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A new dawn for androgens: Novel lessons from 11-oxygenated C19 steroids

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Abstract

The abundant adrenal C19 steroid 11 β -hydroxyandrostenedione (11OHA4) has been written off as a dead-end product of adrenal steroidogenesis. However, recent evidence has demonstrated that 11OHA4 is the precursor to the potent androgenic 11-oxygenated steroids, 11-ketotestosterone and 11-ketodihydrotestosterone, that bind and activate the human androgen receptor similarly to testosterone and DHT. The significance of this discovery becomes apparent when considering androgen dependent diseases such as castration resistant prostate cancer and diseases associated with androgen excess, e.g. congenital adrenal hyperplasia and polycystic ovary syndrome. In this paper we describe the production and metabolism of 11-oxygenated steroids. We subsequently discuss their androgenic activity and highlight the putative role of these androgens in disease states.

Keywords: adrenal androgens, castration resistant prostate cancer (CRPC); congenital adrenal hyperplasia (CAH); polycystic ovary syndrome (PCOS); 11-ketotestosterone (11KT); 11-ketodihydrotestosterone (11KDHT)

Abbreviations:

3 α HSD, 3 α -hydroxysteroid dehydrogenase; 3 β HSD1/2, 3 β -hydroxysteroid dehydrogenase type1/2; 11 β HSD1/2, 11 β -hydroxysteroid dehydrogenase type 1/2; 11KA4, 11-ketoandrostenedione; 11KDHT, 11-ketodihydrotestosterone; 11KT, 11-ketotestosterone; 11OHA4, 11 β -hydroxyandrostenedione; 11OHT, 11 β -hydroxytestosterone; 17 β HSD3, 17 β -hydroxysteroid dehydrogenase type 3; 17OH-PREG, 17 α -hydroxypregnenolone; 17OH-PROG, 17 α -hydroxyprogesterone; 21OHD, steroid 21-hydroxylase deficiency; A4, androstenedione; ACTH, adrenocorticotrophic hormone; ADT, androgen deprivation therapy; AKR1C3, aldoketo reductase 1C3; AR, androgen receptor; AST, androsterone; CAH, congenital adrenal hyperplasia; CRH, corticotropin releasing hormone; CRPC, castration resistant prostate cancer; CYB5A, cytochrome b₅; CYP11A1, cytochrome P450 cholesterol side chain cleavage; CYP11B1, cytochrome P450 11 β -hydroxylase; CYP17A1, cytochrome P450 17 α -hydroxylase/17,-20-lyase; CYP21A2, cytochrome P450 steroid 21-hydroxylase; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulphate; LHRH, luteinizing-hormone-releasing-hormone; Pdiol, 5 α -pregnane-3 α ,17 α -diol-20-one; PCOS, polycystic ovary syndrome; PREG, pregnenolone; SRD5A, steroid 5 α -reductase; StAR, steroidogenic acute regulatory protein; T, testosterone; UGT, uridine 5'-diphospho-glucuronosyltransferase

Contents

1. Introduction
2. Biosynthesis of C19 steroids in the human adrenal
3. Androgenicity of the 11-oxygenated steroids
4. Castration resistant prostate cancer
5. Congenital adrenal hyperplasia
6. Polycystic ovary syndrome
7. Conclusion

1. Introduction

The production of the C19 steroids androstenedione (A4), dehydroepiandrosterone (DHEA) and DHEA sulphate (DHEAS) by the *zona reticularis* of the adrenal cortex is well documented. Although it has long been established that the C19 steroid 11 β -hydroxyandrostenedione (11OHA4) is also an abundant product of human steroidogenesis, this metabolite has been written off as a by-product of steroidogenesis and is seldom included in the adrenal steroidogenic pathway. Recent studies however, revealed that 11OHA4 is not a dead-end product of steroidogenesis, but that it serves as the precursor to androgenic 11-oxygenated steroids, both in the adrenal itself and in the periphery. In this review we evaluate the production of these 11-oxygenated steroids, their androgenic activity and discuss the potential contribution of these steroids to disease states.

