Human Reproduction Update, Vol.23, No.5 pp. 580–599, 2017
Advanced Access publication on June 5, 2017 doi:10.1093/humupd/dmx014

human reproduction update

Non-classic congenital adrenal hyperplasia due to 21-hydroxylase deficiency revisited: an update with a special focus on adolescent and adult women

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Submitted on December 7, 2016; resubmitted on February 8, 2017; editorial decision on March 29, 2017; accepted on May 12, 2017

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BACKGROUND: Non-classic congenital hyperplasia (NCAH) due to 21-hydroxylase deficiency is a common autosomal recessive disorder characterized by androgen excess.

OBJECTIVE AND RATIONALE: We conducted a systematic review and critical assessment of the available evidence pertaining to the epidemiology, pathophysiology, diagnosis and management of NCAH. A meta-analysis of epidemiological data was also performed.

SEARCH METHODS: Peer-reviewed studies evaluating NCAH published up to October 2016 were reviewed. Multiple databases were searched including MEDLINE, EMBASE, Cochrane, ERIC, EBSCO, dissertation abstracts, and current contents.

OUTCOMES: The worldwide prevalence of NCAH amongst women presenting with signs and symptoms of androgen excess is 4.2% (95% confidence interval: 3.2–5.4%). The clinical consequences of NCAH expand from infancy, i.e. accelerated growth, to adolescence and adulthood, i.e. premature pubarche, cutaneous symptoms and oligo-ovulation in a polycystic ovary syndrome (PCOS)-like clinical picture. The diagnosis of NCAH relies on serum 17-hydroxyprogesterone (17-OHP) concentrations. A basal 17-OHP concentration ≥2 ng/ml (6 nmol/l) should be used for screening if more appropriate in-house cut-off values are not available. Definitive diagnosis requires a 17-OHP concentration ≥10 ng/ml (30 nmol/l), either basally or after cosyntropin-stimulation. Molecular genetic analysis of the *CYP21A2* gene, which is responsible for 21-hydroxylase activity, may be used for confirmation purposes and should be offered to all patients with NCAH along with genetic counseling because these patients frequently carry alleles that may result in classic CAH, the more severe form of the disease, in their progeny. Treatment must be individualized. Glucocorticoid replacement therapy may benefit pediatric patients with accelerated growth or advanced bone age or adult women seeking fertility, whereas adequate control of menstrual irregularity, hirsutism and other cutaneous symptoms is best served by the use of oral contraceptive pills and/or anti-androgens. Some women may need ovulation induction or assisted reproductive technology to achieve pregnancy. Patients with NCAH have a higher risk of miscarriage and may benefit from glucocorticoid treatment during pregnancy.

WIDER IMPLICATIONS: Evidence-based diagnostic and treatment strategies are essential for the proper management of women with NCAH, especially considering that these patients may need different therapeutic strategies at different stages during their follow-up and that appropriate genetic counseling may prevent the occurrence of CAH in their children.

Key words: 17-hydroxyprogesterone / 21-hydroxylase deficiency / androgen excess / hirsutism / hyperandrogenism / miscarriage / non-classic congenital adrenal hyperplasia / polycystic ovary syndrome / pregnancy / premature pubarche

Introduction

A number of guidelines on congenital adrenal hyperplasia have been published; most have primarily focused primarily on classic congenital adrenal hyperplasia (CAH). Yet, in many ways non-classic adrenal hyperplasia (NCAH) differs significantly from CAH not only because of its later and dissimilar clinical presentation, but also because of the need for distinct considerations regarding therapy. This review is exclusively focused on NCAH and represents the integrated view of a special expert committee appointed by the Androgen Excess & PCOS (AE-PCOS) Society, Inc., with expertise in diverse fields including pediatric, adult and reproductive endocrinology.

Methods

Panel

The AE-PCOS Board appointed a panel of experts on NCAH, selected from those researchers who had authored many original articles in the field, to review all evidence available about NCAH. Panel members and Board Directors constituted the Writing Committee. Prior to efforts on this review, selected members of the AE-PCOS committee declared all interests and activities potentially resulting in conflicts of interest. One senior member had to quit the committee because she was contemporaneously chairing a committee preparing guidelines for a different scientific society. All the other members of the committee declared no conflicts of interest because of similar activities or financial interests.

Data selection and statistical analysis

Peer-reviewed studies evaluating NCAH published up to October 2016 were reviewed. Multiple databases were searched including MEDLINE, EMBASE, Cochrane, ERIC, EBSCO, dissertation abstracts and current contents. This review focused on the epidemiology, pathophysiology, diagnosis and treatment of the disease. Some studies were eliminated because the data were either not related to the focus of the systematic review, insufficient for epidemiological analysis or reported in previous publications. All data sources were analyzed while recognizing positive publication bias.

A meta-analysis was performed to obtain pooled prevalence estimates on NCAH prevalence in subjects with signs and symptoms of androgen excess. The terms of the MEDLINE search for this meta-analysis were: (prevalence OR frequency OR frequencies OR prevalences) AND (non classic OR non-classic OR late-onset OR late onset) AND (adrenal hyperplasia OR 21-hydroxylase OR 21α-hydroxylase OR CYP21 OR CYP21A2). The search was completed by examining the references listed in the articles identified. After study identification, screening, selection and inclusion, a random-effects model was applied considering the heterogeneity of the studies in terms of age, race, ethnicity and hyperandrogenic symptoms of the populations being described. Double arcsine transformations were applied to stabilize the variance (Doi *et al.*, 2015). The forest plot in Fig. 1 depicts the pooled prevalence estimate as a diamond, with the lateral points indicating confidence intervals. The left hand column included study identifiers and the right-hand columns included plots and corresponding numerical information for the prevalence found in each of these studies, with squares and horizontal lines representing confidence

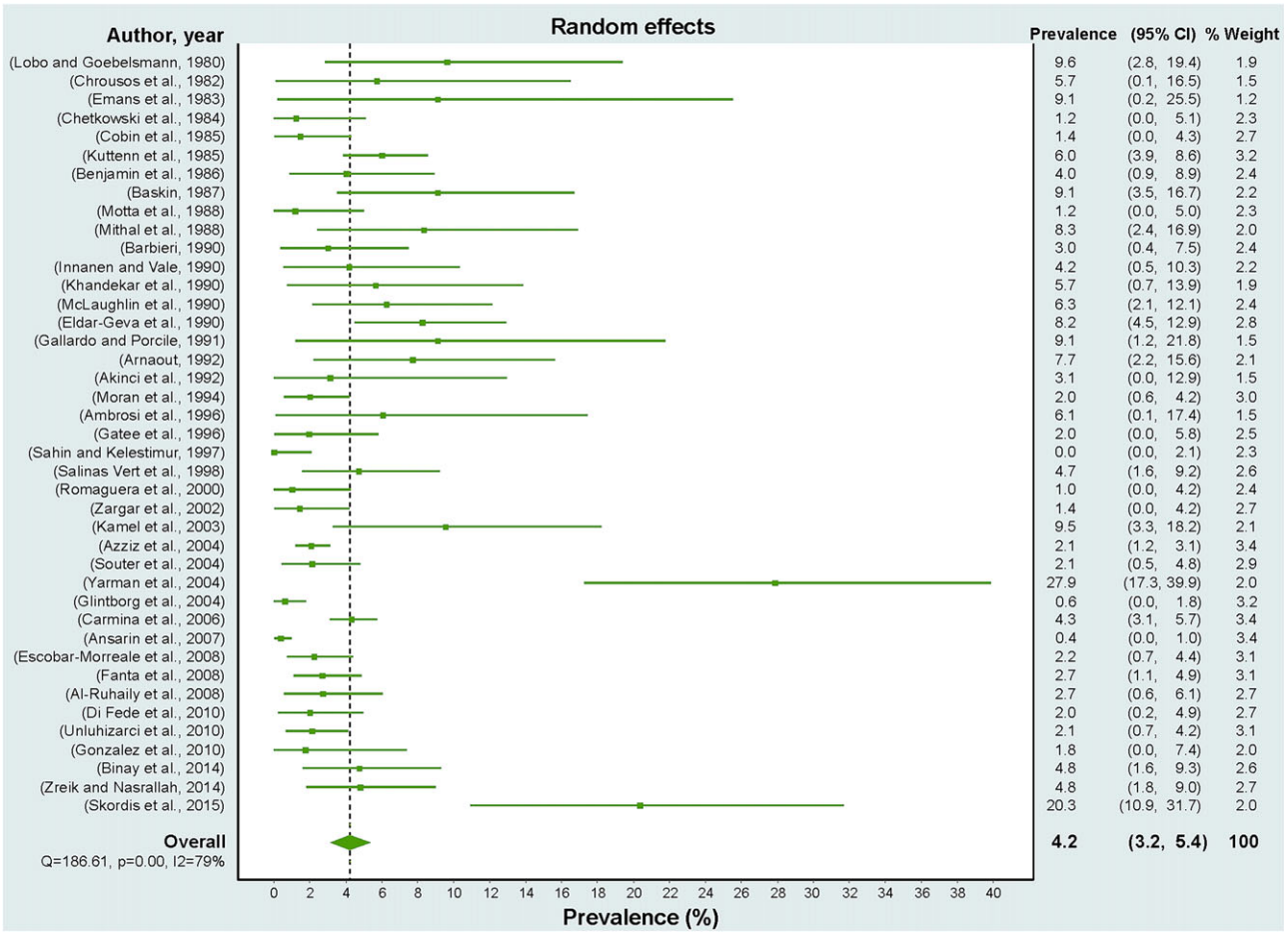

Figure 1 Meta-analysis of the prevalence (%) of non-classic congenital adrenal hyperplasia (NCAH) among patients presenting with signs and symptoms of androgen excess. The terms of the MEDLINE search were (prevalence OR frequency OR frequencies OR prevalences) AND (non classic OR non-classic OR late-onset OR late onset) AND (adrenal hyperplasia OR 21-hydroxylase OR 21α-hydroxylase OR CYP21 OR CYP21A2). This was completed by a hand search of the references lists of the articles identified. The overall characteristic of the studies included in the meta-analysis are summarized in Table I. PRIMA flow-chart of the search and funnel plot of the studies included in the meta-analysis are included in Fig. 2. Data were submitted to a random effects model using MetaXL 3.0 software (Doi *et al.*, 2015).

intervals. Publication bias was assessed by a funnel plot representing the double arcsine transformation of the prevalence against the standard error (Sterne and Egger, 2001). MetaXL 3.0 software was used for the meta-analysis (Doi *et al.*, 2015). The overall characteristics of the studies included in the meta-analysis are summarized in Table I. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (Moher *et al.*, 2009) recommended flow-chart and funnel plot of the studies included in the meta-analysis are depicted in Fig. 2.

