A guide to understanding the steroid pathway: New insights and diagnostic implications

Ronda F. Greaves [a,b,*], Ganesh Jevalikar [c], Jacqueline K. Hewitt [b,d,e], Margaret R. Zacharin [b,d,e]

[a] School of Medical Sciences, RMIT University, Victoria, Australia
[b] Murdoch Children's Research Institute, Melbourne, Australia
[c] Medanta Medicity Hospital, Gurgaon Haryana, India
[d] Department of Endocrinology & Diabetes, The Royal Children's Hospital, Victoria, Australia
[e] Department of Paediatrics, University of Melbourne, Victoria, Australia

ARTICLE INFO

ABSTRACT

Steroid analysis has always been complicated requiring a clear understanding of both the clinical and analytical aspects in order to accurately interpret results. The literature relating to this specialised area spans many decades and the intricacies of the steroid pathway have evolved with time. A number of key changes, including discovery of the alternative androgen pathway, have occurred in the last decade, potentially changing our understanding and approach to investigating disorders of sexual development. Such investigation usually occurs in specialised paediatric centres and although preterm infants represent only a small percentage of the patient population, consideration of the persistence of the foetal adrenal zone is an additional important consideration when undertaking steroid hormone investigations. The recent expanded role of mass spectrometry and molecular diagnostic methods provides significant improvements for accurate steroid quantification and identification of enzyme deficiencies. However analysis of steroids and interpretation of results remain complicated. This review aims to provide an insight into the complexities of steroid measurement in children and offers an updated guide to interpretation, of serum and urine steroids through the presentation of a refined steroid pathway.

© 2014 The Canadian Society of Clinical Chemists. Published by Elsevier Inc. All rights reserved.

Introduction

Since the development of the early radioimmunoassays (RIA) for serum steroids [1] and gas chromatography (GC) metabolomic methods for urine steroids [2–5] in the 1960's, there have been significant advances in our understanding and quantification of these remarkable hormones. Recent refinements in mass spectrometry and molecular techniques have resulted in a paradigm shift in steroid analysis. Appreciation of the advantages and limitations of the analytical methods aids clinical interpretation. Even so, due to the number and similarity of many steroids the interpretation of results remains complicated; particularly in neonates. A clear understanding of steroid structure, the genetic basis of steroid biosynthesis and the relationship between steroids in the pathway is essential for accurate interpretation. With the recognition of the newly discovered alternative androgen pathway and the advent of mass spectrometry in the clinical diagnostic laboratory, it is timely to examine the current advances in steroid biosynthesis and analysis.

This review aims to provide an insight into the complexities of steroid measurement in children and offers an updated guide to the interpretation of serum and urine steroids through the presentation of a refined steroid pathway.

Structure and naming of steroids

Historically many of the current trivial steroid hormone names owe their origin to scientific endeavours of the early 20th century[6,7]. The isolation and naming of the first steroids related to their broad function, such as the names coined for "estrone" (estr(us)=fertile female; and one = ketone), "androsterone" (andro = male; ster = sterol; and one = ketone) and "testosterone" (testo = testes; ster = sterol; and one = ketone)[8]. As the repertoire of steroids of clinical interest expanded individual research groups began to name steroids either alphabetically or numerically in order of isolation[9–12]. The names assigned inevitably varied between these groups[13]. Some examples which still persist colloquially today include Kendall's compound "A" (11 dehydrocortisone), "B" (corticosterone), "E" (cortisone), and "F" (cortisol) and Reichtein's compound "S" (11 deoxy cortisol). Even when groups took steps to harmonise the names[13], the approach proved inadequate as new steroids were purified. In addition, these names did not recognise the steroids' important structural features. Hence it became evident that the alphabetical naming system was clumsy and a solution was sought.

* Corresponding author at: School of Medical Sciences, RMIT University, PO Box 71, Bundoora, Victoria 3083, Australia.
E-mail address: ronda.greaves@rmit.edu.au (R.F. Greaves).

The 1989 recommendations by the Joint Commission on Biochemical Nomenclature (JCBN) for The Nomenclature of Steroids serve as the foundation for our current formal naming rules[14–16]. Understanding the basis for names (trivial and expanded chemical names) of individual steroids provides insight into the steroid pathway; and avoids confusion with similar sounding names. However, with continued routine use of trivial names some confusion persists.

A common example where mistakes arise, due to similar spelling, is with the three androgens androstenediol, androstanediol and androstenedione. In this example each compound is a 19 carbon (C19) steroid, but each differs in double bonds, hydroxyl and/or keto groups. Androstenediol (C19 with a double bond and two hydroxyl groups) is the substrate of 3β-hydroxy steroid dehydrogenase (HSD) enzyme whilst androstenedione (C19 with a double bond and two keto groups) is the product of this enzyme activity from a slightly different but distinct section of the steroid pathway. Moreover, androstanediol (C19 with no double bonds and two hydroxyl groups) is not directly related to activity of the 3β-HSD enzyme and is a metabolite of the "alternative" steroid pathway. Whilst the trivial names are routinely used, this example highlights the benefits of appreciating the basis of the steroid structure (Fig. 1), understanding the steroid naming rules (Table 1), and the steroid pathway (Fig. 2), along with the action and location of the enzymes (Table 2). Together these provide clarity to aid interpretation of complex patient results.

Steroid synthesis

All steroids are synthesised from cholesterol. Tissues that can convert cholesterol with the cytochrome P450 side chain cleavage (P450scc) enzyme have the ability to produce steroids. In humans several organs are capable of steroidogenesis: adrenal cortex (zona glomerulosa, zona fasciculata and zona reticularis); Leydig cells of the testes; granulosa and theca cells of the ovary; and syncytiotrophoblasts of the placenta. It is also possible that some steroidogenesis takes place in the human brain[17]. Hence, steroid expression can be ubiquitous or tissue specific.

The local production of steroids is governed by the expression of enzymes and cofactors. For example the absence of 17α-hydroxylase activity diverts pregnenolone to aldosterone in the zona glomerulosa. Similarly, the presence of cytochrome b5 is required for 17,20-lyase activity in the zona reticularis, leading to
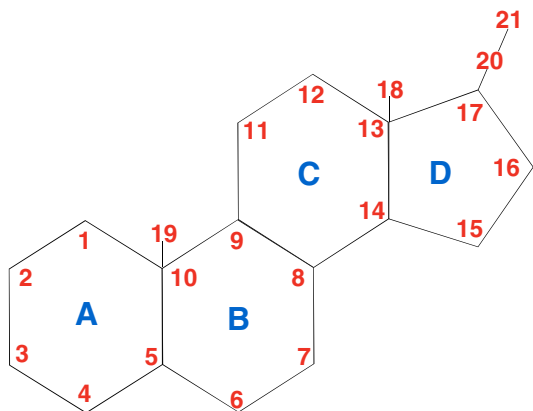

Fig. 1. Basic steroid structure showing a fully saturated 21 carbon steroid with the alphabetical naming of the individual rings and the numbering sequence of the carbon atoms. All steroids share the same basic 17 carbon structure with the presence of four linked rings (three six sided and one five sided) known as the cyclopentanophenanthrene (or cyclopentanoperhydrophenanthrene) ring. The rings are alphabetically labelled with the carbon atoms are numbered sequentially. Cholesterol is recognised as the parent steroid and contains 27 carbon atoms, whereas the three main groups of steroids of interest in clinical endocrinology consist of 18, 19 or 21 carbon atoms, representing the estrane, androstane and pregnane skeleton.

Table 1
Steroid naming rules, illustrating the steps to naming of the common steroids measured for the investigation of DSD in childhood.

Basic steroid naming rules
1. Choose a core name that most closely matches the steroid molecule. This will be either "andro" for carbon 19 steroids, "estra" for carbon 18 steroids and "preg" for carbon 21 steroids.
2. Add a suffix to this name to the number of unsaturated double bonds and specify the location of the unsaturated double bonds as a prefix number. The addition of an "an" or "ane" to the name indicates no double bonds whilst the addition of an "ene", "diene" or "triene" indicates one, two or three double bonds respectively. Note that these additions are commonly written both with and without the "e" at the end.
3. Indicate the number of hydroxyl groups prefixing with the position number of each group and also the orientation of the group to the plain of paper i.e. alpha or beta. The addition of "ol", "diol", "triol" or "tetrol" indicates one, two, three or four hydroxyl groups respectively. The relative location is given immediately before the naming addition.
4. Indicate the number (if any) of keto groups, prefixing with the position of each group. No addition to the name is given if there are no keto groups. The addition of "one", "dione", "trione" or "tetrone" indicates one, two, three or four keto groups respectively. The relative location is given immediately before the naming addition.
5. Prefixing important section of the steroid to highlight its relevance. Steroids may be prefixed with compounds for any structurally important component elements e.g. delta 4 androstenedione or 17 β oestradiol.
6. Suffixing standard derivatives i.e. steroids coupled to another compound. In general these standard derivatives are suffixed to the steroid name with the best known example being dehydro-epiandrosterone sulphate (DHEAS).

Note A — Position of hydrogen bonds: When the hydrogen bonds are designate as alpha (α) the orientation is below the plane of the paper, whilst the beta (β) orientation is above the plane of the paper. The configuration of hydrogen at position 5 (i.e. the ring-junction between A and B) should always be designated as either alpha or beta by placing this numeral and letter being placed immediately before the stem name. The trivial steroid name often includes "allo" for steroids with the alpha configuration at position 5 e.g. allo-tetrahydro-cortisol.