2. Biosynthesis of C19 steroids in the human adrenal

The biosynthesis of C19 adrenal steroids occurs primarily in the *zona reticularis* of the adrenal cortex (Fig. 1). The enzymes involved in these biosynthetic pathways were recently reviewed in detail by Turcu et al. (Turcu et al., 2014). C19 steroids, like all steroid hormones, are derived from the 27-carbon molecule cholesterol. Steroid biosynthesis commences with the transfer of cholesterol from the outer to inner mitochondrial membrane by steroidogenic acute regulatory protein (StAR). Thereafter, cholesterol undergoes the cleavage of the side chain to produce pregnenolone (PREG), a C21 steroid, via the action of the cytochrome P450 side-chain cleavage enzyme (CYP11A1). PREG is further metabolised by the 17 α -hydroxylase activity of CYP17A1 to form 17 α -hydroxypregnenolone (17OH-PREG). Thereafter, the 17,20-lyase activity of CYP17A1, augmented by cytochrome b₅, cleaves the 17-20 carbon bond yielding the C19 steroid DHEA (Auchus, 2004; Miller and Auchus, 2011; Rainey et al., 2002; Turcu et al., 2014). This Δ^5 pathway is the preferred route to C19 steroid production in humans as the 17,20 lyase activity of human CYP17A1 does not efficiently convert 17 α -hydroxyprogesterone (17OH-PROG) to A4 (Auchus et al., 1998).

In humans, a dramatic increase in adrenal C19 steroid production is observed during adrenarche, with clinical signs physiologically observed between the ages of 6 and 10 (Auchus and Rainey, 2004; Rainey et al., 2002; Rege and Rainey, 2012). It is

worth noting that premature adrenarche can represent a forerunner of metabolic disease (Idkowiak et al., 2011).

Interestingly, the process of adrenarche occurs much more gradually than previously assumed (Remer et al., 2015) and encompasses the formation of a distinct *zona reticularis*, in which the enzyme expression is altered from that of the *zona fasciculata*, resulting in the production of C19 steroids (Turcu et al., 2014). During adrenarche, the expression of cytochrome b₅, which augments the 17,20-lyase activity of CYP17A1, is upregulated in the *zona reticularis*, while the expression of 3 β -hydroxysteroid dehydrogenase type 2 (3 β HSD2) is downregulated. This combination of expression promotes the flux through the Δ^5 pathway leading to DHEA production without the conversion of the Δ^5 PREG and 17OH-PREG to their respective Δ^4 metabolites by 3 β HSD2. While some of the resulting DHEA is released directly into circulation, much of this steroid is first sulfonated by the activity of SULT2A1, resulting in the release of DHEAS into circulation (Auchus and Rainey, 2004; Mueller et al., 2015; Rainey and Nakamura, 2008; Rege and Rainey, 2012). DHEA is also converted to A4 by 3 β HSD2. It has been proposed that A4 production occurs in a layer of cells between the *zona fasciculata* and the *zona reticularis* which expresses CYP17A1, cytochrome b₅ and 3 β HSD2 (Nakamura et al., 2011; Nguyen et al., 2016; Rainey and Nakamura, 2008). Despite low expression of 3 β HSD2 in the *zona reticularis*, A4 is produced in this zone and may be facilitated by cytochrome b₅, which has also been shown to upregulate the activity of 3 β HSD (Goosen et al., 2013, 2011; K. Storbeck et al., 2013).

The expression of aldo-keto reductase 1C3 (AKR1C3), also known as 17 β -hydroxysteroid dehydrogenase type 5 (17 β HSD5), in the *zona reticularis* also results in the conversion of DHEA and A4 to androstenediol and testosterone, respectively. Androstenediol, in turn, is converted to testosterone by 3 β HSD2. The adrenal gland is a major source of circulating androgens in women, with the levels of circulating androgens decreased by >90% in female patients with adrenal insufficiency (W Arlt et al., 1999). Conversely the adrenal only makes a minor contribution to circulating testosterone levels in men. However, at a tissue-specific level both men and women generate active androgens from DHEA (Arlt et al., 1998; Wiebke Arlt et al., 1999).