Process

Each chapter of this review was prepared by at least two investigators and then reviewed by all authors of the committee. Analyzed papers included individual studies, systematic reviews, hand searches and abstracts. Criteria for inclusion/exclusion of the published papers related to each chapter were agreed upon by at least two reviewers in each area and arbitrated by a third when necessary. Levels of evidence were assessed and graded from A to D (www.nhmrc.gov.au/publications/synopses/cp65syn.htm). A or B level was considered necessary for recommendations regarding diagnosis and therapy of NCAH patients. The final manuscript was reviewed and approved by the AE-PCOS Society Board. Institutional Review Board approval was not obtained because the study reviewed publicly available medical literature.

Epidemiology of NCAH

Hyperandrogenic non-classic congenital adrenal hyperplasia (NCAH) can be associated with three distinct enzyme defects: (a) defects of 21-hydroxylase (21-OH) activity, catalyzed by cytochrome P450c21A2, encoded by *CYP21A2*; (b) defects of 11β-hydroxylase (11-OH) activity, catalyzed by cytochrome P450c11, encoded by *CYP11B1;* and (c) defects of 3β-hydroxysteroid dehydrogenase (3β-HSD) activity, catalyzed by type II 3β-HSD, encoded by *HSD3B2*.

Table I Prevalence of non-classic congenital adrenal hyperplasia (NCAH) in series of women presenting with signs and symptoms of androgen excess.

References	Country	Sex	Age	Complaint	Series size	NCAH	
					(*n*)	(*n*)	(%)
Lobo and Goebelsmann (1980)	United States	Female	Premenopausal	Hirsutism and oligomenorrhea	52	5	9.6
Chrousos *et al.* (1982)	United States	Female	Premenopausal	Hirsutism	35	2	5.7
Emans *et al.* (1983)	United States	Female	Adolescent	Androgen excess	22	2	9.1
Chetkowski *et al.* (1984)	United States	Female	Premenopausal	Hirsutism	83	1	1.2
Cobin *et al.* (1985)	United States	Female	Premenopausal	PCOS and hirsutism	139	2	1.4
Kuttenn *et al.* (1985)	France	Female	Premenopausal	Hirsutism	400	24	6.0
Benjamin *et al.* (1986)	United States	Female	Premenopausal	PCOS	100	4	4.0
Baskin (1987)	United States	Female	Adolescents and adults	Hirsutism and/or amenorrhea	77	7	9.1
Motta *et al.* (1988)	Italy	Female	Adolescents and adults	Hirsutism	85	1	1.2
Mithal *et al.* (1988)	India	Female	Premenopausal	Hirsutism	60	5	8.3
Barbieri (1990)	United States	Female	Adult	Clinical hyperandrogenism	100	3	3.0
Innanen and Vale (1990)	Canada	Female	Premenopausal	Mild to moderate hirsutism	72	3	4.2
Khandekar *et al.* (1990)	India	Female	Premenopausal	Hirsutism	53	3	5.6
McLaughlin *et al.* (1990)	Ireland	Female	Premenopausal	Clinical hyperandrogenism	96	6	6.3
Eldar-Geva *et al.* (1990)	Israel	Female	Premenopausal	Clinical hyperandrogenism	170	14	8.3
Gallardo and Porcile (1991)	Chile	Female	Premenopausal	Hirsutism	33	3	9.1
Arnaout (1992)	France	Female	Premenopausal	Hirsutism	65	5	7.7
Akinci *et al.* (1992)	Turkey	Female	Adolescent	Hirsutism	32	1	3.1
Moran *et al.* (1994)	Mexico	Female	Premenopausal	Hirsutism	250	5	2.0
Ambrosi *et al.* (1996)	Italy	Female	Premenopausal	Hirsutism	33	2	6.1
Gatee *et al.* (1996)	United Arab Emirates	Female	Premenopausal	Hirsutism	102	2	1.9
Sahin and Kelestimur (1997)	Turkey	Female	Premenopausal	PCOS	83	0	0.0
Salinas Vert *et al.* (1998)	Spain	Female	Premenopausal	Hirsutism	127	6	4.7
Romaguera *et al.* (2000)	Puerto Rico	Female	Premenopausal	Hirsutism	100	1	1.0
Zargar *et al.* (2002)	India	Female	Pre- and postmenopausal	Hirsutism	142	2	1.4
Kamel *et al.* (2003)	Turkey	Female	Premenopausal	Hirsutism	63	6	9.5
Azziz *et al.* (2004)	United States	Female	Premenopausal	Clinical hyperandrogenism	873	18	2.1
Souter *et al.* (2004)	United States	Female	Premenopausal	Minimal unwanted hair growth	188	4	2.1
Glintborg *et al.* (2004)	Denmark	Female	Premenopausal	Hirsutism	340	2	0.6
Yarman *et al.* (2004)	Turkey	Female	Premenopausal	Hirsutism and polycystic ovaries	61	17	27.9
Carmina *et al.* (2006)	Italy	Female	Premenopausal	Clinical hyperandrogenism	950	41	4.3
Ansarin *et al.* (2007)	Iran	Female	Premenopausal	Hirsutism	790	3	0.4
Escobar-Morreale *et al.* (2008)	Spain	Female	Premenopausal	Clinical hyperandrogenism	270	6	2.2
Fanta *et al.* (2008)	Czech Republic	Female	Premenopausal	Clinical and biochemical hyperandrogenism	298	8	2.7
Al-Ruhaily *et al.* (2008)	Arabia	Female	Premenopausal	Hirsutism	148	4	2.7
Di Fede *et al.* (2010)	Italy	Female	Premenopausal	Mild hirsutism	152	3	2.0
Unluhizarci *et al.* (2010)	Turkey	Female	Premenopausal	Hirsutism, hyperandrogenism	285	6	2.1
Gonzalez *et al.* (2010)	United States	2:1 Males	Adolescent	Alopecia	57	1	1.8
Binay *et al.* (2014)	Turkey	Female	Premenopausal	Hirsutism and hyperandrogenemia	126	6	4.8
Zreik and Nasrallah (2014)	Lebanon	Female	Adolescents and adults	Hirsutism	146	7	4.6
Skordis *et al.* (2015)	Cyprus	Female	Children	Premature pubarche	59	12	20.3

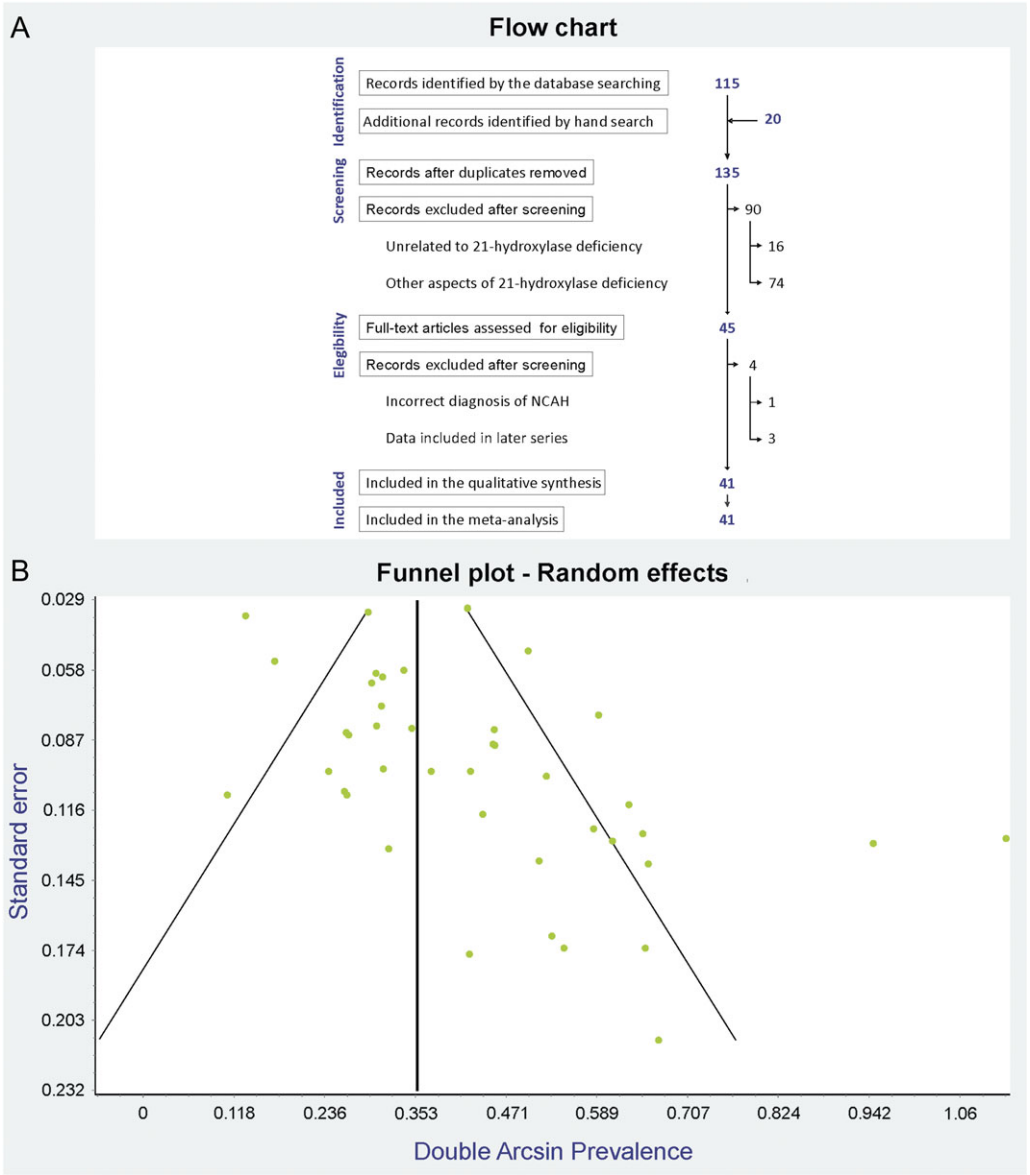

Figure 2 PRISMA flow-chart (**A**) and funnel plot (**B**) of the studies included in the meta-analysis of the prevalence of NCAH among patients presenting with signs and symptoms of androgen excess. For the calculations of standard errors and of double arcsine in the funnel plot, prevalences were introduced as per unit.