Note B — Position of double bonds: To specify where the double bond is always use the lower number of the ring structure. If double bond along the ring junction e.g. between positions 5 and 6, label as 5. If it is between positions 5 and 10, label as 5 (10).

Note C — Isomerisation. An isomer is a chemical with the same molecular formula but with a different structure and an epimer is also an isomer but with only one change to the structure e.g. epi-testosterone which is the result of a change in configuration of the OH group at position 17 of the steroid molecule. Whereas the terms cis (on the same side) and trans (on the opposite side) relate to a change in the position of a functional group.

Note D — Use of the term delta (Δ). Whilst many trivial names for steroids now relate to the steroid structure, the full chemical name or at least additional information about an important structural characteristic/component is often used for identification and analysis. As such with the example of androstenedione, it is often referred to as delta 4 androstenedione to recognise the important change in the position of the double bond from position five with the conversion from DHEA which has the double bond at position four.

Note E — Highlighting specific chemical components. Frequently specific sections of the steroid compound are named to highlight a function property. Examples include 17β for estradiol, 17-keto for a number of androgens and 16-hydroxy for steroids of the foetal adrenal pathway are often added as descriptors of the important functional components.

production of dehydroepiandrosterone (DHEA). Many tissues, such as fat and skin, also have enzymes that convert one steroid to another but this is typically not classified as steroidogenesis as they lack the P450scc enzyme. Instead these intracrine tissues utilise steroid products such as DHEA which are present in the circulation[18]. Tissue specific locations of steroid enzymes are detailed in Table 2.

Steroidogenic enzymes groups

The various steps of steroidogenesis are catalysed by several enzymes. These enzymes are essentially classified into two groups, the cytochrome P450 (CYP) enzymes and the hydroxysteroid dehydrogenase (HSD) enzymes.

The cytochrome P450 enzymes are heme containing oxidative enzymes. Their name is derived from their spectrophotometric characteristic of strong absorbance at 450 nm. Based on their intracellular location, CYPs are further classified into type 1 (mitochondrial) and type 2 (endoplasmic reticulum). Type 1 CYPs receive electrons from
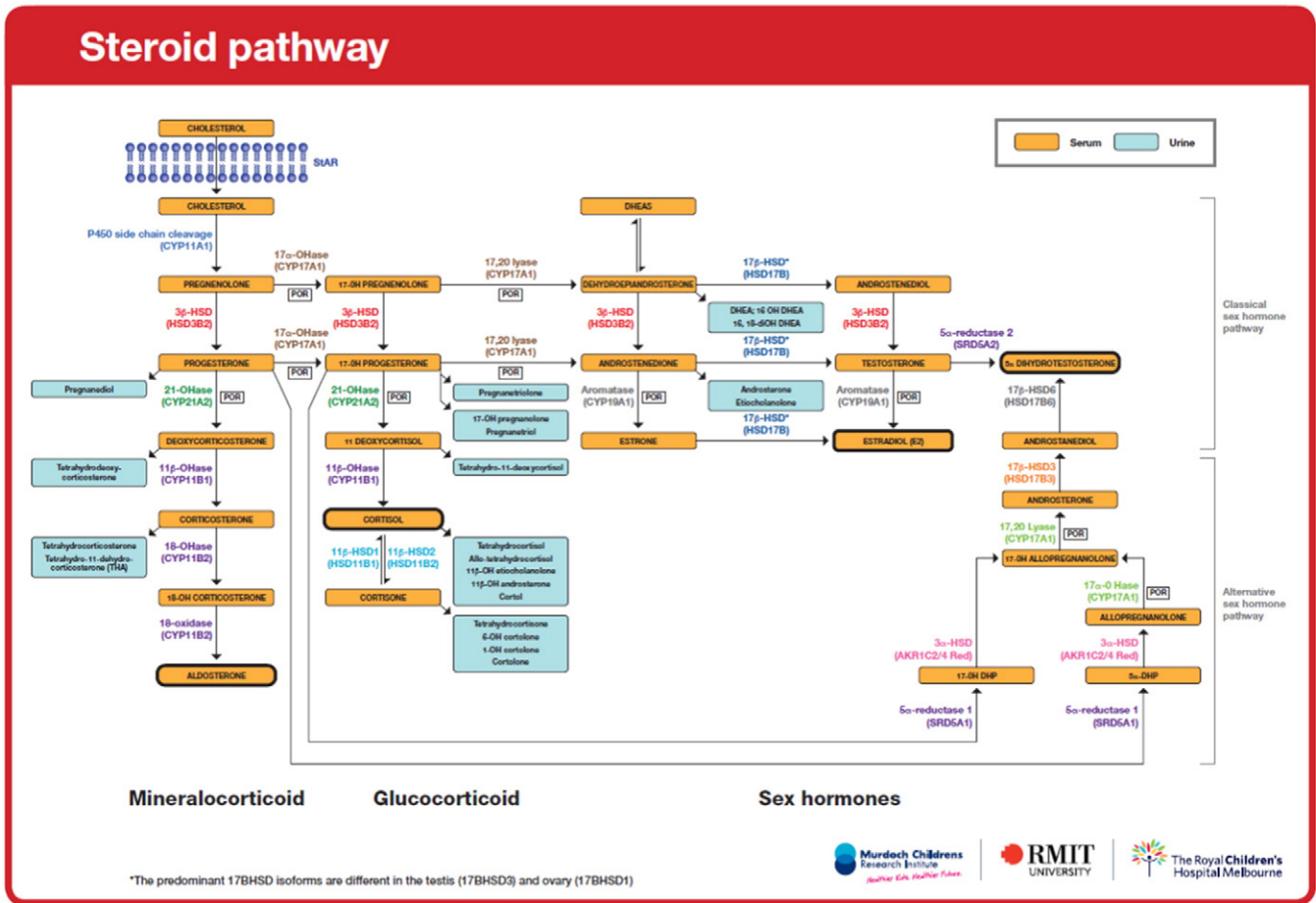
Fig. 2. Steroid pathway. This simplified pathway demonstrates 1) the relationship of the classical pathway in conjunction with the addition of the new alternative pathway to androgen production; 2) the relationship of steroids and their metabolites in serum (orange background) and urine (blue background) steroids; and 3) relationship of enzymes with the gene. Legend: CYP = cytochrome P450 enzyme; DHEA = dehydro-epiandrosterone; DHP = Dihydro-progesterone; HSD = hydroxyl steroid dehydrogenase; OHase = hydroxylase; POR = cytochrome P450 oxido reductase; SDR = short chain dehydrogenase/reductase.

NADPH via ferredoxin reductase and ferredoxin, whereas type 2 CYPs receive electrons via P450 oxidoreductase (POR). CYPs involved in steroidogenesis include P450scc, 11-hydroxylase and aldosterone synthase (type 1) and 17-hydroxylase/17,20-lyase, 21-hydroxylase and aromatase (type 2).

There are two types of HSDs, short chain dehydrogenase/reductase (SDR) and aldo-keto reductase (AKR). Examples of SDR enzymes are 11β-HSD 1 and 2 and 17β-HSD 1, 2 and 3. Examples of AKR enzymes include 3α-HSD, 3β-HSD 1 and 2 and 17β-HSD type 5. The HSDs use NADH/NAD$^+$ or NADPH/NADP$^+$ as cofactors. Reactions catalysed by HSDs are bi-directional, but the predominant action in vivo depends on local factors like pH and the concentration of cofactors.

Steroid groups of clinical interest

Naturally occurring steroids of clinical interest can be categorised based on the number of carbon atoms in the steroid skeleton[19,20].

• The carbon 18 steroids (estranes) are generally referred to as the female sex steroids and are produced primarily by the ovary in females and the adrenal gland in both genders.
• The carbon 19 steroids (androstanes) are the male sex steroids which are produced primarily by the Leydig cells of the testes and to a lesser extent by the adrenal gland in males, whereas in females, ovarian theca cells secrete between 5 and 25% of circulating testosterone with the major androgen production seen in the adrenal gland.

• The carbon 21 steroids (pregnanes) are produced primarily in the adrenal gland and cascade to produce the functional steroids aldosterone and cortisol.

Once steroids are synthesised and enter the circulation, they can be further metabolised, altering their activity.

Steroid metabolism

The classical steroid pathway quoted in numerous texts clearly highlights the cascade of steroids found in blood. Urine steroids are generally not mentioned in the various versions of this classical pathway as they are considered inactive products. However, these urine metabolites have been measured for decades by GC analysis for confirmation of inborn errors of steroid metabolism and adrenal tumours. These urine metabolomic profiles still regularly complement serum steroid analysis[21–24]. Despite this clear relationship, interpretation of urine steroids is frequently seen as challenging, as the pathway linking the blood steroids with their complementary urine metabolite(s) is infrequently provided in the literature. These urine steroid metabolites represent the products of phase one and two metabolism by the liver[25].

The first phase of steroid metabolism involves reduction, oxidation or hydroxylation of the steroid. Estrogens have a distinct metabolism to C19 and C21 steroids due to their aromatic A ring, which results in hydroxylation, primarily to estrone. For the androgens, glucocorticoids, mineralocorticoids and progestins with an ene-one moiety in the A ring, initial reduction followed by

Table 2
Enzymes and genes involved in steroidogenesis.