In addition to the C19 steroids described above, the human adrenal produces significant quantities of the 11-oxygenated C19 steroid 11OHA4 (Axelrod et al., 1973; Dorfman, 1954; Holownia et al., 1992; Jeanloz et al., 1953; Rege et al., 2013; Touchstone et al., 1955), which is formed by the CYP11B1-catalysed 11 β -hydroxylation of A4 (Fig 1) (Schloms et al., 2012; Swart et al., 2013; Turcu et al., 2016). Interestingly, the adrenal produces more 11OHA4 than A4 under both normal (Table 1) and adrenocorticotropin (ACTH)-stimulated conditions (Rege et al., 2013). Low quantities of the 11-oxygenated steroids 11-ketoandrostenedione (11KA4), 11 β -hydroxytestosterone (11OHT) and 11-ketotestosterone (11KT) are also produced by the human adrenal (Table 1). 11KA4 is produced by the conversion of 11OHA4 by low levels of 11 β -hydroxysteroid dehydrogenase type 2 (11 β HSD2) expressed in the adrenal (Rege et al., 2013; K.-H. Storbeck et al., 2013). 11KA4 is in turn a substrate for AKR1C3, yielding 11KT (K.-H. Storbeck et al., 2013). Although it has been proposed that 11OHA4 is converted to 11OHT by AKR1C3 (Rege et al., 2013; Turcu et al., 2014), we have shown that neither AKR1C3 nor 17 β -hydroxysteroid dehydrogenase type 3 (17 β HSD3) catalyses this reaction (K.-H. Storbeck et al., 2013; unpublished data). Instead, 11OHT can be produced by the CYP11B1 catalysed 11 β -hydroxylation of testosterone (Schloms et al., 2012). 11OHT is, in turn, a substrate for 11 β HSD2, yielding 11KT.

Turcu et al recently showed that in patients with steroid 21-hydroxylase deficiency (21OHD) the levels of 11KT correlate well with those of 11OHT, suggesting that 11OHT is the primary precursor to 11KT. Furthermore, this study found a correlation between T and 11KT in woman, again suggesting that the 11 β -hydroxylation of T to 11OHT provides the precursor for 11KT production in the adrenal. Moreover, the circulating pool of 11OHT and 11KT was found to be similar in men and women, thereby confirming that the adrenal is likely the primary site of 11OHT and 11KT biosynthesis and that gonadal T is not an important precursor (Turcu et al., 2016). Even though the adrenal expresses low levels of 11 β HSD2, differences in the concentration of the individual 11-oxygenated steroids in the adrenal vein and inferior vena cava suggest that while 11OHA4 and 11OHT are products of the adrenal, 11KA4 and 11KT may be formed in peripheral target tissues of androgen action rather than in the adrenal glands (Rege et al., 2013; Turcu et al., 2016).

3. Androgenicity of the 11-oxygenated steroids

Studies in 1950s and 1960s revealed that both 11OHA4 and 11KA4 (often referred to as adrenosterone in older literature), which were known to be products of the human adrenal, had negligible androgenic activity (Byrnes and Shipley, 1955; Dorfman and Dorfman, 1963; Rosemberg and Dorfman, 1958). Unlike 11OHA4, A4 was recognised as an important precursor to active androgens leading to the suggestion that the 11 β -hydroxylation of A4 served as a mechanism to inactivate this androgen precursor (Bélanger et al., 1993; Goldzieher et al., 1978; Labrie et al., 1988). A general loss of interest in the function of 11-oxygenated C19 steroids in mammals followed and 11OHA4 and its metabolites have, in most cases, been left out of the steroidogenic scheme, despite 11OHA4 being an abundant product of adrenal steroidogenesis (Table 1) (Rege et al., 2013). In contrast to the case in mammals, the role of 11-oxygenated steroids in teleost fish was firmly established. In these fish, which produce varying levels of 11OHA4, 11KA4, 11OHT and 11KT (Jiang et al., 1998, 1996; Liu et al., 2000), 11KT functions as the primary androgen (Borg, 1994).