The vast majority of NCAH patients seeking medical attention have the 21-OH deficient type associated with *CYP21A2* mutations (Azziz and Zacur, 1989) (*Level of Evidence: A*). In contrast, NCAH due to mutations of *CYP11B1* and *HSD3B2* are extremely rare (Azziz *et al.*, 1991; Joehrer *et al.*, 1997; Lutfallah *et al.*, 2002; Moran *et al.*, 1999). Thus, when referring to NCAH, we will be solely referring to the 21-OH deficiency variety unless otherwise noted.

There are at least three approaches for determining the prevalence of NCAH. The first is based on the prevalence of carriers or genetic markers in a population. Speiser and colleagues (Speiser *et al.*, 1985) used this approach 30 years ago when they evaluated HLA-B genotype data in families containing multiple family members affected by NCAH together with the results of quantitative hormonal tests. These investigators estimated that the prevalence of NCAH was far more common than the classic form of 21-OH deficiency, with a prevalence of 3.7% (or 4:100) in Ashkenazi Jews, 1.9% in Hispanics, and 0.1% (or 1:1000) in the diverse Caucasian population (Speiser *et al.*, 1985).

Another approach to understanding the prevalence of NCAH can arise from population screening for the disorder. Unfortunately, neonatal screening is relatively insensitive for identifying children affected with NCAH, and consequently population-based based data are still lacking.

Because the exclusion of NCAH is mandatory as part of the scheme to diagnose PCOS (Azziz *et al.*, 2006, 2009), a large amount of data on the prevalence of NCAH among hyperandrogenic women is available (Table I). In North America, a prevalence of NCAH of 1–2% in populations of White and Hispanic hyperandrogenic patients has been found (Chetkowski *et al.*, 1984; Moran *et al.*, 1994; Romaguera *et al.*, 2000; Sanchez *et al.*, 2001). In contrast, studies in Spain, France, Italy and Canada have yielded frequencies between 3% and 6% (Kuttenn *et al.*, 1985; Motta *et al.*, 1988; Innanen and Vale, 1990; Escobar-Morreale *et al.*, 2008), and other studies in the Middle East and India have demonstrated prevalences of 5–10% (Mithal *et al.*, 1988; Eldar-Geva *et al.*, 1990; Khandekar *et al.*, 1990; Arnaout, 1992; Zreik and Nasrallah, 2014). Similar to the classic form of the disorder, NCAH in Black patients is relatively uncommon (Moran *et al.*, 2000) (*Level of Evidence: C*).

Using these data, and understanding that hyperandrogenism affects ~10% of the female population (Azziz *et al.*, 2006, 2009), it may be estimated that 21-OH deficient NCAH affects between 1:1000/2000 (e.g. in Anglo-Saxons) and 1:100 (e.g. in Ashkenazi Jewish and certain Middle Eastern and Indian sub-continent) women in the general population, and between 1% and 10% of hyperandrogenic women depending on the ethnicity of the population being studied (*Level of Evidence: C*).

A much more precise estimate of worldwide prevalence of NCAH may be obtained by a meta-analysis of the large number of studies published in hyperandrogenic women. Here we report the results of a meta-analysis of all studies published to date and assessing the prevalence of NCAH among women presenting with symptoms of androgen excess. The worldwide prevalence of NCAH among hyperandrogenic women was 4.2% (95% confidence interval: 3.2–5.4%) (Table I, Figs 1 and 2) and appears to be higher than that suspected using only North American data (*Level of Evidence: A*).

Genetics of NCAH

NCAH is an autosomal recessive disorder due to mutations in the 21-hydroxylase (*CYP21A2*) gene resulting in a 30–50% reduction in the activity of the enzyme. This gene is mapped to a complex genetic region at chromosome 6p21.3 where it lies in close proximity to a highly homologous pseudogene, *CYP21A1P*. *CYP21A2* and *CYP21A1P* are arranged in tandem repeats with the *C4A* and *C4B* genes, which encode complement component 4. The tenascin (*TNX*) and serine threonine nuclear protein kinase (*RP*) genes are also located in this region. These four genes, *RP*, *C4*, *CYP21* and *TNX*, form a unit or module known as RCCX. Most alleles carry two RCCX modules such that one has *CYP21A2* and the other has *CYP21A1P*.

To date, over 200 *CYP21A2* mutations have been reported (http://www.hgmd.cf.ac.uk; http://www.cypalleles.ki.se). Yet, despite the large number of reported mutations, approximately 10 mutations account for the majority of affected alleles. The complexity of this locus leads to nonallelic homologous recombination resulting into gene conversion events in which the functional gene acquires deleterious *CYP21A1P* sequences. Unequal crossover and misalignment during meiosis generate duplications and deletions of the RCCX modules. Haplotypes with three or four RCCX modules have been described (Parajes *et al.*, 2008). Another example of misalignment is a *CYP21A1P/CYP21A2* chimera in which a portion of the *CYP21A1P* gene is fused to a portion of the *CYP21A2* gene (Chen *et al.*, 2012). Rarely, CAH can be associated with uniparental disomy (Parker *et al.*, 2006). The de-novo mutation rate is ~1%.

Most NCAH individuals are compound heterozygotes showing different mutations on each allele (Tables II and III). The majority of patients carry one mutation causing severe enzyme deficiency in one allele and one mutation encoding a mild defect in the other. The NCAH phenotype roughly reflects the enzymatic activity encoded by the milder mutation. Approximately 25–50% of individuals with NCAH are reported to have mild mutations on both alleles (Speiser *et al.*, 2000; Bidet *et al.*, 2009; Livadas *et al.*, 2015). Mild mutations associated with NCAH include P30L, V281L, P453S, and R339H among others. Although the P30L mutation is typically associated with the NCAH patient phenotype, *in-vitro* functional studies indicate that P30L causes greater loss of function than the V281L and P453S mutations. Not surprisingly, the P30L mutation has also been identified in individuals with simple-virilizing CAH reflecting a greater degree of androgen excess (Barbaro *et al.*, 2015) (*Level of evidence: A*).

More recently, mutations located in non-coding genomic regions have been detected in patients with NCAH. For example, the promoter of the pseudogene, *CYP21A1P*, has 20% of the transcriptional activity of the *CYP21A2* promoter due to the presence of specific nucleotide variants, −126C > T, −113G > A, −110T > C, and −103A > G. Microconversions involving this region decrease *CYP21A2* gene transcription and are associated with NCAH. Additional mutations in non-coding regions of *CYP21A2* associated with NCAH include a variant in the steroidogenic factor-1 binding site located in the 5′-untranslated regulatory region and a variant located in the 3′-untranslated region (Araujo *et al.*, 2007) (*Level of evidence: B*).

As would be anticipated, specific *CYP21A2* mutations occur more frequently among particular ethnic groups. The mild V281L mutation associated with NCAH is the most common affected allele among Ashkenazi Jews. In other ethnic groups, severe mutations associated with CAH dominate the picture, with a large deletion being the most common mutation among Native Americans (40%) and Anglo-Saxons (28%) and the intron 2 splicing mutation being the only mutation reported for the Yupik Eskimos and being among the most common affected alleles in Iranians and Native Americans (Wilson *et al.*, 2007) (*Level of evidence: B*).

Using computational analyses, mutations associated with classic CAH have been found to disrupt essential enzyme functions such as membrane anchoring or substrate binding (Pallan *et al.*, 2015). Several mechanisms have been proposed for mutations associated with NCAH. Mutations associated with NCAH can be located in hydrophobic clusters, which abolish the hydrophobicity and destabilize the local environment. For the V281L mutation, the increase in chain length leads to a steric disruption, which minimally impairs enzyme activity (Haider *et al.*, 2013). Other mutations associated with NCAH may disrupt interactions with the P450 oxidoreductase protein interfering with electron transfer. Mutations that alter hydrogen bonding can destabilize the tertiary protein structure leading decreased enzyme activity (New *et al.*, 2013) (*Level of evidence: B*).