Enzyme	Gene	Chromosome locus	Tissue/organs of expression	Major function	Role in human steroidogenesis
P450 scc	CYP11A1	15q23-q24	All layers of adrenal cortex, Leydig cells, theca cells, brain	22-hydroxylation 20-hydroxylation 20,22-desmolase	Converts cholesterol to pregnenolone
3β-HSD1	HSD3B1	1p13.1	Placenta, breast, liver, brain	3β-dehydrogenase Δ5-Δ4 isomerase	Peripheral conversion of Δ5 compounds to Δ4
3β-HSD2	HSD3B2	1p13.1	All layers of adrenal cortex, Leydig cells, theca cells	3β-dehydrogenase Δ5-Δ4 isomerase	Conversion of Δ5 compounds to Δ4 in adrenal and gonads
17-Hydroxylase/ 17,20-lyase[a]	CYP17A1	10q24.3	ZF, ZR, Leydig cells, theca cells, brain	17α-hydroxylase 17,20 lyase	Conversion of pregnenolone and progesterone to 17-hydroxylated products, conversion of 17-OH-preg to DHEA and 17-OHP to androstenedione[b]
P450-Oxidoreductase	POR	7q11.2	Widely expressed in human tissues	Electron transfer	Electron donor for 17-hydroxylase, 21-hydroxylase and aromatase
21-Hydroxylase(21α-hydroxylase)	CYP21A2	6p21.1	ZG, ZF	21-Hydroxylation	Conversion of progesterone to DOC and 17-OHP to 11-deoxycortisol
11β-Hydroxylase	CYP11B1	8q21-q22	ZF, to a lesser extent in ZR, brain	11β-Hydroxylation	11-Deoxycortisol to cortisol, 11-DOC to corticosterone
Aldosterone synthase	CYP11B2	8q21-q22	ZG, brain	11β-Hydroxylation 18-Hydroxylation 18-Oxidation	DOC to aldosterone in 3 reactions
17β-HSD1	HSD17B1	17q11-q21	Placenta, granulosa cells	17β-Ketosteroid reductase	Oestrone to oestradiol
17β-HSD2	HSD17B2	16q24.1-q24.2	Endometrium, placenta, ovary	17β-Hydroxysteroid dehydrogenase	Oestradiol to oestrone, testosterone to androstenedione, DHT to 5α androstanediol
17β-HSD3	HSD17B3	9q22	Leydig cells	17β-Ketosteroid reductase	Androstenedione to testosterone,
17β-HSD5	HSD17B5 (AKR1C3)		ZR, foetal adrenal, liver, prostate	17β-Ketosteroid reductase	Androstenedione to testosterone
17β-HSD6	HSD17B6	12q13.3	Prostate, probable role in alternative pathway	Dehydrogenase	Andostanediol to DHT
P450Aromatase	CYP19A1	15q21.1	Granulosa cells, placenta, fat, growing bones	Oxidative demethylation	Androstenedione and testosterone to oestradiol
5α-reductase1	SRD5A1	5p15	Scalp, peripheral tissues	5α-Reduction	Metabolism of multiple steroids, peripheral conversion of testosterone to DHT
5α-reductase2	SRD5A2	2p23	Foetal genital skin, prostate	5α-Reduction	Testosterone to DHT
Reductive 3α-HSDs	AKR1C 1,2,3,4	10p14-p15	Multiple tissues	3α-Ketosteroid reductase, 17β-ketosteroid reductase 20α-Reduction of pregnanes	Inactivation of multiple steroids in liver, reduction of 5α-DHP to allopregnanolone in brain
11β-HSD1	HSD11B1	1q32-q41	Liver, testis, lung, fat, PCT	Reduction (in vivo)	Cortisone to cortisol
11β-HSD2	HSD11B2	16q22	Distal nephron, placenta	Dehydrogenase	Cortisol to cortisone

Abbreviations: scc — side chain cleavage; HSD — hydroxysteroid dehydrogenase; ZG — zona glomerulosa; ZF — zona fasciculate; ZR — zona reticularis; 17-OH-preg — 17-hydroxy-pregnenolone; 17-OHP — 17-hydroxyprogesterone; DHEA — dehydroepiandrosterone; DOC — deoxycorticosterone; DHT — di-hydrotestosterone; DHP — dihydroprogesterone; PCT — proximal convoluted tubule.

[a] 17,20-Lyase activity is enhanced in the presence of cytochrome b5.
[b] 17-OHP is a weak substrate for 17,20-lyase activity, AKR1C3 action.

hydroxylation occurs[26]. This process involves two sets of enzymes to generate the major steroids represented in urine:

- 5α or β-reductase. The first step in this process is reduction of the double bond located between positions 4 and 5 by 5α-reductase (type 1 or 2) or 5β-reductase. This provides the mechanism to generate the common 5 α and β isomers seen in the urine steroid metabolome.
- 3α-HSD. Steroids reduced at position 5 become substrates for 3α-HSD. Hydroxylation of steroids with a keto group at position 3 results in formation of the tetra hydro steroid products.

The sequential reduction at carbon position 4/5 followed by hydroxylation of the carbon position 3 keto group generates a number of important urine metabolites (e.g. tetrahydrocortisol, tetrahydrocortisone, androsterone, etiocholanolone and androstanediol). Following on from these first two steps some C19 and C21 compounds can be further metabolised by 17β-HSD or 20α/β-HSD respectively.

For the C21 steroids with a 5β configuration, further reduction can occur via side chain cleavage at position 20 by the enzyme 20α or 20β-HSD. In this process the tetrahydro products are hydroxylated at position 20 to form cortol (from cortisol), cortolone (from cortisone) and pregnanetriol (from 17-hydroxyprogesterone). Side chain cleavage of the 17-hydroxylated C21 compounds can generate C19 compounds (e.g. tetrahydrocortisol to 11β-hydroxyaetiocholanolone); this mechanism is not involved in the metabolism of progesterone, aldosterone and corticosterone as they are not 17-hydroxylated. On the other hand, the C19 steroids, androsterone and etiocholanolone can be further metabolised by 17β-HSD[26].

The second phase of steroid metabolism is reversible and involves conjugation with sulphate or glucoronide to reduce their hydrophobicity in preparation for excretion[25]. In the process of phase one metabolism most steroids now have an alcohol (i.e. OH) group at position three. The addition of a glucuronide or sulphate moiety to the oestrogen, androgen or pregnane molecule at this position has the effect of decreasing its hydrophobicity, allowing for excretion. The majority of steroids are glucuronidated and only a few, such as DHEA, are sulphated in this process. Of interest, this conjugation process has been proposed as a mechanism to transport inactive steroids to specific receptors where a local de-conjugation enzyme will reverse the process[25]. In addition to the steroid metabolites produced by the liver, small amounts of DHEA and cortisol are excreted in urine unchanged.

Important urinary metabolites of steroids are shown in Table 3 and also in Fig. 2.

Steroid synthesis and metabolism in the foetus and neonate

The process of steroid production and metabolism described so far relates to children and adults. Both gender and age influence steroid production. The most significant changes in steroid levels and pattern occur during the neonatal period, at adrenarche, puberty, pregnancy and also menopause. Of these milestones, interpretation of steroids in the neonate remains the most challenging. To understand some of the complications with interpretation in the neonate, a background understanding of the ontogeny of steroids is helpful.

Steroid hormones are produced by the foetus from the first trimester, with evidence of production at eight weeks gestational age. DHT is responsible for formation of male external genitalia, including labioscrotal fusion during the eighth to twelfth weeks of gestation, in addition to phallic growth which progresses throughout gestation but becomes more apparent in the third trimester[27,28]. At 20 weeks gestation the foetal adrenal gland can potentially function to produce cortisol and aldosterone; although the activity of the enzymes differ compared with the adult adrenal[29]. Little cortisol is produced in the foetus and maternal cortisol appears to be inactivated by placental 11β-HSD2[30]. By the third trimester most of the steroid enzymes, with the important exception of 3β-HSD are active; refer to Fig. 2 for clarification.

The foetal adrenal gland has its own characteristic enzyme pattern which includes very low activity of 3β-HSD and an additional sulphatase step[23]. This is significant as 3β-HSD, together with CY11A1, is responsible for metabolism at the start of the cascade to produce aldosterone, cortisol, oestradiol and testosterone. Production of the glucocorticoid cortisol is essential for the final stage of foetal lung maturity. Without this stimulation the lung remains immature and respiratory distress syndrome is the clinical result. This cortisol secretion in the neonate is pulsatile and diurnal variation does not commence until 8–12 weeks of age in term infants and preterm infants born >31 weeks gestation[31].

Foetal adrenal enzyme function persists, at least to 40 weeks gestation, even if preterm delivery has occurred[32]. At term the weight and size of the foetal adrenal gland are significantly greater than the adult adrenal gland[29,33]. This gland is primarily composed of a central reticular zone, known as the foetal zone. This zone is lost in early postnatal life and replaced by the "permanent" zone by six weeks of age in the full term neonate[29].

As 3β-HSD is responsible for the conversion of pregnenolone to progesterone the result of this low activity of 3β-HSD is preferential conversion of prenenolone to DHEA (a 3β-hydroxy-5-ene steroid).

This results in altered steroid metabolism, including production of 16 hydroxy steroids. Sulphate conjugation of steroids predominates in the foetus over glucuronidation, due to immaturity of the liver. As the foetal adrenal zone has a high sulphurylating activity, DHEA and other foetal steroids are conjugated with sulphates in this process. Of interest, in direct contrast to the foetal adrenal zone, the placenta has high 3β-HSD and sulphatase activity. This is relevant for steroid analysis of full and preterm neonates. Fig. 3 provides a simplified representation of the interaction of the foetal adrenal with the placenta and also the maternal circulation.