Interestingly, despite the seeming lack of interest in the role of 11-oxogenated C19 steroids, 11KA4 is widely promoted as a prohormone in dietary supplements (usually referred to as 11-oxoandrostenedione or 11-OXO), capable of reducing body fat and increasing muscle mass (Brooker et al., 2009; de la Torre et al., 2015). To our knowledge, evidence for the effect of 11KA4 on muscle mass is taken from studies conducted in fish (Lone and Matty, 1982). It has also been proposed that 11KA4 functions as an inhibitor of 11 β -hydroxysteroid dehydrogenase type 1 (11 β HSD1), which catalyses the conversion of cortisone to cortisol. A reduction in cortisol, a catabolic hormone, is thought to be beneficial for increasing muscle mass. 11KA4 supplements are therefore sometimes promoted as 'selective cortisol modulators' (Brooker et al., 2009). We have shown that 11KA4 is in fact a substrate for 11 β HSD1 (Swart et al., 2013), and any inhibition is therefore likely due to competition between the substrates. Due to the anabolic nature of 11KA4, presumably by conversion to 11KT, and its ability to modulate cortisol levels, 11KA4 is considered a performance

enhancing drug and a number of doping control methods have been developed in order to detect its usage (Brooker et al., 2009; de la Torre et al., 2015).

Despite the prominent androgenic role played by 11KT in fish and the claimed androgenic effects of supplements containing 11KA4, very few studies have considered the potential androgenic activity of the 11-oxygenated steroids in mammalian systems. In 2008, Yazawa et al. showed that CYP11B1 expression could be induced in both Leydig cells and ovarian theca cells from immature mice by treatment with human chorionic gonadotropin, leading to the production of 11OHT and 11KT (Yazawa et al., 2008). More importantly, this study was the first, to our knowledge, to investigate the androgenic activity of 11OHT and 11KT, which were up until that point only considered fish androgens, via a mammalian androgen receptor (AR). They found that 11KT demonstrated similar activity to testosterone, while 11OHT demonstrated poor androgenic activity, similar to that of A4. After confirming that 11OHA4, 11KA4, 11OHT and 11KT are products of the human adrenal, Rege et al. used MDA-kb2 cells, containing a human AR and an androgen receptor-driven luciferase reporter to show that 11OHT and 11KT both have similar, albeit slightly lower, androgenic activity to that of testosterone via the human AR (Table 2). They confirmed that 11OHA4 has no androgenic activity, and that 11KA4 is only slightly androgenic (Rege et al., 2013). In the same year, our group showed that 11KT is a partial AR agonist at 1 nM, comparable to T. We found that the 11OHA4 metabolites, 11K-5 α -dione, 11OHT and 11OHDHT also demonstrated partial agonist activity at 1 nM, albeit less than that of 11KT. Significantly, we found that 11-ketodihydrotestosterone (11KDHT), the 5 α -reduced product of 11KT, acted as a full AR agonist at 1 nM, comparable to DHT (Table 2) (K.-H. Storbeck et al., 2013). While developing a cell based androgen screening model Campana et al. recently confirmed that 11KT is a full AR agonist whereas 11OHT is only a partial AR agonist. In their test system 11KA4 elicited only a low, but detectable response, while no response was observed for 11OHA4 (Campana et al., 2016).

We subsequently conducted a comparative study of the androgenic activity of testosterone, DHT, 11KT and 11KDHT. First, we showed that 11KT and 11KDHT bind to the human AR with affinities similar to that of T and DHT. Next, we used a selective androgen response element-driven luciferase reporter to assess the potencies and efficacies of the four androgens via the human AR. We found that

both the potencies and efficacies of 11KT and 11KDHT are comparable to that of T and DHT, respectively (Table 2). To our knowledge, this was the first study to show that 11KDHT is equipotent to DHT, which is considered the most potent natural androgen in mammals. Finally, we confirmed that 11KT and 11KDHT, like T and DHT, were able to induce AR-regulated gene expression and cell growth in two androgen dependent prostate cancer cell lines (Pretorius et al., 2016).