In general, *CYP21A2* mutations on both alleles will be identified when cosyntropin-stimulated 17-OHP concentrations are greater than 15 ng/ml (45 nmol/l). However, some individuals with diagnostic genotypes have adrenocorticotrophic hormone (ACTH)-stimulated 17-OHP values

Table II ***CYP21A2*** **mutations associated with NCAH due to 21-hydroxylase deficiency when present in homozygosis or double heterozygosity.**

Nucleotide changes*	Protein	Exon	Clinical phenotype	Activity *in vitro*†	References
g.89C>T	p.P30L	1	NCAH/SV	60%	Tusie-Luna *et al.* (1991)
g.140A>G	p.Y47C	1	NCAH	–	Tardy and Morel (2007)
g.185A>T	p.H62L	1	NCAH	45%	Ezquieta *et al.* (2002), Pinto *et al.* (2003), Soardi *et al.* (2008)
g.734A>G	p.H119R	3	NCAH	32%	Capoluongo *et al.* (2008b, d), Concolino *et al.* (2009a)
g.739A>C	p.K121Q	3	NCAH/SV	14%	Riepe *et al.* (2008)
g.749G>A	p.R124H	3	NCAH	–	Usui *et al.* (2004)
g.772C>T	p.R132C	3	NCAH	35%	Minutolo *et al.* (2011), Taboas *et al.* (2014)
g.817T>C	p.C147R	3	NCAH/SV	–	Robins *et al.* (2006)
g.929C>T	p.R149C	4	NCAH	36	Minutolo *et al.* (2011), Taboas *et al.* (2014)
g.929G>C	p.R149P	4	NCAH	23%	Chu *et al.* (2014)
g.981T>C	p.L166P	4	NCAH	–	Robins *et al.* (2006)
g.987C>A	p.T168N	4	NCAH	–	Vrzalova *et al.* (2010)
g.996T>A	p.I171N	4	NCAH	–	Barbaro *et al.* (2006)
g.1153T>A	p.I194N	5	NCAH	33%	Capoluongo *et al.* (2008c), Concolino *et al.* (2009a)
g.1343C>T	p.R224W	6	NCAH	52%	Concolino *et al.* (2007, 2009b)
g.1362T>C	p.I230T	6	NCAH	63%	Tardy *et al.* (2010)
g.1380A>G	p.R233G	6	NCAH	8%	Robins *et al.* (2006), Barbaro *et al.* (2015)
g.1588T>C	p.V249A	7	NCAH	–	Concolino *et al.* (2010)
g.1683G>T	p.V281L	7	NCAH	50%	Speiser *et al.* (1988), Tusie-Luna *et al.* (1990), Barbat *et al.* (1995)
g.1689A>C	p.M283L	7	NCAH	–	Ezquieta *et al.* (2002)
g.1689A>G	p.M283V	7	NCAH	16%	Minutolo *et al.* (2011), Taboas *et al.* (2014)
g.1744C>A	p.S301Y	7	NCAH	–	Stikkelbroeck *et al.* (2003)
g.1752G>A	p.V304M	7	NCAH	46%	Lajic *et al.* (2002)
g.1981C>A	p.L317M	7	NCAH	–	Deneux *et al.* (2001)
g.1981C>G	p.L317V	7	NCAH	–	Bojunga *et al.* (2005)
g.2012A>G	p.D322G	8	NCAH	18%	Loidi *et al.* (2006), Bleicken *et al.* (2009), Minutolo *et al.* (2011)
g.2058G>A	p.R339H	8	NCAH	50%	Helmberg *et al.* (1992)
g.2063C>T	p.R341W	8	NCAH	5%	Barbaro *et al.* (2015)
g.2064G>C	p.R341P	8	NCAH/SV	0.7%	Pinto *et al.* (2003), Barbaro *et al.* (2006)
g.2138C>T	p.R366C	8	NCAH	37%	Robins *et al.* (2006), Barbaro *et al.* (2015)
g.2147C>T	p.R369W	8	NCAH/SV	46%	Tardy *et al.* (2010)
g.2286C>G	p.N387K	9	NCAH	–	Wasniewska *et al.* (2009)
g.2296G>A	p.A391T	9	NCAH	38%	Robins *et al.* (2006, 2007)
g.2344G>A	p.D407N	9	NCAH	73%	Capoluongo *et al.* (2008a), Concolino *et al.* (2009b)
g.2512G>A	p.E431K	10	NCAH	–	Dain *et al.* (2006), Minutolo *et al.* (2011)
g.2524C>T	p.R435C	10	NCAH	–	Deneux *et al.* (2001)
g.2578C>T	p.P453S		NCAH	50-68%	Helmberg *et al.* (1992), Owerbach *et al.* (1992), Nikoshkov *et al.* (1997)
g.2597C>A	p.P459H	10	NCAH	–	Wang *et al.* (2007)
g.2640G>T	p.M473I	10	NCAH	85%	Robins *et al.* (2006), Barbaro *et al.* (2015)
g.2657G>T	p.R479L	10	NCAH	–	Zeng *et al.* (2004)
g.2665C>T	p.P482S	10	NCAH	70%	Balsamo *et al.* (2000), Barbaro *et al.* (2004)
g.2669G>C	p.R483P	10	NCAH	–	Wedell and Luthman (1993), Nikoshkov *et al.* (1998)
g.2669G>A	p.R483Q	10	NCAH	1.1%	Stikkelbroeck *et al.* (2003), Robins *et al.* (2007)

Updated from http://www.cypalleles.ki.se/cyp21.htm (last accessed Nov 1 2016) and Haider *et al.* (2013).
NCAH, non-classic congenital adrenal hyperplasia; SV, simple virilizing classic congenital adrenal hyperplasia.
*Numbering starts from A in the initiation codon. †Using 17-OHP as substrate.

Table III ***CYP21A2*** **genotypes associated with NCAH due to 21-hydroxylase deficiency in different series.**

Country (*n*)	Multi-center (34)	France (161)	Argentina (159)	United States (43)	United States (545)	Brazil (114)	Greece (280)
References	Speiser *et al.* (2000)	Bidet *et al.* (2009)	Marino *et al.* (2011)	Finkielstain *et al.* (2012)	New *et al.* (2013)	Moura-Massari *et al.* (2013)	Livadas *et al.* (2015)
V281L/V281L	20.6	25	43.4	27.9	38.5	47.5	38.1
V281L/Intron 2	17.6	17.7	15.1	7.0	19.8	16.2	20.6
V281L/LG	5.9	9.7	8.2	11.6	17.4	11	7.8
V281L/R356W	2.9	2.4	1.9	2.3	2.4	5.1	1.4
V281L/I172N	11.8	5.6	6.9	5.0	5.7		12.1
V281L/Q318X	5.9	5.6	3.8	7.0	3.7		5.0
V281L/exon 3	2.9	0.8	0.6		1.0	1.0	8.5
V281L/P453S		7.2	5.0	2.3		7.1	23.7
V281L/P30L		1.6	1.3		1.3		18.0
V281L/intron 2, V281L			1.9	2.3		1.0	1.4
V281L, R483fs/LG						1.0	
V281L/intron 2, Q318X			0.6				
V281L/Intron 2, R356W						1.0	
V281L,P453S/P453S		0.8					
V281L/W22X							2.1
V281L/R483P		2.4					1.4
V281L/5′gene conversion		3.2					
V281L/W19X		0.8					
V281L/exon 6			1.3		1.3		
V281L/L307fs	2.9	0.8					
V281L/S460_P465del		0.8					
V281L/R408C		0.8					
V281L/P482S				2.3			3.6
V281L/R426P				2.3			
V281L/complex rearrangement	5.9	0.8					
V281L/I172N,V281L		0.8					
P453S/P453S							2.9
P453S/W22X							0.07
P453S/intron 2	2.9	0.8	0.6				4.3
P453S/exon 3							2.1
P453S/I172N				2.3			1.4
P453S/LG			1.3				0.01
P453S/exon 6		0.8					
P453S/R356W	2.9						
P453S/Q318X	2.9						
P453S/Q318X, R356W		0.8					
P453S/P482S							2.2
P30L/P30L					0.4		5.8
P30L/I172N		0.8		2.3	1.3		5.0
P30L/intron 2		0.8		2.3	2.8		7.8

Continued

Table III *Continued*

Country (*n*)	Multi-center (34)	France (161)	Argentina (159)	United States (43)	United States (545)	Brazil (114)	Greece (280)
References	**Speiser et al. (2000)**	**Bidet et al. (2009)**	**Marino et al. (2011)**	**Finkielstain et al. (2012)**	**New et al. (2013)**	**Moura-Massari et al. (2013)**	**Livadas et al. (2015)**
P30L/V281L,R483Q		0.8					
P30L/R356W					0.4		0.07
P30L/Q318X					0.4		0.01
P30L/P453S	2.9						5.8
P30L/exon 6					0.2		
P30L/intron 2			1.2				5.0
P30L/exon 3					0.4		5.0
P30L/LG				2.3	2.3		3.5
P30L/L307fs							0.07
P30L + other mutations	8.8						
I172N/P482S							2.8
Intron 2/P482S							0.01
Intron 2/promoter							0.01
Intron 2/I172N				2.3			
Intron 2/LG						1.0	
I172N/I172N				2.3			
R356W/P482S							0.07
R435C/Q318X		0.8					
I172N, R356W, P453S*	2.9						

Data are frequencies (%).
Abbreviations: LG, large deletion; intron 2, IVS2-13A/C>G (splicing mutation); exon 3, 8-bp deletion (G110Efs); exon 6, I236N + V237E + M239K; L307fs, T insertion in exon 7; *, alleles were not fully segregated.

between 10 and 15 ng/ml (30–45 nmol/l). In some instances, molecular genetic analysis may be necessary to confirm the diagnosis of NCAH (Ambroziak *et al.*, 2016).

Pathophysiology of NCAH

The pathophysiology of classic 21-hydroxylase deficiency is well known (Merke, 2015). More severe forms of 21-hydroxylase deficiency (salt-wasting and simple virilizing CAH) are the consequence of the impairment of cortisol biosynthesis with accumulation of steroid intermediates (mainly 17-OHP). The resulting reduction of circulating cortisol leads to increased ACTH production and to hyperstimulation of adrenal androgen pathway. The excessive ACTH stimulation also determines the hypertrophy of fasciculata and reticularis zones, leading to adrenal hyperplasia and to adrenocortical nodularity in some cases (Falhammar and Torpy, 2016) (*Level of evidence: A*).