Persistence of the foetal adrenal zone, despite early delivery, has been a confounder for interpretation of steroid results. Advice given in such instances should be to repeat analysis post the equivalent of term corrected age e.g. at one and three months of age[21,34]. The need for this advice highlights our still limited understanding of steroid production in the foetus and the preterm neonate. The more recent recognition of the existence of the alternative section of the sex steroid pathway has raised consideration of its presence and relevance in the human foetus, preterm infant and neonate.

The alternative steroid pathway

The "alternative" or "backdoor" sex steroid pathway, identified in 2003 in the pouch young of the Australian Tammar wallaby, is now recognised to have potential implications for human sex development [35]. Formation of dihydrotestosterone (DHT) is central to both the classical and alternative androgen pathways. In humans, the classical pathway to formation of DHT involves its conversion from testosterone by the enzyme 5α-reductase, whereas this mechanism is bypassed in the alternative pathway. Whilst the full relevance of this pathway is still to be elucidated, the presence of this additional section in humans is now established and effectively rewrites the "classical" steroid pathway that has been presented in biochemical and medical textbooks for decades[28,35].

A clear presentation of the alternative steroid pathway has been provided by Flück and colleagues[36]. In this representation 17α-hydroxyprogesterone is converted to 5α-pregnane-3α,17α-diol-20-one which is in turn converted to androsterone by the 17,20-lyase activity of CYP17A1[36]. Androsterone is then converted to androstanediol which is oxidised to DHT by oxidative 3β-HSD in target tissues[28]. In many ways this additional section of the steroid pathway is not surprising as there have always been a proportion of unexplained disorders of sex development (DSD) which has baffled scientists and clinicians[36]. Some of these may be explained in time through research into this pathway.

We present here in Fig. 2 an updated novel version of the steroid pathway which relates the serum with the urine steroid metabolites along with fully integrating the classical with the alternative steroid pathway.

Laboratory investigation of steroid biosynthesis

For the clinical scientist the fundamental basis for interpreting steroid results relies on a clear appreciation of the analytical method, the structure of related steroids, their relationship in the steroid pathway and clear "normal" ranges. However analysis of steroids and interpretation of results has remained complicated due to the number and similarity of steroids, plus the matrices in which steroids are measured. In addition, analytical performance characteristics, such as specificity, sensitivity and accuracy of the method requires consideration when interpreting results. The following questions and related discussion are provided to briefly reflect on the advantages and limitations of the biochemical techniques used to investigate steroid related pathologies.

Table 3
Urinary metabolites of steroid hormones.

Hormone	Metabolites
Cortisol	Tetrahydrocortisol (5β)
	Allo-tetrahydrocortisol (5α)
	Cortol (20 α and β)
	11-Hydroxyaetiocholanolone
Cortisone	Tetrahydrocortisone (5β)
	6-Hydroxytetrahydrocortisone
	1-Hydroxycortolone
	11-Oxo-androsterone
Dehydroepiandrosterone	Androsterone (5 α)
	Aetiocholanolone (5β)
17-Hydroxyprogesterone	17-Hydroxypregnanolone
	Pregnanetriol
	15,17-Dihydroxy-prenanolone
Corticosterone	Tetrahydrocorticosterone
	Tetrahydro-11-dehydro-corticosterone
11-Deoxycortisol	Tetrahydro-11-deoxycortisol

R.F. Greaves et al. / Clinical Biochemistry 47 (2014) 5–15

Table 4
The enzyme defects associated with congenital adrenal hyperplasia (CAH). CAH due to 21 hydroxylase and 11 β hydroxylase deficiency are the most common. P450 is cytochrome P450.
Note, CYP11β2 deficiency is not considered a form of CAH.

Enzyme defect	CYP21	CYP11β1	CYP17	HSD3β	CYP11A
Alternative name	21-OH deficiency	11β-OH deficiency	17α-OH deficiency	3β-hydroxysteroid dehydrogenase deficiency	Lipoid/20,22-desmolase deficiency 11 α OH deficiency STAR
Chromo-some	6p	8q	10q	1p	8p 15q
Frequency	1:11,800 to 21,800 ≈90% of cases	≈5% of cases	Rare	Rare	Rare
Clinical features	Ambiguous genitalia in females	Ambiguous genitalia in females Increased blood pressure	No puberty in females Under-virilized males Increased blood pressure	Ambiguous genitalia mild in females Under-virilized males	No puberty in females Under-virilized males
Diagnostic serum steroids	↑17 OHP	↑11 Deoxy cortisol ↑ DOC	↑ Preg-nenolone ↑ DOC ↑ Cortico-sterone	↑ DHEAS ↓ Andro-stenedione ↑Ratio of substrate-to product ↑17 OH pregnenolone ↑ Progestrone	↓ Serum adrenal steroids — as the block is at the beginning of the pathway
Diagnostic urine steroids	↑17β OH pregnanolone ↑ PT ↑ PTL	↑ PT ↑ TH-11 deoxy cortisol ↑ TH-DOC	↑ TH-DOC ↑ TH-corticosterone	Adrenal foetal steroids persist ↑ P'T beyond the corrected neonatal period is diagnostic.	↓ Urine adrenal steroid metabolite output — as the block is at the beginning of the pathway

Abbreviations: 17 hydroxy progesterone — 17OHP; dehydroepiandrostane sulphate — DHEAS; deoxycorticosterone — DOC; hydroxy — OH; pregnanetriol — PT; pregnanetriolone — PTL; pregnenetriol — P'T; Tetrahydro — TH.

Question 1: what sample should I collect?

Steroid hormones are routinely measured in blood (serum or plasma), urine and more recently saliva. Whilst serum is the classical matrix for immunoassay, it could not be used in the early chromatographic techniques due to insufficient sensitivity. Urine steroids on the other hand, are often higher in concentration than blood[37]. Urine is still analysed predominately by GCMS whilst blood is analysed by either immunoassay or LC–MSMS[38–40].

Serum. Of particular importance for paediatric samples is the volume of blood required for analysis[41]. To ensure fitness for the intended clinical purpose, which includes achieving the desired sensitivity, some methods require large sample volumes. With significant improvements in technology, these sensitivity issues have largely been addressed, mitigating this issue. However, the volume of blood collected from neonates remains a critical concern to avoid issues of iatrogenic anaemia.
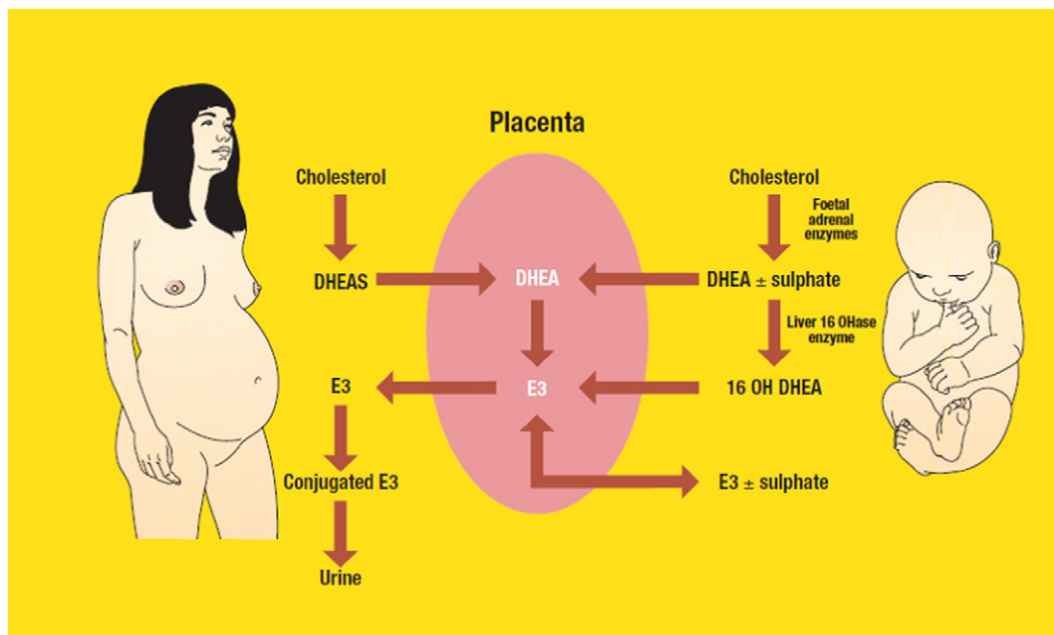

Fig. 3. The foetal placental unit and the production of steroids, represented by the foetal adrenal gland, foetal liver and the placenta. Dehydroepiandrostane sulphate (DHEAS) is produced by the foetal adrenal gland and then in the absence of a significant amount of 3β-HSD2 is metabolites by the liver to produce the 16 hydroxylated (OH) steroids. 16 OH DHEA is then utilised by the placenta to make oestriol (E3) which is then excreted in the maternal urine. Note 1: Liver sulphation of steroids predominates over glucuronidation in the foetal liver — e.g. Δ5 sulphated steroids (including pregnenolone sulphate and 17α-OH pregnenolone sulphate are produced). Note 2: Another metabolite of DHEA is 15,16-OH DHEA which will then lead to the production of oestetrol (E4) by the placenta.