It is therefore apparent that there is a growing body of evidence supporting not only the production of 11-oxygenated steroids by the human adrenal, but also the androgenic activity of these steroids and their metabolites (Pretorius et al., 2016; Rege et al., 2013; K.-H. Storbeck et al., 2013). While the role of these steroids in normal human physiology will take time to elucidate, there is increased interest in the role that these steroids may play in disease conditions which are either dependent on adrenal C19 steroids or associated with androgen excess, as will be discussed below.

4. Castration resistant prostate cancer

Prostate cancer is an androgen-dependent and -driven disease. As such, the first line of treatment for advanced prostate cancer is androgen deprivation therapy (ADT) which is accomplished by either surgical (Orchiectomy) or chemical castration using luteinizing-hormone-releasing-hormone (LHRH) agonists or antagonists (Sharifi, N., Gulley, J.L., Dahut, 2005). This approach significantly lowers the levels of circulating T (Heidenreich et al., 2014; Zlotta and Debruyne, 2005) which is the precursor to the most potent natural androgen, DHT. While ADT is initially effective, the cancer often later re-emerges in the form of castration resistant prostate cancer (CRPC), which is eventually fatal. Subsequent treatment, targeting androgen synthesis and action, has revealed that in most cases CRPC remains androgen dependent. These include the clinical results obtained with the CYP17A1 inhibitor, abiraterone (Attard et al., 2009a, 2009b; Fizazi et al., 2012) and the AR antagonist enzalutamide (Scher et al., 2012). Various mechanisms for the resurgence of androgen action have been proposed and include AR gene amplification, AR splice variants and AR mutations within CRPC cells (Holzbeierlein et al., 2004; Knudsen and Penning, 2010; Yuan and Balk, 2009; Zlotta and Debruyne, 2005). Furthermore, studies have shown that while ADT results in a 90 to 95% reduction in serum T

levels, intraprostatic DHT levels are only decreased by approximately 50% (Luu-The et al., 2008), thereby implicating steroids other than T as precursors to DHT. It has since been confirmed that a variety of adrenal steroids can serve as precursors to DHT in tissue such as the prostate, containing the necessary enzymatic machinery. After ADT, the transcriptional profile of key steroidogenic enzymes in the prostate is altered. The expression of 3β HSD1, steroid 5α -reductase type 1 (SRD5A1) and AKR1C3 is increased, while that of SRD5A2 is downregulated (Shaw et al., 2016; Stanbrough, 2006; Titus et al., 2005).

The adrenal C19 steroids, DHEA, DHEAS and A4 are substrates for the so called "alternate 5α -dione pathway", which bypasses T to produce DHT via 5α -androstanedione (5α -dione) (Fig. 2) (Auchus, 2004; Chang and Sharifi, 2012; Chang et al., 2011; Luu-The et al., 2008; Miller and Auchus, 2011; Sharifi and Auchus, 2014, 2012; Sharifi, 2012). In this pathway, SRD5A1 preferentially metabolises A4 to 5α -dione rather than T to DHT (Chang et al., 2011; Thigpen et al., 1993). 5α -dione is subsequently converted to DHT by the action of AKR1C3 (Chang et al., 2011; Knudsen and Penning, 2010).

While there is no doubt that the abovementioned pathways play a significant role in CRPC, the intratumoral androgen pool may not be limited to classical androgens such as testosterone and DHT. We have recently shown that prostate cancer cell lines are able to metabolise the abundant adrenal steroid 11OHA4, yielding the potent 11-oxygenated androgens 11KT and 11KDHT (Fig. 2) (Storbeck et al 2013). 11OHA4 is metabolised by the same enzymes responsible for the conversion of the adrenal steroid A4 to DHT, namely AKR1C3 and SRD5A1. The 11OHA4 pathway however, also requires 11β HSD2 which is expressed in prostate cancer cells (Dovio et al., 2009; Page et al., 1994). The preferred route for 11OHA4 metabolism appears to be the conversion of 11OHA4 to 11KA4 by 11β HSD2, followed by the conversion of 11KA4 to 11KT by AKR1C3. 11KT in turn, is reduced by SRD5A1 to produce 11KDHT. Interestingly, this suggests that the alternate 5α -dione and 11OHA4 pathways may be complementary, with the 5α -dione pathway bypassing T, while the 11OHA4 pathway favours the production of 11KT. The low levels of 11OHT, which is a partial AR agonist, may also contribute to the intratumoral androgen pool. 11OHT can be converted to 11OHA4 by 17β -hydroxysteroid dehydrogenase type 2