In contrast, the pathophysiology of non-classic 21-hydroxyase deficiency may be more complex (Fig. 3). In fact, while a few patients present with increased ACTH and have the same pathophysiological mechanisms of CAH (Chrousos *et al.*, 1982), in the majority of the affected subjects with NCAH, ACTH production is normal (Azziz *et al.*, 1994). Cortisol response to ACTH may be normal or slightly impaired but some patients with NCAH may have an over-responsive glucocorticoid response to ACTH stimulation (Carmina *et al.*, 1984; Carmina and Lobo, 1990; Azziz *et al.*, 1994; Carmina, 1995). However, adrenal androgen secretion and its response to ACTH are increased in NCAH (Carmina *et al.*, 1984; Carmina and Lobo, 1990; Azziz *et al.*, 1994; Carmina, 1995). Interestingly, in these patients, DHEAS serum levels are generally normal while androstenedione, testosterone and DHT are elevated but similar to the levels found in PCOS patients (Carmina *et al.*, 1984; Carmina and Lobo, 1990; Levin *et al.*, 1991; Azziz *et al.*, 1994; Carmina, 1995) (*Level of evidence: A*).

It has been difficult to understand how increased adrenal androgen secretion may be maintained in the absence of increased ACTH. It has been suggested that in NCAH, excessive adrenal androgen secretion mainly results from altered enzyme kinetics due to *CYP21A2* missense mutations (Witchel and Azziz, 2010). The mutated enzyme is transcribed and translated into a protein, but the enzyme protein is less efficient than the wild type. The net result is an increased precursor to product ratio, independent of ACTH levels. This concept is important in treatment because normalization of 17OHP levels may lead to excessive glucocorticoid administration.

Other mechanisms may contribute to hyperandrogenism of NCAH patients including ovarian dysfunction and peripheral synthesis of androgens from steroid precursors (Fig. 3). Polycystic ovarian

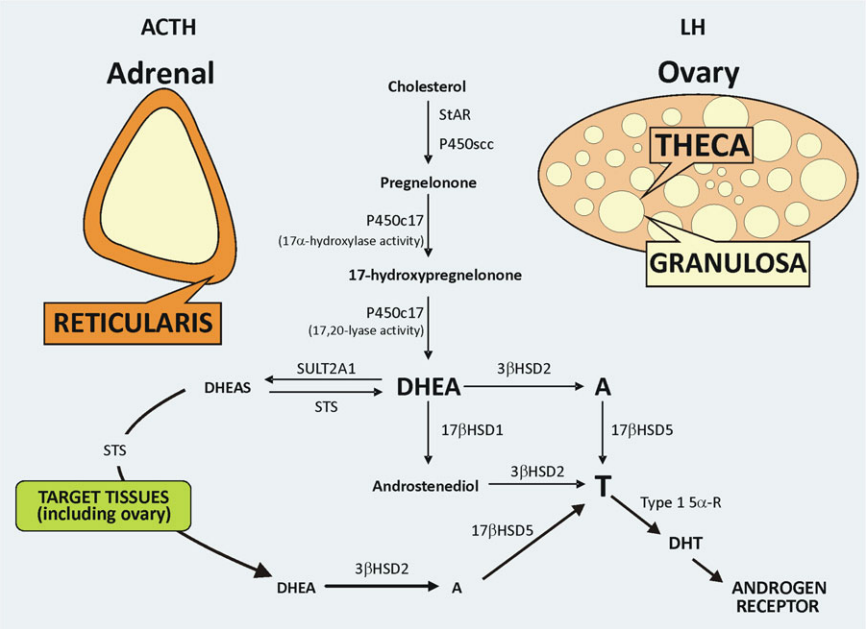

Figure 3 Mechanisms possibly contributing to androgen excess in NCAH. 3βHSD2, 3β-hydroxysteroid dehydrogenase type 2; 5α-R, 5α-reductasetype 1; 17βHSD5, 17β-hydroxysteroid dehydrogenase type V; A, androstenedione; ACTH, adrenocorticotropin; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone-sulfate; DHT, dyhydrotestosterone; HPA, hypothalamic–pituitary–adrenal; HPO, hypothalamic–pituitary–ovary; P450c17, 17α-hydroxylase/17,20-lyase; P450ssc, cholesterol side chain cleavage enzyme; StAR, steroidogenic acute regulatory protein; STS, steroid sulfatase; SULT2A1, sulfotransferase; T testosterone.

morphology is a frequent finding in women with NCAH (Levin *et al.*, 1991; Carmina and Lobo, 1994; Carmina, 1995; Pall *et al.*, 2010; Witchel and Azziz, 2010) and ovarian dysfunction may contribute to androgen excess in these women (*Level of evidence: B*), as demonstrated by the improvement of clinical and biochemical hyperandrogenism after ovarian suppression (Carmina and Lobo, 1994). The exaggerated adrenal secretion of progesterone and androgens might disrupt the hypothalamic–pituitary–ovarian axis favouring rapid GnRH pulse frequency and LH hypersecretion contributing to ovarian androgen excess (Blank *et al.*, 2007). Chronic androgen excess, and glucocorticoid administration around puberty in certain cases, may favor abdominal visceral adiposity, insulin resistance and its metabolic consequences, and exacerbate adrenal and ovarian androgen production (Escobar-Morreale and San Millan, 2007; Kim and Merke, 2009).

In addition, the role of a backdoor pathway in the hyperandrogenism of NCAH has been suggested (Auchus, 2004). Both progesterone and 17-OHP may be converted into more potent androgens such as dihydrotestosterone (DHT) by the backdoor pathway (Kamrath *et al.*, 2012) (Fig. 3). This pathway involves 5αpregnane-3α-, 17α-diol-20-one (Pdiol) synthesis after the 5α- and 3α reduction of 17-OHP by 5α-R type 1 and reductive 3αHSD type 1–4 enzymes (AKR1C). Then, Pdiol is cleaved to androsterone by the 17α-hydroxylase and 17, 20-lyase activities of P450c17, and androstanediol is synthesized from the latter by type 3/6 17β-hydroxysteroid dehydrogenase. Finally, 3α-hydroxysteroid dehydrogenase can oxidize androstanediol to DHT in target tissues (Kamrath *et al.*, 2012; Turcu *et al.*, 2014) (*Level of evidence: B*).

In summary, hyperandrogenism in NCAH results from (Fig. 3):

(1) Adrenal hyperactivity depending on altered enzyme kinetics without increased ACTH circulating levels.
(2) Increased peripheral conversion to androgens of circulating excessive levels of steroid metabolites.
(3) Increased ovarian androgen secretion determined by the appearance of a secondary PCOS-like phenotype in NCAH patients.

Clinical presentation of NCAH

Clinical presentation of NCAH during childhood and adolescence

Most children with NCAH are asymptomatic in the prepubertal years and exhibit normal external genitalia at birth and throughout early childhood. Premature pubarche may be the first clinical presentation with onset reported as early as 6 months of age (*Level of evidence: A*). In one study including 25 females younger than 10 years-old, 92% had premature pubarche as presentation of NCAH (Moran *et al.*, 2000). In another study, as many as 60% of children with NCAH presented with premature adrenarche (Kohn *et al.*, 1982). In fact, the prevalence of NCAH in children with premature adrenarche has been reported to vary from 5% to 20% (Dacou-Voutetakis and Dracopoulou, 1999).

Children with NCAH may demonstrate rapid linear growth, bone age advancement and tall stature (Oberfield *et al.*, 2011). Short stature has also been reported in subjects with NCAH, especially when

glucocorticoid therapy was begun prior to the onset of puberty (Speiser et al., 2010). In a cohort of 30 adult NCAH patients, 7% had a predicted adult height standard deviation score (SDS) of −2.0 or less (Finkielstain et al., 2012). Among 141 women with NCAH studied by Livadas and colleagues (Livadas et al., 2015), only 1 (0.7%) demonstrated short stature (defined as height less than the third percentile for sex and age using local population standards). Overall, while some NCAH patients may be at risk for short stature, this risk appears to be relatively small and most children reach a relatively normal final height for family standards (Speiser et al., 2010) (*Level of evidence: B*). This likely is due to the fact that these children have not been exposed to prolonged suppressive effects of glucocorticoid treatment on linear growth (Weintrob et al., 1997).

Female adolescents may present with severe acne, hirsutism, and even alopecia (Moran et al., 2000; New, 2006) (*Level of evidence: B*). Moreover, clitoromegaly can be found in 11% of adolescents (Moran et al., 2000). In a study of 220 adolescents, menstrual irregularities (56%) or even primary amenorrhea (9%) were the presenting sign of NCAH (Moran et al., 2000). The long-term outcome of these findings will be discussed later with respect to fertility in this population.

In male adolescents, gynecomastia may be the presenting symptom. Wasniewska et al. (2008) reported two boys presenting with either prepubertal or pubertal gynecomastia. In both boys, gynecomastia completely regressed 5–8 months after the institution of glucocorticoid replacement therapy. They suggested that NCAH due to 21-hydroxylase deficiency should be kept in mind in the differential diagnosis of either prepubertal or pubertal gynecomastia (Wasniewska et al., 2008).

Limited data exist in patients with NCAH regarding obesity, metabolic syndrome, and exercise intolerance. It has been suggested that children with classic CAH have increased fat mass (Speiser et al., 1992). The BMI distribution in German patients with CAH, although not NCAH patients, was skewed towards larger figures when compared with appropriate controls (Volkl et al., 2006). Similarly, increased abdominal adiposity was noted in adolescents and young adults with classic CAH due to 21-hydroxylase deficiency with increased visceral to subcutaneous adipose tissue ratios when compared with matched controls (Kim et al., 2015). Finally, adolescents with congenital adrenal hyperplasia exhibit impaired exercise performance and an enhanced systolic blood pressure response to exercise that appears to be related to glucocorticoid therapy (Marra et al., 2015). Extrapolation of these findings to patients with NCAH must be considered cautiously.

Clinical presentation of NCAH in adult women

Clinical symptoms in adult women with 21-hydroxylase deficient NCAH encompass two principal areas: those due to hypothalamic–pituitary–ovarian dysfunction and those due to dermatologic signs of hyperandrogenism. Other clinical symptoms in adulthood may include metabolic dysfunction and anatomic defects of the adrenal cortex.