Urine. A timed or spot urine sample may be used to obtain accurate results for diagnosis[40]. Although a 24 hour urine collection is optimal, the difficulties associated with an accurately timed complete collection are recognised. By comparison, random samples are much easier to collect. Due to the added difficulty of obtaining urine from neonates, especially females, urine steroids can be extracted from wet (faeces free) nappies as an alternative collection method. In neonates and infants up to 2 to 3 months of age secretion of cortisol is pulsatile and does not exhibit diurnal variation. The circadian rhythm of steroids however requires consideration in the interpretation of steroid hormone results from approximately three months of age[39].

Blotter samples. Blotter samples are routinely collected for blood as part of newborn screening collection protocols. Methods utilising urine blotter samples have also been developed. This approach is useful for remotely collected samples due to their transportability. A reference point is required to determine concentration e.g. to haemoglobin for blood and creatinine for urine blotters. For investigation of enzyme defects related to the steroid pathway, the use of ratios is suggested[42].

Saliva samples. Steroid hormone analysis in saliva has received increased interest recently, particularly for the measurement of cortisol. Purported advantages of saliva hormone analysis include its less invasive mode of collection in comparison to blood and the assessment of "free" i.e. active fraction of the steroid. Immunoassay and mass spectrometry methods have been developed with similar reported methodological advantages and limitations to other matrices. In young children, the volume of saliva collected can be a limiting factor. Various collection methods have been considered to optimise sample volume, which can be dichotomised into "no stimulation" and "stimulation" of the salivary glands. The volume of saliva collected and potential concentration of hormones varies between collection methods.

The matrix of choice is clear for the steroids commonly analysed, but is less clear for steroids associated with the alternative steroid pathway. Whether the collection of a blood or urine sample is the most appropriate for analysis of the alternative pathway steroids remains a question still to be addressed.

Question 2: will the sample be extracted prior to analysis?

One of the traditional methods used to quantitate steroids in biological matrices was RIA. In the early days of measurement by RIA, liquid–liquid extraction (LLE) of steroids from plasma or serum with organic solvents (e.g. ether, dicholoromethane or hexane:ether) was widely used[43–45]. The aqueous phase was then frozen by immersing the test tube in a dry ice bath in order to easily decant the steroid-containing organic phase[43–45]. Solid phase extraction (SPE) was introduced in the 1980s as an alternative to liquid extraction[46]. With LLE or SPE the extracted organic layer containing the steroid of interest was evaporated to dryness under a stream of nitrogen. The dried extract was then reconstituted with an assay buffer. The aims of such pre-treatment steps are to: 1) clean up the sample to improve specificity; and/or 2) concentrate the sample to improved sensitivity.

Samples destined for mass spectrometry analysis routinely require extraction. Most samples for steroid analysis by immunoassay techniques, with the exception of those collected from neonates, are not extracted. However, the question related to extraction is important for immunoassay analysis in neonates due to the presence of a number of potentially interfering foetal steroids. Extraction with solvents such as ether removes the more polar (e.g. 16 hydroxylated) steroids associated with the foetal adrenal gland, which are thought to interfere in

immunoassay methods[41]. When available mass spectrometry analysis of neonatal samples is preferred.

Question 3: will the sample be run by immunoassay or mass spectrometry?

Many steroids are now routinely measured by automated immunoassay techniques, such as cortisol, oestradiol and testosterone whilst manual techniques (such as RIA and ELISA) still remain the only immunoassay options for other steroids including 17OHP and DHT. Immunoassays have the advantage of turn-around time and simplicity allowing for the analysis of a large number of samples. However, sensitivity of automated immuno assays for some populations such as sex steroids measured in neonates and pre-pubertal children makes this technology limiting.

Chromatographic analysis of steroids has come to age with the coupling of liquid chromatography to MS and its introduction into routine laboratories for serum and urine steroid quantitation[47,48]. This "recent push towards adoption of mass spectrometry for clinical assays came not from analysts but from neonatologists and endocrinologists with little knowledge of the technique, but dissatisfaction with the status quo" [3]. With advancements in mass spectrometry technology, many serum steroids such as testosterone can now be accurately quantified by liquid chromatography coupled with tandem mass spectrometry detection (LC–MS/MS)[49–52]. These methods have the advantage of simultaneous measurement of steroids. Fig. 4 provides a simplified two dimensional outline demonstrating the specificity of LC–MS/MS for serum steroid commonly analysed. The mass selection of precursor (first MS) and product (second MS) ions in part provides this specificity.

Question 4: what about interference from related steroids?

Method performance characteristics such as cross reactivity i.e. interferences, in immunoassay methodology are clearly documented by commercial manufacturers[53]. Mass spectrometry is not however without its own problems and work to improve agreement between methods is ongoing[54,55]. Strategies to improve LC–MSMS steroid
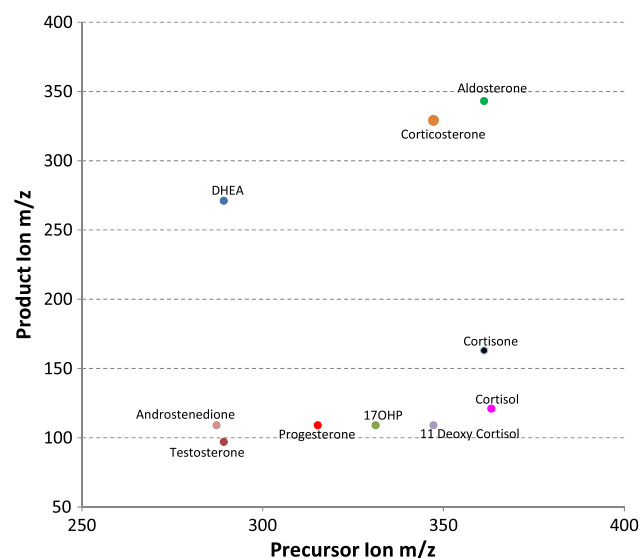
Fig. 4. Demonstration of specific precursor and product ions based on mass to charge ratio of LC–MSMS detection in positive electrospray ionisation mode for multiple serum steroid analytes. The selection of each of these precursor and product ions is a significant component of specificity. Absolute selectivity will also depend on the chromatographic separation of structurally related compounds (isomers and isobars) in the sample. DHEA = dehydroepiandrosterone; 17OHP = 17 hydroxy progesterone; m/z = mass to charge ratio. Adapted from reference [48] (permission inherently granted).

methods have been widely considered, such as: to separate stereo isomers, e.g. separation of the 5 α and β matched reduced metabolites; the removal of phospholipids from the sample — as phospholipids can cause ion alteration in some instances leading to decreased sensitivity; and standardisation to improve the agreement of results between laboratories for samples[48,56–58]. Whether the method is immunoassay or mass spectrometry based, an important question is whether the method has been validated for neonatal samples. This is a key consideration due to the difference in the steroid pattern exhibited in this group.

Question 5: how were your reference intervals determined?

Blood and urine steroid age related reference intervals have been established by a number of groups. Sample numbers in general are limited and age divisions tend to vary between these groups[24, 59–64]. Interpretation of clinical measurements and laboratory results in preterm infants remains a significant issue due to scarce age appropriate data[21,34,65,66].

Question 6: how good is your assay?

"Fitness for purpose" is now a frequently used phrase in medical laboratory testing literature. It essentially assesses whether the intended assay is appropriate for the assigned patient. All laboratories accredited to ISO15189 and manufacturers have validated their assay and the limitations are usually clearly described. However, this can be a difficult question to answer for neonates and preterm infants.

External quality assurance (also known as proficiency testing) is an important component for a laboratory to assess their performance against peers. Testing is available for most of the common serum steroids as well as urine steroids. Results are usually reviewed within the laboratory, and the laboratory may choose to provide this information to interested parties as a "proof" of ongoing proficiency.

Question 7: can you also offer molecular diagnostic testing?

Genetic testing complements chromatography–mass spectrometry analysis of steroids. Real time PCR is now routinely used to amplify DNA (or cDNA fragments from the mRNA) associated with the expression of steroid enzymes[18]. By far, the most frequent gene encoding a steroid enzyme assessed, is CYP21, encoding the enzyme 21-hydroxylase. Laboratories offering a fully accredited diagnostic service are limited worldwide. Next generation sequencing is transforming the diagnostic potential of genomics, with the projected assessment of all genes encoding the steroid pathway enzymes. In addition, non-invasive prenatal diagnosis (as early as 6 weeks of gestation) for CAH using cell-free foetal DNA in maternal plasma is now a diagnostic option[67].

Common steroid patterns in paediatric patients

Normal physiological results

Steroid levels and the pattern observed in serum and urine samples change with age. These changes mark key events in maturity and are most marked in the neonatal period, onset of adrenarche and puberty.

Neonates

The neonatal period is defined as the first four weeks of life. Biochemically, cortisone and its metabolites predominate over cortisol and related metabolites in the newborn. When cortisol production and metabolism first predominates, it is initially pulsatile and without its circadian rhythm which is established later (i.e. at 2–3 months of age)[30].

There are however differences in steroid hormones, depending on the period of gestation prior to delivery. Preterm infants i.e. babies born prior to 37 weeks gestation and especially very (<32 weeks) and extremely (<28 weeks) preterm infants display a different pattern of steroids due to persistence of the foetal adrenal zone[32]. The persistence of this foetal zone, despite early delivery, confounds result interpretation.