(17 β HSD2) and then to 11KT by the 11OHA4 pathway, or it can be converted directly to 11KT by the action of 11 β HSD2 (Storbeck et al 2013).

The products of the 11OHA4 pathway, 11KT and 11KDHT, have recently been confirmed to be potent AR agonists comparable to T and DHT, respectively (Table 2) (Pretorius et al., 2016). Furthermore, we have shown that these steroids are able to induce the expression of well-known AR-regulated genes (*KLK3*, *TMPRSS2* and *FKBP5*) in two androgen dependent prostate cancer cell lines, LNCaP and VCaP. 11KT and 11KDHT were also able to induce cell growth in both cell lines. Finally, proteomic analysis of VCaP cells revealed that both 11KT and 11KDHT modulated the expression of known AR-regulated proteins. Collectively, this data confirmed that 11KT and 11KDHT are *bone fide* androgens and that their contribution to the development and progression of CRPC warrants further investigation (Pretorius et al., 2016).

While it is clear that the intratumoral concentration of androgens is dependent on the conversion of inactive adrenal precursors to active androgens, the subsequent inactivation of androgens is equally as important. Androgens are inactivated by either the 3 α HSD catalysed reduction of 5 α -reduced steroids and/or by uridine 5'-diphospho-glucuronosyltransferase (UGT) catalysed glucuronidation (Bélanger et al., 2003). We therefore measured the metabolism of both 11KT and 11KDHT in LNCaP and VCaP cells. Interestingly, we found that while T and DHT were rapidly inactivated by both cell lines, the metabolism of 11KT and 11KDHT occurred at a significantly lower rate. This observation suggests that 11KT and 11KDHT have the potential to remain active longer than T and DHT, further implicating these 11-oxygenated steroids as a putative driving force behind the development and progression of CRPC (Pretorius et al., 2016).

5. Congenital adrenal hyperplasia

Congenital adrenal hyperplasia (CAH) is a collection of inherited conditions characterized by deficiencies in cortisol biosynthesis. The ensuing loss of negative feedback by cortisol on the hypothalamus and pituitary gland results in the excessive secretion of corticotropin releasing hormone (CRH) and ACTH, which in turn leads to hyperplasia of the adrenal cortex. This autosomal recessive condition is caused by mutations in genes encoding enzymes mediating adrenal steroidogenesis. The

deficiencies can be complete or partial, leading to a wide array of clinical presentations (Turcu and Auchus, 2015; White and Speiser, 2000).

The most prominent cause of CAH is 21OHD, which is considered one of the most common autosomal recessive diseases, and accounts for over 90% of all CAH cases (Speiser and White, 2003). The loss of CYP21A2 function results in the accumulation of cortisol precursors which are subsequently diverted toward androgenic pathways (Fig. 3). Significant features associated with the androgen excess observed in 21OHD include *in utero* virilisation in females as well as epiphyseal maturation, premature pubarche, subfertility and rapid somatic growth in both sexes (Cabrera et al., 2016; Claahsen-van der Griten et al., 2008; Reisch et al., 2009; Speiser and White, 2003; Stikkelbroeck et al., 2003).

Treatment of 21OHD includes the administration of glucocorticoids and mineralocorticoids in order to re-establish the negative feedback on ACTH secretion (White and Speiser 2000). However, treatments often fail to blunt the early morning rise of ACTH, leading to physicians resorting to non-physiologic doses of glucocorticoids, which results in side effects such as bone loss, obesity and features of metabolic syndrome (Arlt et al. 2010; Finkelstein et al. 2012). Monitoring and optimizing clinical treatment therefore remains a challenge (Auchus and Arlt, 2013; Han et al., 2014).