Adult women with NCAH generally present with hirsutism, acne and/or androgenic alopecia. In various studies, the prevalence of hirsutism ranged from 60% to 80% (Bidet et al., 2009; Moran et al., 2000; Pall et al., 2010; Finkielstain et al., 2012), acne was found in approximately one-third of cases (Moran et al., 2000), and alopecia was seen in 2–8% (Moran et al., 2000; Livadas et al., 2015) (*Level of evidence: A*). Clitoromegaly has been reported in 6–20% of adult women with NCAH (Moran et al., 2000).

Over 30–50% of patients with NCAH show overt ovulatory and menstrual dysfunction (Moran et al., 2000; Livadas et al., 2015) (*Level of evidence: B*). Polycystic ovarian morphology (PCOM) may also be present. In one study, enlarged ovaries were found in 44% of 25 adult women with NCAH and PCOM was referred (but the number of follicles was not measured) in 80% of these patients (Carmina, 1995). In another study, PCOM was found in only 24% of NCAH patients (Pall et al., 2010).

Even though adrenal hyperplasia and adenomas have been reported in patients with NCAH, the actual prevalence of such pathology remains largely unknown (Azziz and Kenney, 1991). However, the finding of a small increase in the prevalence of genotypically verified CAH, including NCAH, in patients presenting with adrenal incidentalomas in a recent meta-analysis further suggests an association between both disorders (Falhammar and Torpy, 2016) (*Level of evidence: B*). Accordingly, patients with adenomas identified incidentally should be first evaluated for NCAH/CAH before any attempt of surgical treatment (Jaresch et al., 1992). Tumor size may improve with corticosteroid therapy (Kiedrowicz et al., 2015) (*Level of evidence: D*). Whether or not adrenal incidentalomas in patients with NCAH require follow-up is still unclear, even though in the authors' clinical experience progression to malignancy or to syndromes of hormone hypersecretion are very unlikely (*Level of evidence: C*). In a series of parents of patients with classic CAH, 10/249 (4%) were found to have cryptic/asymptomatic NCAH and one female patient was found to have a small adrenal myelolipoma (Nandagopal et al., 2011).

Some investigators have reported that adult males and females with NCAH demonstrate evidence of mild insulin resistance (Bayraktar et al., 2004), although others have been unable to confirm these findings (Moran et al., 2000). One recent study reported that adult patients with NCAH demonstrated an increased risk of metabolic and cardiovascular morbidities (Falhammar et al., 2015). Not only were obesity and type 2 diabetes much more common in the NCAH patients than in the general population, but the odds ratio for cardiovascular disease (mainly for stroke) was also elevated (2.9). However, the number of NCAH patients ($n = 75$) was relatively small and the number of events were too few to accurately assess the actual prevalence of cardiovascular events in adult subjects with NCAH. Nevertheless, and even though these preliminary data need confirmation in larger follow-up studies and in other populations, it appears reasonable and safe to extend to NCAH subjects the current recommendations for the assessment and management of cardiometabolic dysfunction also observed in PCOS (Wild et al., 2010).

Fertility and reproductive outcome in women with NCAH

Despite the adrenal and ovarian androgen excess, the majority of women with NCAH will conceive spontaneously (Feldman et al., 1992; Moran et al., 2006) (*Level of evidence: B*). Nonetheless, in one study, the rate of a singleton live birth was higher in NCAH women diagnosed and treated for their disorder prior to conceiving than in those patients who conceived spontaneously (86% vs. 69%, respectively); there were no differences in the rate of ectopic pregnancy, preterm birth, stillbirths, twins or multiple pregnancies (Moran et al., 2006). Spontaneous miscarriages have been reported in 25% of pregnancies occurring before the diagnosis of NCAH was made, a figure that decreases to 10% in women in whom diagnosis was performed

and treatment was started before pregnancy (Moran *et al.*, 2006; Bidet *et al.*, 2010), suggesting that prompt diagnosis and therapy may reduce the rate of pregnancy loss in NCAH (*Level of evidence: B*).

Between 10% and 30% of NCAH women of reproductive age complain of infertility (Moran *et al.*, 2000, 2006; Bidet *et al.*, 2010) (*Level of evidence: B*). Anovulation is the main cause of subfertility in NCAH women, which is most evident by the positive outcome in response to ovulation induction (Reichman *et al.*, 2014; Lekarev *et al.*, 2015). However, persistent elevated progestogen concentrations (due to excess circulating levels of progesterone and 17-OHP of adrenal origin) may also result in an unfavorable cervical mucus and a persistent decidualized or hypo- or atrophic endometrium (Reichman *et al.*, 2014).

Clinical presentation of NCAH in adult males

Data regarding adult male patients with NCAH due to 21-hydroxylase deficiency are extremely limited. Only a few papers including case reports and small series are available in the literature. It would be expected that NCAH occurs equally in men and women, since it is an autosomal recessive disease. But among the 440 patients with NCAH patients, only 96 were males (New, 2006). Accordingly, the great majority of male patients are asymptomatic and most are identified during genetic screening carried out for purposes of genetic counseling. Only a few seek medical advice because of prepubertal or pubertal manifestations (Witchel, 2013).

Testicular adrenal rest tumors (TARTs) are the most important cause of infertility in male patients with CAH due to 21-hydroxylase deficiency and are more frequent in the salt-wasting form (Cabrera *et al.*, 2001). While TART may present also in males with NCAH due to 21-hydroxylase deficiency (Chrousos *et al.*, 1981; Falhammar *et al.*, 2012; Nandagopal *et al.*, 2011), most data suggest that TART are quite uncommon in these patients (Pinkas *et al.*, 2010). Hence, the routine measurement of 17-OHP in the evaluation of male infertility is not recommended (*Level of evidence: B*).

Diagnosis of NCAH

Measurement of basal and cosyntropin-stimulated 17-hydroxyprogesterone concentrations

The clinical picture does not permit a definite diagnosis of NCAH (Table IV, *Level of evidence: A*). Moreover, the NCAH phenotype may be highly variable even within a family sharing the same *CYP21A2* genotype (Bidet *et al.*, 2009). Hence, the diagnosis of NCAH relies mostly on the finding of serum 17-OHP concentrations above 10 ng/ml (30 nmol/l) either in basal conditions or after cosyntropin-stimulation (New *et al.*, 1983; Dewailly *et al.*, 1986; Azziz and Zacur, 1989) (*Level of evidence: A*).

Screening is conducted by measuring this steroid precursor early in the morning during the follicular phase of the menstrual cycle. A basal 17OHP level cut-off value of 2 ng/ml (6 nmol/l) is recommended for screening, although ideally the reference value should be established at each laboratory since lower figures must be valid based on specific methodology used for 17-OHP determinations (Escobar-Morreale *et al.*, 2008) (Table IV). An increased basal 17-OHP result less than 10 ng/ml (30 nmol/l) must be confirmed by the finding of 17OHP above 10 ng/ml (30 nmol/l) after stimulation of adrenal function by an intravenous bolus of cosyntropin (Azziz *et al.*, 1994; Trapp and Oberfield, 2012).

The cosyntropin-stimulation test should be performed in the morning during the follicular phase of the menstrual cycle. 17-OHP concentrations are measured 30 and/or 60 min after a 250 μg intravenous bolus of cosyntropin. The 10 ng/ml (30 nmol/l) cut-off value corresponds approximately to 3-fold the upper normal limit of 17-OHP concentrations observed in normal women after cosyntropin-stimulation. If available, *CYP21A2* genotyping should follow the biochemical diagnosis of NCAH, not only for diagnostic confirmation but also for detecting compound heterozygosity for severe alleles that may result in CAH cases in the progeny of these patients (Table IV). Of note, normal basal and ACTH-stimulated 17-OHP responses do not exclude a carrier status for mild or severe *CYP21A2* mutations (Armengaud *et al.*, 2009) (Table IV).

For children presenting with premature pubarche, consideration of the diagnosis of NCAH is warranted when elevated basal 17-OHP, androstenedione, and testosterone concentrations are elevated and/or bone age is advanced. It has been suggested that a cut-off value of basal 17-OHP > 2 ng/ml is adequately sensitive and specific to identify prepubertal children with NCAH (Armengaud *et al.*, 2009).

The effectiveness of this diagnostic approach in adolescents and adults is supported by several studies (Azziz *et al.*, 1999; Bidet *et al.*, 2009; Nandagopal *et al.*, 2011). However, the election of a cut-off basal 17-OHP value for the screening of 21-hydroxylase deficient NCAH is matter of debate. In a study by Azziz *et al.* (1999), eight healthy controls, 20 patients with genetically confirmed NCAH and 284 patients with hyperandrogenism were investigated with the aim

Table IV Evidence based recommendations for the diagnosis of NCAH (*Level of evidence A or B*).

(1) We recommend screening for NCAH due to 21-hydroxylase deficiency in all patients presenting with signs and symptoms of androgen excess except in specific ethnic populations in which the prevalence of this disorder is negligible.
(2) We recommend the clinical diagnosis of NCAH due to 21-hydroxylase deficiency to require a circulating 17-OHP concentration above 10 ng/ml (30 nmol/l) either basally or after cosyntropin-stimulation.
(3) We recommend *CYP21A2* genotyping in patients presenting with circulating 17-OHP concentration above 10 ng/ml (30 nmol/l) with the aim of confirming the diagnosis and identifying severe alleles that may increase the risk of CAH in the offspring of NCAH patients.
(4) We suggest establishing local assay-specific 17-OHP cut-off values for the screening of NCAH due to 21-hydroxylase deficiency. If these are not available, conducting cosyntropin-stimulation in patients presenting with basal 17-OHP above 2 ng/ml (6 nmol/l) would identify most patients with the disorder.
(5) We recommend *against* making any conclusion about the *CYP21A2* carrier status of any person based on the results of a cosyntropin-stimulation test.

of establishing the cut-off value of basal 17-OHP concentrations for the screening of NCAH due to 21-hydroxylase deficiency. A cosyntropin-stimulated 17-OHP level above 10 ng/ml was used as the criterion for the diagnosis. They suggested that a basal 17-OHP level of 2 ng/ml should be used to maximize detection of NCAH.