The recently recognised alternative section of the sex steroid pathway is thought to be the most relevant to preterm infants, neonates and also patients with 21-hydroxylase deficiency. Dysfunction of the alternative steroid pathway has been associated with the development of disorders of sex development [36], indicating that the function of this steroid pathway is essential for foetal sex development. In congenital adrenal hyperplasia, the relative activity of the alternative steroid pathway is increased in the neonatal and infant periods[68]. Studies suggest an exaggerated ratio of androsterone (formed via the classical and alternative pathways) to etiocholanolone (formed almost entirely by the classical pathway), when the alternative section of the steroid pathway is functioning[68]. Future metabolomic studies will further elucidate the choice of steroids and the ideal matrix for measurement.

Adrenarche

The onset of adrenarche is a gradual process that occurs at around seven to eight years of age and is seen earlier in girls than in boys[20, 64,69]. This event has recently been linked to increased intra-adrenal production of cortisol. Urine cortisol metabolite excretion declines from 3 to 4 years of age until 7–8 years of age where it increases prior to adrenarche[70]. The increase in cortisol inhibits the activity of 3β-HSD type 2 (produced by the zona reticularis of the adrenal gland) which is thought to be a precursory step for adrenarche[71]. This inhibition results in stimulation of DHEA and associated adrenal androgens and consequent pubarche[72]. Conversely, the two enzymes related to sex steroid formation (17 β-HSD and 5α-reductase) do not appear to be related to adrenarche[64]. Biochemically we observe increased levels of serum DHEAS, androstenedione and cortisol plus associated increases in adrenal androgen and cortisol metabolites in the urine; such as androsterone and tetrahydo-cortisol.

Puberty

Visually, the uniformly accepted five stages of puberty have been clearly defined by Tanner[73,74]. Biochemically the onset of puberty is marked by gender specific changes in gonadotropins and steroid hormones. As puberty progresses there is an evolution of the pattern of steroids and their metabolites which reflects the physical changes i.e. an increase in testosterone in males and increase in oestradiol in females. Testosterone and oestradiol are frequently requested measurements by clinicians during early stages of puberty although they add little to assessment of clinical parameters of growth and development of pubertal characteristics, except in the case of apparent pubertal arrest.

Gender differences in cortisol excretion occur from 11 years of age. Discordance between genders is associated with a sharper rise for a longer period in males compared to females. Cortisol levels in females have been shown to attenuate by approximately 16 years of age, whereas cortisol levels in males continue to increase until at least 18 years. Additionally of note, the ratio of α THF to THF (indicative of 5α-reductase activity) is lower in females compared to males from this time[70].

Pathophysiology

Pituitary and steroid hormone analysis in paediatrics is primarily used as part of the differential diagnosis of disorders of sex development (DSD)[21,34,75]. Congenital adrenal hyperplasia (CAH) is the most common genetically determined and inherited endocrine disorder in both children and adults. The neonatal period, onset of adrenarche and puberty are key periods where clinical assessments are made. Some forms of CAH are easily defined e.g. 21 and 11β-hydroxylase deficiency but early defects of adrenal enzyme biosynthesis can be more difficult in the neonate e.g. 17α-hydroxylase, 17β-HSD deficiency.

Over 50% of males with under-virilization and clinical disorders of severe hypospadias remain without a clear diagnosis. In addition, it is evident that some preterm female infants appear to have unexplained transient virilization[21,34]. Research to answer the question of the relevance of the alternative steroid pathway in preterm infants may help elucidate some of these unanswered questions.

CAH

CAH as a disease was fully described in the 20th century with the paradox of hyperplasia of the adrenal glands and overproduction of adrenal androgens, with impaired capacity of cortisol production. 1950 saw the first report of effective treatment of CAH with suppression of 17 keto steroids by cortisone[76]. 21-hydroxylase deficiency accounts for approximately 95% of cases, with a reported incidence of 1 in 12,000 births for the classical form and 1 in 2500 for the non-classical (i.e. late onset) form, although it is seen more frequently in some communities e.g. Ashkenazi Jews and Yupik speaking Eskimos of Western Alaska [77–79]. Universal newborn screening has been recommended to detect classical 21-hydroxylase deficiency[80].

Affected babies are at risk of life threatening adrenal crisis due to glucocorticoid and mineralocorticoid deficiency, and associated adrenaline insufficiency[79]. Increased virilization is evident in female infants at birth due to the shunting of the steroid pathway, leading to increased adrenal androgen production, due to the enzymatic block. Male infants do not manifest this tell-tale sign and often first present in adrenal crisis with associated hyponatraemia and hyperkalaemia in the first two weeks of life. Some will be diagnosed at post mortem[81]. A diagnosis of 21-hydroxylase deficiency can be made by demonstrating an increase in the enzyme substrate, 17 hydroxy progesterone (17OHP) in serum or whole blood. Diagnostic metabolites (17 hydroxy pregnanolone, pregnanetriol and pregnanetriolone) are also evident in the urine of affected untreated individuals[82–84]. In addition, oxygenated metabolites of 21-deoxycorticosterone have more recently been recognised as reliable markers for 21-hydroxylase deficiency in the early neonatal period[85]. Table 4 provides a summary of the enzyme defects resulting in CAH.

17β-HSD, 5α-reductase deficiency and the alternative pathway

Outside of CAH, biochemically the DSD commonly encountered biochemically are 5α reductase deficiency and 17β hydroxy steroid dehydrogenase deficiency. Whilst excess DHT causes virilisation of genetic females (46, XX disorder), deficiency of DHT in males, often associated with 5α-reductase deficiency, causes 46, XY disorders[27,86]. 46, XY DSD caused by 5α-reductase deficiency have significant clinical implications, as marked virilisation can occur at the time of puberty, due to increased testosterone substrate acting with a partially active enzyme, and increased activity of isoenzyme 5α-reductase 1[87]. This can be associated with change of gender identity from female to male in a child initially raised in the female gender. A similar case exists for 17β hydroxy steroid dehydrogenase, with virilisation at the time of puberty. Due to the significance of this outcome, it is recommended that 17β hydroxy steroid dehydrogenase and 5α-reductase deficiency should be excluded when considering female gender assignment at birth in an infant with 46, XY DSD and limited virilisation[88].

Unfortunately, determination of serum DHT levels has been challenging due to inherent assay difficulties. It has been presumed that the recent introduction of LC–MSMS techniques would solve this, but to date whilst there are reports of methods in the literature, in practice many groups continue to struggle with ionisation of this steroid.

Cortisol–cortisone

The failure to regenerate cortisol from cortisone is known as cortisone reductase deficiency and relates to a mutation in the gene encoding 11β-HSD type 1[69]. NADPH is a co-factor for this enzyme and mutations associated with its production have also been demonstrated in patients with apparent cortisone reductase deficiency[89].

Investigations into female hirsutism with oligomenorrhoea may warrant consideration of cortisone reductase deficiency[20,90–92]. Biochemically urine metabolomic profiles characterise the pattern of cortisol to cortisone metabolites. To calculate the ratio of metabolites in urine the sum of tetrahydro-cortisol plus cortol to the sum of tetrahydro-cortisone plus cortolone is performed[20]. Both the alpha and beta isomers of each compound should be included in this calculation. Simplistically the ratio of β-THF plus α-THF to β-THE will suffice. With the increased use of mass spectrometry analysis of serum steroids the ratio of cortisol to cortisone can also be calculated.

Of relevance for the interpretation of steroid profiles is the body mass index. Notably, in patients with an increased body mass index, an exaggerated levels of cortisol and related metabolites exists. This is due to the presence of 11β-HSD type 1 in adipose tissue which results in the reduction of cortisone to cortisol. In the urine of these patients an exaggerated ratio of cortisone to cortisol is evident due to the presence of 11β-HSD type 2 in the kidney. In addition, various studies hypothesise a relationship between other steroid enzymes found in adipose tissue and the effect of an increased BMI may have on altered expression of sex steroids in relation to the timing of adrenarche and puberty[93–95].

Conclusion

Laboratory techniques for steroid analysis have progressed significantly over the last twenty plus years. In an already complicated area, the interpretation of steroid results potentially is now more challenging with our new understanding of the steroid cascade, its clinical relevance to DSD, advances in laboratory technology and interpretation of steroid results. The future brings a combination of next generation sequencing (genomics) in association with mass spectrometry (metabolomics) to provide the automated platforms to link genotypic with phenotypic analysis of disorders of sexual development.

Financial disclosure

The authors have no financial relationships relevant to this article to disclose.

Conflict of interest

All authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this manuscript.

Contributor statement/disclosure summary

There is no conflict of interest that could be perceived as prejudicing the impartiality of this manuscript. All authors listed contributed to this work. Dr Greaves wrote the first draft of this manuscript and designed the first version of the steroid pathway. Dr Jevalikar performed the background search for the work in Tables 2 and 3 and together with Dr Hewitt and Prof Zacharin contributed to the refinement of the steroid pathway presented in this manuscript. Professor Zacharin critically reviewed each draft of the manuscript. All authors reviewed, edited and approved the final manuscript.

References

[1] Abraham GE. Solid-phase radioimmunoassay of estradiol-17 beta. J Clin Endocrinol Metab 1969;29:866–70.

[2] Horning EC, Brooks CJ, Vanden Heuvel WJ. Gas phase analytical methods for the study of steroids. Adv Lipid Res 1968;6:273–392.

[3] Shackleton C. Clinical steroid mass spectrometry: a 45-year history culminating in HPLC–MS/MS becoming an essential tool for patient diagnosis. J Steroid Biochem Mol Biol 2010;121:481–90.