The significant accumulation of 17OH-PROG, a substrate of CYP21A2, which is a hallmark of 21OHD is used both for diagnostic purposes and to monitor the success of treatment (Kamrath et al. 2012). The accumulation of 17OH-PROG is due to both the loss of CYP21A2 function and the inability of human CYP17A1 to efficiently catalyse the conversion of 17OH-PROG to A4 (Fig. 3). Even though the Δ^5 steroid 17OH-PREG is the preferred substrate for the 17,20-lyase activity of human CYP17A1, the excessive accumulation of 17OH-PROG can overcome this substrate presence and in so doing contribute to the excess production of androgens observed in 21OHD (Auchus et al., 1998; Turcu and Auchus, 2015). The resulting adrenal androgen precursors are further converted to T and DHT, both in the adrenal and in the peripheral tissue. To a lesser extent, estrogens such as estradiol and estrone are also produced in peripheral tissues (White and Speiser, 2000).

The so called “backdoor pathway” is an additional pathway for DHT biosynthesis, where adrenal 17OH-PROG can be converted to DHT without DHEA, androstenedione or testosterone as intermediates and appears to play an important role during male sexual differentiation (Arlt et al., 2004; Auchus, 2004; Flück et al., 2011; Wilson et al., 2003). Accumulation of 17OH-PROG, as in patients with 21OHD, could reopen the backdoor pathway, with some evidence from urinary steroid profiling that this actually takes place in patients with CAH due to 21-hydroxylase deficiency (Kamrath et al., 2012). In this pathway 17OH-PROG is not metabolised by cytochrome P450 steroid 21-hydroxylase (CYP21A2), but is instead reduced by SRD5A1, yielding 17OH-dihydroprogesterone (Wilson et al., 2003). 17OH-dihydroprogesterone is subsequently metabolised by 3 α -hydroxysteroid dehydrogenase activity to form 5 α -pregnane-3 α ,17 α -diol-20-one (pdiol), which is a substrate for the 17,20-lyase activity of CYP17A1. The resulting androsterone (AST) is reduced by AKR1C3 to form 3 α -adiol. Finally, 3 β -epimerase activity converts 3 α -adiol to DHT (Auchus, 2004).

The blockage caused by CYP21A2 deficiency has also been shown to result in the accumulation of additional steroids, including 16 α -hydroxyprogesterone, 11 β -hydroxyprogesterone and 21-deoxycortisol (Turcu et al., 2015). Recently, Turcu and colleagues showed that the levels of four 11-oxygenated C19 steroids, 11OHA4, 11KA4, 11OHT and 11KT, are significantly (3- to 4-fold) elevated in both male and female patients with classic 21OHD when compared to age-matched controls (Table 2). The authors suggest that since 11KT is an active androgen, this steroid may be clinically relevant to the hyperandrogenism associated with 21OHD. Significantly, it was shown that 11KT tended to correlate inversely with T in males with 21OHD, strongly suggesting that 11KT was able to suppress gonadotropins and T production from the testes in men (Turcu et al., 2016). This finding supports the idea that 11KT is capable of eliciting physiological effects. Furthermore, the authors suggest that the ratio of T to 11KT might be ideal for monitoring the treatment of men with 21OHD (Turcu et al., 2016). Indeed, as the 11-oxygenated steroids are primarily derived from the adrenal, they may be superior biomarkers of adrenal androgen hyperandrogenism in 21OHD than other C19 steroids which have multiple origins. For example, A4 and T are produced by both the adrenals and the gonads which may explain why there is no good correlation between these steroids and the clinical

evidence of androgen excess in 21OHD patients (Krone et al., 2000; Speiser et al., 1992; Turcu et al., 2016).