However, Escobar-Morreale *et al.* (2008) found that the most appropriate cut-off value for the diagnosis of NCAH due to 21-hydroxylase deficiency was a basal follicular phase 17-OHP above 1.7 ng/ml (5.1 nmol/l), which had 100% sensitivity and 88.6% specificity, in a series of 270 consecutive women with hyperandrogenism submitted to cosyntropin-stimulation, of whom six had NCAH confirmed by *CYP21A2* molecular genetic testing. The authors suggested that the cut-off value should be established for every laboratory, or, if this is not possible, the cut-off value should be lowered to 1.7 ng/ml (5.1 nmol/l) from 2 ng/ml (6 nmol/l) as the upper limit of the normal range. Of note, these suggestions are reinforced by the finding of basal plasma 17-OHP levels below 2 ng/ml (6 nmol/l) in 13 of the 161 NCAH patients reported by Bidet *et al.* (2009).

Unluhizarci *et al.* (2010) investigated the prevalence of NCAH due to 21-hydroxylase deficiency in 285 Turkish women with hirsutism or hyperandrogenism in a nationwide study. When cosyntropin-stimulated 17-OHP levels exceeding 10 ng/ml (30 nmol/l) was accepted as hormonal criterion, 2.1% of the women had NCAH. The diagnosis was confirmed by *CYP21A2* genotyping in all patients. Because the lowest 17-OHP level in NCAH women in that study was 2.2 ng/ml (6.7 nmol/l), the 2 ng/ml (6 nmol/l) cut-off value appeared to be adequate in this setting.

In a multi-center study including 220 women with NCAH due to 21-hydroxylase deficiency, defined by basal and/or cosyntropin-stimulated 17-OHP concentrations above 10 ng/ml (30 nmol/l), the median ACTH-stimulated 17-OHP level was 37 ng/ml (113 nmol/l) yet 10% of the patients had basal 17-OHP values below 2 ng/ml (6 nmol/l) (Moran *et al.*, 2000). According to this study, 10% of NCAH patients would be undiagnosed if the cut-off for basal 17-OHP level was established at a 2 ng/ml (6 nmol/l) concentration.

In conclusion, we suggest using a 2 ng/ml (6 nmol/l) cut-off value of basal 17OHP concentrations for the screening of NCAH in cases where in-house cut-off values are not available (Table IV, *Level of evidence: A*).

Genetic aspects of diagnosis

The *CYP21A2* locus is quite complicated, precluding its molecular genetic analysis as the first line diagnostic test (*Level of evidence: A*). However, it is essential for genetic counseling since many patients with NCAH carry a severe allele that might result in CAH in their progeny (Table IV, *Level of evidence: A*).

Since one allele can carry multiple mutations, merely identifying two *CYP21A2* mutations in the absence of diagnostic hormone test results is insufficient to confirm the diagnosis of CAH. Multiple genetic testing strategies such as PCR-based mutation detection methods, DNA sequencing, and multiplex ligation-dependent probe amplification may be needed to accurately ascertain the mutations in an affected individual. In some instances, it is necessary to confirm that mutations are on opposite alleles. To segregate the specific alleles, *CYP21A2* genetic analyses can be obtained from parents to discriminate the specific maternal and paternal mutations.

During a study that involved comprehensive genetic testing of 145 unrelated patients with CAH, 10/249 (4%) parents were found to have genotype analyses diagnostic for NCAH (Nandagopal *et al.*, 2011). All 10 parents were compound heterozygotes for a severe mutation on one allele and a mild mutation on their other allele; their affected children had inherited the severe mutation. These parents were largely asymptomatic, but ~50% of the mothers reported subfertility.

Genetic analysis can be a useful adjunct to newborn screening for family studies, and to accurately distinguish between heterozygous mutation carriers and affected individuals (Ambroziak *et al.*, 2016). Importantly, the caveats for molecular diagnosis include the complexity of this genetic locus, identification of multiple mutations on a single allele, detection of different *CYP21A2* mutations within a family, and cryptic NCAH in other family members (*Level of evidence: B*).

In Fig. 4, an algorithm for the diagnosis of NCAH is shown.

Treatment of NCAH

Prepubertal and adolescent patients

Treatment with glucocorticoids for NCAH should be considered only for pre- and peripubertal children who have inappropriately early onset or rapid progression of pubarche or bone age (Table V, *Level of evidence: B*). Routine treatment with GnRH analogs and GH are not recommended and should be considered experimental (Trapp and Oberfield, 2012). In adolescents and young adults with NCAH, treatment is reserved for those who demonstrate important or clinically significant hyperandrogenism. However, recent data suggest that replacement with glucocorticoids should be considered when major surgery or trauma in childhood or even when 'fatigue' occurs, particularly in those NCAH patients with a decreased cortisol response to cosyntropin. A cortisol cut-off value of 18 μg/dl (497 nmol/l), below which glucocorticoid therapy may be considered, has been recommended in children and adolescents (Stoupa *et al.*, 2015) (*Level of evidence: C*).

Adult women

In Fig. 5, an algorithm for the treatment of NCAH is shown.

Treatment of hyperandrogenism

Theoretically adrenal androgen excess could be treated either by suppressing ACTH production with glucocorticoids and therefore lowering the excessive androgen production by the adrenal glands or by blocking the effects of androgens on their receptors with anti-androgens (Escobar-Morreale *et al.*, 2012). However, in adult women with NCAH, reduction of circulating androgens may also be obtained by blocking ovarian androgen secretion by the use of estrogen–progestin combination preparations or GnRH agonists (Carmina and Lobo, 1994; Trapp and Oberfield, 2012). Most available data suggest that, in these patients, both peripheral androgen blockade or ovarian androgen suppression are more effective than glucocorticoids in reducing circulating androgens (Frank-Raue *et al.*, 1990; Spritzer *et al.*, 1990) (Table V, *Level of evidence: B*). One controlled randomized trial on a series of 30 patients compared the effectiveness of cyproterone acetate (CPA) versus hydrocortisone (Spritzer *et al.*, 1990). Based on the hirsutism score, CPA was more effective than hydrocortisone (hirsutism improved in 54% and 24% patients, respectively). In another randomized trial

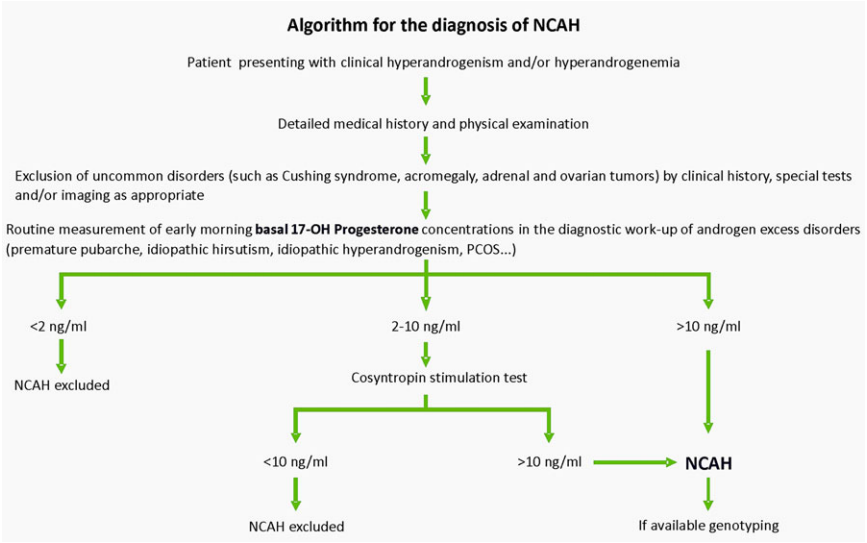

Figure 4 Algorithm for the diagnosis of NCAH. 17-OH progesterone, 17-hydroxyprogesterone.

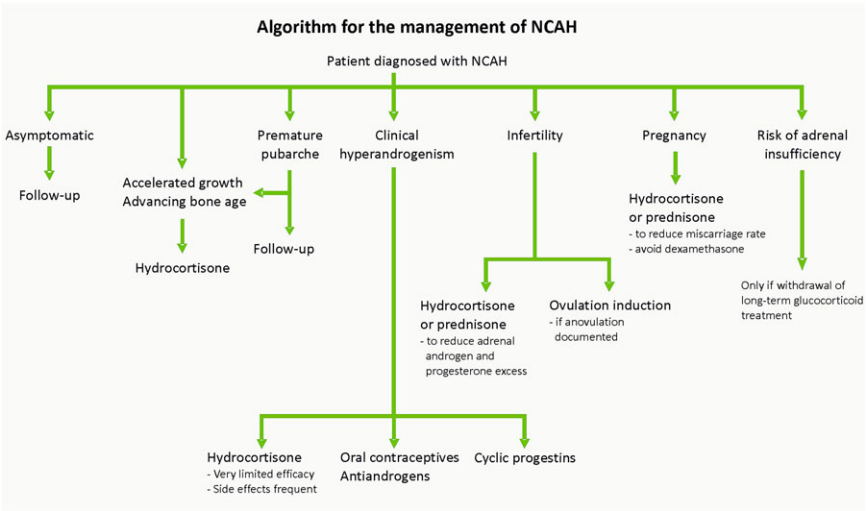

Figure 5 Algorithm for the treatment of NCAH.

including 28 patients, CPA associated with ethinylestradiol was found to be superior to dexamethasone (hirsutism improved in 66% and 31% patients, respectively) (Frank-Raue *et al.*, 1990). In countries where CPA is not available, spironolactone could be used as an alternative androgen blocking drug. Although the anti-mineralocorticoid action of spironolactone could be a concern when treating patients with NCAH, to date this hypothetical risk has not been documented. A concern regarding the use of spironolactone, or for that matter any anti-androgen in a female experiencing unprotected coitus, would be the risk of impaired virilization of the external genitalia of a male fetus in the event that an unanticipated pregnancy occurred. Generally, when fertility is not an immediate concern, estrogen–progestin combination preparations represent the most commonly used treatment of adult women with NCAH (Escobar-Morreale *et al.*, 2012).