[4] Mitchell FL, Shackleton CHL. The investigation of steroid metabolism in early infancy. Adv Clin Chem 1969;12:141–215.

[5] Sweeley CC, Horning EC. Microanalytical separation of steroids by gas chromatography. Nature 1960;187:144–5.

[6] Starling EH. The Croonian lectures on the correlation of the functions of the body. Lancet 1905;2:339–41.

[7] Liljestrand G. The Nobel Prize in physiology or medicine 1950: award ceremony speech. [Available at] http://www.nobelprize.org/nobel_prizes/medicine/laureates/1950/press.html. [Accessed on 02/02/2014].

[8] Freeman ER, Bloom DA, McGuire EJ. A brief history of testosterone. J Urol 2001;165:371–3.

[9] Kendall EC. The development of cortisone as a therapeutic agent. Nobel lecture, December 11, 1950; 1950 [Available at http://www.nobelprize.org/nobel_prizes/medicine/laureates/1950/kendall-lecture.pdf, Accessed on 01/02/2014].

[10] Reichstein T. Chemistry of the adrenal cortex hormones. [Available at] http://www.nobelprize.org/nobel_prizes/medicine/laureates/1950/reichstein-lecture.pdf. [Accessed on 01/02/2014].

[11] Wintersteiner O, Pfiffner JJ. Chemical studies on the adrenal cortex. J Biol Chem 1936;116:291–305.

[12] Julian PL, Meyer EW, Karpel WJ, Waller IR. Sterols. XI. 17α-hydroxy-11-desoxycorticosterone (Reichstein's substance S). J Am Chem Soc 1950;72:5145–7.

[13] Reichstein T, Shoppee CW. The hormones of the adrenal cortex. Vitam Horm 1943;1:345–413.

[14] IUPAC-IUB Joint commission on biochemical nomenclature (JCBN). The nomenclature of steroids. Eur J Biochem 1989;186:429–58.

[15] IUPAC-IUB Joint commission on biochemical nomenclature (JCBN). The nomenclature of steroids. Pure Appl Chem 1989;61:1783–822.

[16] IUPAC-IUB Joint commission on biochemical nomenclature (JCBN). The nomenclature of steroids. [Available at] http://www.chem.qmul.ac.uk/iupac/steroid/. [Accessed on 02/02/2014].

[17] Haining R. Cytochrome P450 reactions in the human brain. Handbook of neurochemistry and molecular neurobiology. US: Springer; 2007. p. 43–91.

[18] Luu-The V. Assessment of steroidogenesis and steroidogenic enzyme functions. J Steroid Biochem Mol Biol 2013;137:176–82.

[19] Griffin JF, Duax WL, Weeks CM. Atlas of steroid structure volume 2: published online in 2010. Biochemical education. New York: IFI/Plenum Data Corp; 2010 754. http://dx.doi.org/10.1016/0307-4412(85)90054-8 [ISBN 0-306-66102-0].

[20] Carey RM. Adrenal disease update 2011. J Clin Endocrinol Metab 2011;96:3583–91.

[21] Greaves R, Kanumakala S, Read A, Zacharin M. Genital abnormalities mimicking congenital adrenal hyperplasia in premature infants. J Paediatr Child Health 2004;40:233–6.

[22] Greaves R, Poomthavorn P, Zacharin M. 11β-hydroxylase deficiency masked by alternative medicines. J Paediatr Child Health 2006;42:652–4.

[23] Honour JW. Steroid profiling. Ann Clin Biochem 1997;34:32–44.

[24] Wudy SA, Hartmann MF. Mass spectrometry in the diagnosis of steroid-related disorders: clinical applications. In: Ranke MB, Mullis P-E, editors. Diagnostics of endocrine function in children and adolescents4th ed. 2011. p. 379–401.

[25] McNamara KM, Nakamura Y, Miki Y, Sasano H. Phase two steroid metabolism and its roles in breast and prostate cancer patients. Front Endocrinol 2013;4:116. http://dx.doi.org/10.3389/fendo.2013.00116.

[26] Andrew R. Clinical measurement of steroid metabolism. Best Pract Res Clin Endocrinol Metab 2001;15(1):1–16.

[27] Auchus R, Chang A. 46, XX DSD: the masculinised female. Best Pract Res Clin Endocrinol Metab 2010;24(2):219–42.

[28] Auchus RJ. The alternative pathway to dihydrotestosterone. Trends Endocrinol Metab 2004;15:432–8.

[29] Shackleton CHL. Steroid synthesis and catabolism in the fetus and neonate. Endocrinology of Steroid Hormones. Oxford: Blackwell; 1985. p. 441–77.

[30] Midgley PC, Holownia P, Smith J, Moore M, Russell K, Oates N, et al. Plasma cortisol, cortisone and urinary glucocorticoid metabolites in preterm infants. Biol Neonate 2001;79:79–86.

[31] Vermes I, Dohanics J, Toth G, Pongracz J. Maturation of the circadian rhythm of the adrenocortical functions in human neonates and infants. Horm Res 1980;12:237–44.

[32] Midgley PC, Russell K, Oates N, Shaw JC, Honour JW. Activity of the adrenal fetal zone in preterm infants continues to term. Endocr Res 1996;22:729–33.

[33] Rainey WE, Rehman KS, Carr BR. The human fetal adrenal: making adrenal androgens for placental estrogens. Semin Reprod Med 2004;22(4):327–36.

[34] Greaves R, Hunt R, Zacharin M. Transient anomalies in genital appearance in some extremely preterm female infants may be the result of foetal programming causing a surge in LH and the over activation of the pituitary-gonadal axis. Clin Endocrinol (Oxf) 2008;69:763–8.

[35] Wilson JD, Auchus RJ, Leihy MW, Guryev OL, Estabrook RW, Osborn SM, et al. 5α-androstane-3α, 17β-diol is formed in tammar wallaby pouch young testes by a pathway involving 5α-prenane-3α, 17α-diol-20-one as a key intermediate. Endocrinology 2003;144:575–80.

[36] Flück CE, Meyer-Böni M, Pandey AV, Kempná P, Miller WL, Schoenle EJ, et al. Why boys will be boys: two pathways of fetal testicular androgen biosynthesis are needed for male sexual development. Am J Hum Genet 2011;89:201–18.

[37] Biogiovanni AM. Urinary pregnanetriol. A practical determination in clinical medicine. Clin Endocrinol (Oxf) 1960;1:700–4.

[38] Rossi C, Calton L, Hammond G, Brown HA, Wallace AM, Sacchetta P, et al. Serum steroid profiling for congenital adrenal hyperplasia using liquid chromatography–tandem mass spectrometry. Clin Chim Acta 2010;411(3–4):222–8.

[39] Jerjes WK, Cleare AJ, Peters TJ, Taylor NF. Circadian rhythm of urinary steroid metabolites. Ann Clin Chem 2006;43:287–94.

[40] Caulfield MP, Lynn T, Gottschalk ME, Jones KL, Taylor NF, Malunowicz EM, et al. The diagnosis of congenital adrenal hyperplasia in the newborn by gas chromatography/mass spectrometry analysis of random urine specimens. J Clin Endocrinol Metab 2002;87(8):3682–90.

[41] Verras P, Greaves R. Abnormal laboratory results: important issues to consider in the interpretation of paediatric biochemistry. Aust Prescr 2005;28:126–9.

[42] Poomthavorn P, Greaves R, Nugyen T, Warne G, Pitt J. Paper blotter urine steroid profiles by tandem mass spectrometry: a new method for the investigation of 21-hydroxylase deficiency. AACB conference abstract O3, 2006, 27. Clin Biochem Reviews; 2006. p. S20 [Abstract].

[43] Abraham G, Manlimos F, Solis M, Wickman A. Combined radioimmunoassay of four steroids in one ml plasma: II. Androgens. Clin Biochem 1975;8(6):374–8.

[44] Pazzagli M, Forti G, Cappellini A, Serio M. Radioimmunoassay of plasma dihydrotestosterone in normal and hypogonadal men. Clin Endocrinol 1975;4(5):513–20.

[45] Tateishi K, Hamaoka T, Hayashi C. Non-chromatographic radioimmunoassay for testosterone and 5α-dihydrotestosterone in adult female serum using antisera with low cross-reactivities. J Steroid Biochem 1980;13(8):869–72.

[46] Schönesöfer M, Fenner A. A convenient and efficient method for the extraction and fractionation of steroid hormones from serum or urine. J Clin Chem Clin Biochem 1981;19(2):71–4.

[47] Pitt JJ. Principles and applications of liquid chromatography–mass spectrometry in clinical biochemistry. Clin Biochem Rev 2009;30:19–34.

[48] Greaves RF. A guide to harmonisation and standardisation of measurands determined by liquid chromatography–tandem mass spectrometry in routine clinical biochemistry. Clin Biochem Rev 2012;33:123–32.

[49] Shiraishi S, Lee PWN, Leung A, Goh VHH, Swerdloff RS, Wang C. Simultaneous measurement of serum testosterone and dihydrotestosterone by liquid chromatography–tandem mass spectrometry. Clin Chem 2008;54(11):1855–63.

[50] Wang C, Shiraishi S, Leung A, Baravarian S, Hull L, Goh V, et al. Validation of a testosterone and dihydrotestosterone liquid chromatography tandem mass spectrometry assay: interference and comparison with established methods. Steroids 2008;73(13):1345–52.

