8. doi: 10.1159/000102593
32. Kariyawasam D, Nguyen-Khoa T, Gonzalez Briceño L, Polak M. Newborn Screening for Congenital Adrenal Hyperplasia in France. *Medecine/Sciences* (2021) 37:500–6. doi: 10.1051/medsci/2021060
33. Parks GA, Bermudez JA, Anast CS, Bongiovanni AM, New MI. Pubertal Boy With the 3β-Hydroxy Steroid Dehydrogenase Defect. *J Clin Endocrinol Metab* (1971) 33:269–78. doi: 10.1210/jcem-33-2-269
34. Jänne O, Perheentupa J, Viinikka L, Vihko R. Testicular Endocrine Function in a Pubertal Boy With 3β-Hydroxysteroid Dehydrogenase Deficiency. *J Clin Endocrinol Metab* (1974) 39:206–9. doi: 10.1210/jcem-39-1-206
35. Schneider G, Genel M, Bongiovanni AM. Persistent Testicular Δ5 Isomerase 3β Hydroxysteroid Dehydrogenase (Δ5 3β HSD) Deficiency in the Δ5 3β HSD Form of Congenital Adrenal Hyperplasia. *J Clin Invest* (1975) 55:681–90. doi: 10.1172/JCI107977
36. Zachmann M, Forest MG, De Peretti E. 3 Beta-Hydroxysteroid Dehydrogenase Deficiency. Follow-Up Study in a Girl With Pubertal Bone Age. *Horm Res* (1979) 11:292–302. doi: 10.1159/000179067
37. Chang YT, Kappy MS, Iwamoto K, Wang J, Yang X, Pang S. Mutations in the Type II 3 Beta-Hydroxysteroid Dehydrogenase Gene in a Patient With Classic Salt-Wasting 3 Beta-Hydroxysteroid Dehydrogenase Deficiency Congenital Adrenal Hyperplasia. *Pediatr Res* (1993) 34:698–700. doi: 10.1203/00006450-199311000-00026
38. Yoshimoto M, Kawaguchi T, Mori R, Kinoshita EI, Baba T, Tajima T, et al. Pubertal Changes in Testicular 3 Beta-Hydroxysteroid Dehydrogenase Activity in a Male With Classical 3 Beta-Hydroxysteroid Dehydrogenase Deficiency Showing Spontaneous Secondary Sexual Maturation. *Horm Res* (1997) 48:83–7. doi: 10.1159/000185492
39. Alos N, Moisan A, Ward L, Desrochers M, Legault L, Leboeuf G, et al. A Novel A10e Homozygous Mutation in the HSD3B2 French-Canadians: Evaluation

of Gonadal Function After Puberty *. *J Clin Endocrinol Metab* (2000) 85:(5) 1968–74. doi: 10.1093/hmg/4.5.969
40. Bin-Abbas B, Sakati NA, Al-Ashwal A. Congenital Adrenal Hyperplasia Due To3 Beta-Hydroxysteroid Dehydrogenasetype II Deficiency in 4 Saudi Children.Long Term Follow Up. *saudi Med J* (2004) 25:1295–6. doi: 10.1210/jcem-39-1-206
41. Burckhardt M, Udhane SS, Marti N, Schnyder I, Tapia C, Nielsen JE, et al. Human 3 B -Hydroxysteroid Dehydrogenase Deficiency Seems to Affect Fertility But may Not Harbor a Tumor Risk: Lesson From an Experiment of Nature *Eur J Endocrinol* (2015) 173(5):1–12. doi: 10.1530/EJE-15-0599
42. Lolis E, Christofer Juhlin C, Nordenström A, Falhammar H. Extensive Bilateral Adrenal Rest Testicular Tumors in a Patient With 3β-Hydroxysteroid Dehydrogenase Type 2 Deficiency. *J Endocr Soc* (2018) 2:513–7. doi: 10.1210/js.2018-00082
43. Falhammar H, Nyström HF, Ekström U, Granberg S, Wedell A, Thorén M. Fertility, Sexuality and Testicular Adrenal Rest Tumors in Adult Males With Congenital Adrenal Hyperplasia. *Eur J Endocrinol* (2012) 166:441–9. doi: 10.1530/EJE-11-0828
44. Ladjouze A, Yala I, Yahiaoui M, Zerguini D, Tardy V, Mohammedi K, et al. Age at Diagnosis and Outcome in Maghreb Patients With 21-Hydroxylase Deficient Congenital Adrenal Hyperplasia; Urgent Need for Newborn Screening. *SPE Abstracts* (2018) 89:P-P-005. doi: 10.3252/pso.eu.57ESPE.2018
45. Tajima T, Fujieda K, Nakae J, Shinohara N, Yoshimoto M, Baba T, et al. Molecular Analysis of Type II 3 Beta-Hydroxysteroid Dehydrogenase Gene in Japanese Patients With Classical 3 Beta-Hydroxysteroid Dehydrogenase Deficiency. *Hum Mol Genet* (1995) 4:969–71. doi: 10.1093/hmg/4.5.969
46. Martin F, Perheentupa J, Adlercreutz H. Plasma and Urinary Androgens and Oestrogens in a Pubertal Boy With 3β-Hydroxysteroid Dehydrogenase Deficiency. *J Steroid Biochem* (1980) 13:197–201. doi: 10.1016/0022-4731(80)90192-2
47. Donaldson MDC, Thomas PH, Love JG, Murray GD, Mcninch AW, Savage DCL, et al. Presentation, Acute Illness, and Learning Difficulties in Salt Wasting 21-Hydroxylase Deficiency. *Arch Dis Child* (1994) 70:214–8. doi: 10.1136/adc.70.3.214

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