Management of infertility in NCAH

NCAH adult women who have not conceived spontaneously and who demonstrate overt or subclinical ovulatory dysfunction may benefit from glucocorticoids or from ovulation induction (Table V, *Level of evidence: B*).

Several studies, mostly retrospective and including small series, suggest that glucocorticoids improve fertility probably by lowering adrenal

Table V Evidence based recommendations for the treatment of NCAH (*Level of evidence A or B*).

(1) In children or adolescents with NCAH, treatment with glucocorticoids should be considered only for those who have inappropriately early onset of pubarche or rapid progression of bone age.
(2) In adult women with NCAH, ovarian androgen suppression or peripheral androgen blockade are more effective than glucocorticoids for reducing circulating androgens and their effects.
(3) Adult women with NCAH who have not conceived spontaneously and who demonstrate overt or subclinical ovulatory dysfunction may benefit from glucocorticoids or from ovulation induction.

androgen and progesterone excess that impair ovulation and endometrial receptiveness, respectively. In a recent study of 38 patients who presented with oligo-amenorrhea before treatment, 27 achieved regular menstrual cycles with hydrocortisone treatment (average dose: 17.5 ± 7.5 mg/day) (Bidet *et al.*, 2010). Among the 11 patients presenting with amenorrhea before treatment, only three still had amenorrhea after glucocorticoid-only treatment. Plasma testosterone and androstenedione concentrations decreased significantly in all patients.

When hydrocortisone therapy does not lead to normal ovarian function, ovulation induction with clomiphene citrate or exogenous gonadotropins, or assisted reproductive techniques, should be considered following the usual protocol for these treatments (Table V, *Level of evidence: B*).

Management of pregnancy in NCAH

There are no clear guidelines concerning treatment of NCAH during pregnancy. Cross-sectional data suggest that early pregnancy losses might be lower when patients are on glucocorticoid therapy and these data support continuing corticosteroids during pregnancy (Trapp and Oberfield, 2012).

Feldman *et al.* (1992) reported 6 miscarriages in 18 pregnancies (33%) occurring in 10 NCAH patients before diagnosis, and none among pregnancies occurring during hydrocortisone treatment. Moran *et al.* (2006) observed a markedly lower rate of miscarriage after diagnosis compared to prior to diagnosis among 101 NCAH patients and 203 pregnancies (6.2% vs. 25.4%, respectively). However, in this study, the respective benefits of glucocorticoids and ovulation induction were not clearly established. Bidet *et al.* (2010) observed that the risk of miscarriage in the absence of glucocorticoid treatment was significantly higher than with treatment (odds ratio: 4.5, 95% confidence interval: 1.4–14.2). Although these studies suggest that glucocorticoid replacement reduces the miscarriage risk, most of the data about this issue were obtained from infertile anovulatory women, which may have biased the results (*Level of evidence: C*).

Glucocorticoid replacement is usually maintained at a hydrocortisone dose of 20–25 mg/day or a prednisone dose of 2.5–5 mg/day, but no study so far has prospectively addressed the benefits of this practice to prevent early pregnancy loss and/or improve pregnancy outcome in NCAH. Of note, the selection of glucocorticoid is important since dexamethasone crosses the fetoplacental barrier and may have a long-term detrimental impact on fetal intellectual development (Harris and Seckl, 2011; Wallensteen *et al.*, 2016) (*Level of evidence: D*). Therefore, dexamethasone should be avoided during pregnancy in favor of replacement doses of glucocorticoids such as hydrocortisone and prednisone, which are easily metabolized by placental 11 beta-hydroxysteroid dehydrogenase type 2.

Cortisol-binding globulin concentrations increase in pregnancy, and as hydrocortisone administered exogenously is bound by this globulin (Jung *et al.*, 2011), hypothetically the dose of hydrocortisone should be increased in pregnant patients with NCAH. However, in actual practice there are no biochemical markers and few clinical markers that should be followed in pregnancy, and doses are rarely adjusted.

Risk of adrenal insufficiency

As previously reported, there is limited evidence that some patients with NCAH who present with reduced cortisol response to ACTH need glucocorticoid supplementation when undergoing major surgery or if trauma occurs (Stoupa *et al.*, 2015). There are no data demonstrating the benefits of prophylactic supraphysiological loading with glucocorticoids prior to surgery or other elective procedures on the quality of life, on potential morbidity, or on mortality rates, nor have potential secondary effects been investigated (Young *et al.*, 2010). Therefore, the risk/benefit ratio has not been established and routine systematic hydrocortisone loading for elective procedures in NCAH patients cannot be recommended (*Level of evidence: D*).

A review of the literature identified only a few cases of adults with NCAH who suffered acute adrenal insufficiency (Young *et al.*, 2010; Falhammar *et al.*, 2014; Reichman *et al.*, 2014). However, two cases of fatal adrenal crisis have been reported in young NCAH patients (Falhammar *et al.* 2014), although these unfortunate events probably resulted from the discontinuation of long-term chronic glucocorticoid treatment. Therefore, in patients who have been on long-term glucocorticoid replacement and whose treatment is stopped abruptly, the risk of acute adrenal insufficiency may be substantial, and possibly even higher than for other subjects being treated with prolonged glucocorticoid therapy, because of the combination of corticotroph inertia and a relative inefficiency in cortisol secretion.

There is no consensus regarding the recommendation that NCAH patients carry a medical alert identification, available in various forms such as wallet cards, bracelets, necklaces, etc., stating that the patient has NCAH and may be at risk for adrenal insufficiency, and that systematic glucocorticoid treatment should be considered prior to surgery or if acute trauma or acute physical stress occurs. Nevertheless, patients on chronic glucocorticoid therapy may need to carry with them medical alert identification.

Future perspectives on the diagnosis and treatment of NCAH

Most of the studies addressing the biochemical diagnosis of NCAH due to 21-hydroxylase deficiency have relied on direct immunoassays

such as radioimmunoassay or time-resolved fluorescence assay to measure circulating 17-OHP concentrations. As happens with any immunoassay measuring steroid hormones at the nanomolar range, 17-OHP immunoassays suffer from reliability issues because of the lack of sensitivity, specificity and of matrix effects, often overestimating true steroid values (Meier *et al.*, 2004; Rauh, 2009). Such issues may contribute to the discrepancies in the 17-OHP cut-off values suggested for screening of NCAH described above. Hopefully, the use of liquid chromatography coupled with mass spectrometry (LC/MS) assays will improve the accurate measurement of 17-OHP concentrations and also provide the analysis of steroid panels that may discriminate 21-hydroxylase deficiency from other conditions (Turcu *et al.*, 2015).

Regarding treatment, new drugs that simulate physiologic glucocorticoid secretion or provide ease of delivery are under investigation at present, but have been studied primarily in CAH patients. Most recently evaluated is Chronocort®, a modified release formulation of hydrocortisone (Mallappa *et al.*, 2015). Corticotropin-releasing factor receptor antagonists are being investigated in adult females with CAH (Auchus *et al.*, 2015) and subcutaneous hydrocortisone therapy has been tried in a limited number of individuals with CAH who have a rapid clearance of cortisol or those who cannot tolerate oral glucocorticoid therapy (Nella *et al.*, 2016). However, more important than finding new glucocorticoid-like drugs is to determine whether glucocorticoid treatment, aside from its use in patients presenting with accelerated growth, cortisol insufficiency, or fertility issues, is truly necessary in most NCAH patients. In fact, most of the chronic problems developing in NCAH patients (increased risk of obesity, type 2 diabetes and cardiovascular disease) might be related more closely to prolonged glucocorticoid use than to the disorder itself. The pathophysiology of NCAH is quite different from that of CAH and long-term glucocorticoid treatment should be reserved only for a few select patients.

There is also preliminary data suggesting that abiraterone acetate, a steroidal P450c17 inhibitor used in prostate cancer, may be a useful therapeutic agent in adult women with CAH (Auchus *et al.*, 2014). On the contrary, no data are available in patients with CAH regarding the possible use of steroid sulfatase inhibitors. Finally, neurokinin-3 receptor antagonists are being investigated in women with PCOS with the aim of reducing LH pulse frequency and LH and T levels (George *et al.*, 2016). Whether these drugs may be useful also in NCAH is unclear at present.

Authors' roles

E.C. organized the study and participated in the collection of literature data, manuscript writing and critical discussion. He read and approved the final manuscript. D.D. participated in the collection of literature data, manuscript writing and critical discussion. He read and approved the final manuscript. H.F.E.-M. conducted the meta-analysis of the prevalence of NCAH among women with androgen excess and participated in the collection of literature data, manuscript writing and critical discussion. He read and approved the final manuscript. F.K. participated in the collection of literature data, manuscript writing and critical discussion. He read and approved the final manuscript. C.M. participated in the collection of literature data, manuscript writing and critical discussion. He read and approved the final manuscript. S.O. participated in the collection of literature data, manuscript writing and critical discussion. She read and approved the final manuscript. S.W. participated in the collection of literature data, manuscript writing and critical discussion. She read and approved the final manuscript. R.A. participated in the collection of literature data, manuscript writing and critical discussion. He read and approved the final manuscript.

Funding

No specific funding was sought for the study. During the preparation of the manuscript, H.F.E.-M. received grants PI11501686 and PIE1600050 from Instituto de Salud Carlos III, Ministry of Economy and Competitiveness and European Regional Development Fund, Spain.

Conflict of interest

The authors have no competing interests to declare.

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