[51] Kulle AE, Riepe FG, Melchior D, Hiort O, Holterhus PM. A novel ultrapressure liquid chromatography tandem mass spectrometry method for the simultaneous determination of androstenedione, testosterone, and dihydrotestosterone in pediatric blood samples: age and sex specific data. J Clin Endocrinol Metab 2011;95(5):2399–409.

[52] Licea-Perez H, Wang S, Szapacs M, Yang E. Development of a highly sensitive and selective UPLC/MS/MS method for the simultaneous determination of testosterone and 5-dihydrotestosterone in human serum to support testosterone replacement therapy for hypogonadism. Steroids 2008;73(6):601–10.

[53] Honour J. Development and validation of a quantitative assay based on tandem mass spectrometry. Ann Clin Biochem 2011;48(2):97–111.

[54] Asian and Pacific Federation of Clinical Biochemists. Mass Spectrometry Harmonisation Working Group. http://www.apfcb.org/scientific-activities.html. [Accessed 14th April 2014].

[55] Greaves RF, Ho CS, Hoad KE, Joseph J, McWhinney B, Gill JP, et al. Establishment of a common calibrator for mass spectrometry analysis of serum testosterone (Abstract — oral communication). Proceedings of the IFCC World Lab Istanbul 2014, 52, Special Suppl. Clin Chem Lab Med; 2014. p. S180.

[56] Martin-Roussety MNG. Development of an LC–MS/MS method for the simultaneous quantification of androstenedione, dihydrotestosterone, epitestosterone and testosterone in serum samples. [Master of Applied Science Thesis] RMIT University; 2012.

[57] Clarke M, Tuckey R, Gorman S, Holt B, Hart P. Optimized 25-hydroxyvitamin D analysis using liquid–liquid extraction with 2D separation with LC/MS/MS detection, provides superior precision compared to conventional assays. Metabolomics 2013;9(5):1031–40.

[58] Starka L. Epitestosterone. J Steroid Biochem Mol Biol 2003;87:27–34.

[59] Weykamp CW, Penders TJ, Schmidt NA, Borburgh AJ, van de Calseyde JF, Wolthers BJ. Steroid Profile for Urine: Reference Values. Clin Chem 1989;35:2281–4.

[60] Homma K, Hasegawa T, Masumoto M, Takeshita E, Watanabe K, Chiba H, et al. Reference values for urinary steroids in Japanese newborn infants: gas chromatography/mass spectrometry in selected ion monitoring. Endocr J 2003;50:783–92.

[61] Greaves RF, Donath S, Hunt RW. Serum Hormone Profiles over the first six weeks of life for preterm infants born less than 30 weeks gestation. XIIth International Congress of Paediatric Laboratory Medicine Berlin 2011, 44. Clinical Biochemistry; 2011. p. 531.

[62] Greaves R, Hunt R, Chiriano A, Zacharin M. LH and FSH Levels in Extreme Prematurity: Development of Reference Intervals. Pediatrics 2008;121:e574–80.

[63] Fluck CE. Assessing the Function of the Adrenal Cortex. In: Ranke MB, Mullis P-E, editors. Diagnostics of endocrine function in children and adolescents4th ed.; 2011. p. 350–78.

[64] Remer T, Boye KR, Hartmann MF, Wudy SA. Urinary markers of adrenarche: reference values in healthy subjects, aged 3–18 years. J Clin Endocrinol Metab 2005;90(4):2015–21.

[65] Greaves RF, Zacharin MR, Donath SM, Inder TE, Doyle LW, Hunt RW. Establishment of hormone reference intervals for infants born <30 weeks' gestation. Clin Biochem 2014;47:101–8 (in this issue).

[66] Greaves RF. Hormone profiles in extremely preterm infants. Clin Biochem 2014; 47:744–6.

[67] New MI, Tong YK, Yuen T, Jiang P, Pina C, Chan KCA, et al. Noninvasive prenatal diagnosis of congenital adrenal hyperplasia using cell-free fetal DNA in maternal plasma. J Clin Endocrinol Metab 2014;99(6):1118-.

[68] Kamrath C, Hochberg A, Hartmann M, Remer T, Wudy S. Increased activation of the alternative "backdoor" pathway in patients with 21-hydroxylase deficiency: evidence from urinary steroid hormone analysis. J Clin Endocrinol Metab 2012; 97(3):E367–75.

[69] Miller WL, Auchus RJ. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. Endocr Rev 2011;32:81–151.

[70] Wudy SA, Remer T, Hartmann MF. Sexual dimorphism in cortisol secretion starts after age 10 in healthy children: urinary cortisol excretion rates during growth. Am J Physiol Endocrinol Metab 2007;293(4):E970–6.

[71] Arlt W, Martens JW, Song M, Wang JT, Auchus RJ, Miller WL. Molecular evolution of adrenarche: structural and functional analysis of p450c17 from four primate species. Endocrinology 2002;143:4665–72.

[72] Topor LS, Asai M, Dunn J, Majzoub JA. Cortisol stimulates secretion of dehydroepiandrosterone in human adrenocortical cells through inhibition of 3βHSD2. J Clin Endocrinol Metab 2011;96:E31–9.

[73] Marshall WA, Tanner JM. Variations in pattern of pubertal changes in girls. Arch Dis Child 1969;44:291–303.

[74] Marshall WA, Tanner JM. Variations in the pattern of pubertal changes in boys. Arch Dis Child 1970;45:13–23.

[75] Midgley P, Azzopardi D, Oates N, Shaw J, Honour J. Virilisation of female preterm infants. Arch Dis Child 1990;65:701–3.

[76] Wilkins L, Lewis RA, Klein R, Rosemberg E. The suppression of androgen secretion by cortisone in a case of congenital adrenal hyperplasia. Preliminary report, 86. Bulletin of the Johns Hopkins Hospital, Baltimore; 1950. p. 249–52.

[77] Nordenström A, Ahmed S, Jones J, Coleman M, Price DA, Clayton PE, et al. Female preponderance in congenital adrenal hyperplasia due to CYP21 deficiency in England: implications for neonatal screening. Horm Res 2005;63:22–8.

[78] Riepe FG, Sippell WG. Recent advances in diagnosis, treatment, and outcome of congenital adrenal hyperplasia due to 21-hydroxylase deficiency. Rev Endocr Metab Disord 2007;8:349–63.

[79] 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;90:414–21.

[80] 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.

[81] Gozzi T, Harris N, McGown I, Cowley D, Cotterill A, Campbell P, et al. Autopsy diagnosis of 21-hydroxylase deficiency CAH in a case of apparent SIDS. Pediatr Dev Pathol 2005;8:397–401.

[82] Nguyen ATT, Brown JJ, Warne GL. Growth in Congenital Adrenal Hyperplasia. Indian J Pediatr 2006;73:89–93.

[83] Merke DP, Bornstein SR, Avila NA, Chrousos GP. Future Directions in the Study and Management of Congenital Adrenal Hyperplasia due to 21-Hydroxylase Deficiency. Ann Intern Med 2002;136:320–34.

[84] Merke D. Approach to the Adult with Congenital Adrenal Hyperplasia due to 21-Hydroxylase Deficiency. J Clin Endocrinol Metab 2008;93(3):653–60.

[85] Christakoudi S, Cowan DA, Taylor NF. Steroids excreted in urine by neonates with 21-hydroxylase deficiency. 4. Characterization, using GC–MS and GC–MS/MS, of 11oxo-pregnanes and 11oxo-pregnenes. Steroids 2013;78:468–75.

[86] http://emedicine.medscape.com/article/924291-overview. [Accessed on 02/02/ 2014].

[87] Zhu YS, Imperato-McGinley JL. 5alpha-reductase isozymes and androgen actions in the prostate. Ann N Y Acad Sci 2009;1155:43–56.

[88] 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(4):541–7.

[89] Lavery GG, Walker EA, Tiganescu A, Ride JP, Shackleton CH, Tomlinson JW, et al. Steroid biomarkers and genetic studies reveal inactivating mutations in hexose-6-phosphate dehydrogenase in patients with cortisone reductase deficiency. J Clin Endocrinol Metab 2008;93:3827–32.

[90] Biason-Lauber A, Suter SL, Shackleton CH, Zachmann M. Apparent cortisone reductase deficiency: a rare cause of hyperandrogenemia and hypercortisolism. Horm Res 2000;53(5):260–6.

[91] Jamieson A, Wallace AM, Andrew R, Nunez BS, Walker BR, Fraser R, et al. Apparent cortisone reductase deficiency: a functional defect in 11beta-hydroxysteroid dehydrogenase type 1. J Clin Endocrinol Metab 1999;84(10):3570–4.

[92] Lawson AJ, Walker EA, Lavery GG, Bujalska IJ, Hughes B, Arlt W, et al. Cortisone-reductase deficiency associated with heterozygous mutations in 11β-hydroxysteroid dehydrogenase type 1. Proc Natl Acad Sci 2011;108(10):4111–6.

[93] Shi L, Remer T, Buyken AE, Hartmann MF, Hoffmann P, Wudy SA. Am J Physiol Endocrinol Metab 2010;299(6):E990–7.

[94] Ibanez L, Dimartino-Nardi J, Potau N, Saenger P. Premature adrenarche: normal variant or forerunner of adult disease? Endocr Rev 2000;21:671–96.

[95] Tsilchorozidou T, Honour JW, Conway GS. Altered cortisol metabolism in polycystic ovary syndrome: insulin enhances 5α-reduction but not the elevated adrenal steroid production rates. J Clin Endocrinol Metab 2003;88:5907–13.