The prominent adrenal C19 steroids DHEA and DHEAS, on the other hand, are also not good markers of hyperandrogenism in classic 21OHD as they are disproportionately suppressed by glucocorticoid treatment (Finkelstein et al., 2012; Kamrath et al., 2012; Turcu et al., 2016). The finding that 11-oxygenated steroids are elevated in 21OHD therefore not only holds promise for better understanding the effects of androgen excess associated with 21OHD, but also as biomarkers for disease severity and treatment response in both male and female patients.

6. Polycystic Ovary Syndrome

Polycystic ovarian syndrome (PCOS) is a common grouping of conditions, which affects 4-10% of women of reproductive age (Azziz et al., 2004; Knochenhauer et al., 1998) and is characterized by biochemical and/or clinical evidence of androgen excess and chronic anovulation, with or without polycystic appearance of the ovaries on ultrasound, the latter reflecting enhanced folliculogenesis with premature developmental arrest. Importantly, women with PCOS have an increased adverse metabolic risk, with a higher prevalence of insulin resistance, type 2 diabetes, arterial hypertension and surrogate parameters of cardiovascular risk (Azziz et al., 2004; Legro, 2003; Ovalle and Azziz, 2002; Wild, 2002).

The most common clinical feature of PCOS is hyperandrogenism. Biochemical evidence of androgen excess is commonly found in PCOS patients and, in turn, can negatively affect menstrual cycle and ovarian function (Azziz et al., 2004). The origin of excess androgen production in PCOS patients is, however, controversial. Several studies indicate that the ovary exhibits abnormally high T production in the majority of PCOS cases (Stahl and Greenblatt, 1973; Stahl et al., 1973). Stimulation of ovarian androgen production with GnRH agonists elicits higher responses in PCOS patients (Barnes et al., 1989; Ehrmann et al., 1992) and the level of circulating androgens are higher in PCOS patients than in healthy woman even after adrenal suppression by dexamethasone (Lachelin et al., 1982), suggesting an ovarian origin. Still other studies indicate that adrenal androgens contribute to the androgen excess in PCOS. Androgen levels remain higher in PCOS patients than in healthy women

even after ovarian suppression with GnRH agonists (Cedars et al., 1992; Chang et al., 1983; Rittmaster and Thompson, 1990). Conversely, there is also ample evidence for an enhanced adrenal contribution to androgen excess in PCOS. An exaggerated androgen response is observed in PCOS patients after ATCH stimulation (Anapliotou et al., 1990; Azziz et al., 1998; Turner et al., 1992). Moreover, using adrenal vein sampling, it was shown that the increased T seen in PCOS patients could originate from the adrenal as well as from the ovary (Parker et al., 1975; Stahl and Greenblatt, 1973; Stahl et al., 1973). In addition, approximately 50% of women with PCOS were previously shown to have elevated serum levels of DHEAS and 11OHA4, two androgens that are almost exclusively secreted by adrenal glands (Carmina et al., 1986; Stanczyk et al., 1991). Interestingly, brothers of PCOS patients have also been shown to have elevated serum DHEAS (Legro et al., 2002). Recent work has shown that patients with impaired DHEA sulfation due to inactivating mutations in the sulfate donor-generating enzyme PAPSS2 show not only significant androgen excess, resulting from enhanced conversion of DHEA to active androgens, but also an overt PCOS phenotype (Noordam et al., 2009; Oostdijk et al., 2015).

Taken together, evidence therefore suggests that both the ovaries and adrenal contribute to the hyperandrogenism observed in PCOS. However, other studies have highlighted an additional role of the peripheral tissue in androgen activation. For example, increased peripheral conversion of T to DHT has been observed due to up-regulated 5 α -reductase activity (Fassnacht et al., 2003; Stewart et al., 1990).

Even though PCOS can be considered a collection of endocrine disorders which share the common characteristic of hyperandrogenism, serum T alone remains the most commonly measured and widely available marker for the estimation of biochemical androgen excess in PCOS (Azziz et al., 2004). Quantification of multiple androgens, rather than just T may therefore significantly expand our ability to distinguish different forms of PCOS (O'Reilly et al., 2014). Serum A4 is inconsistently measured in PCOS patients as clinicians remain uncertain about its diagnostic value. Limited previous work suggests that 10% of PCOS patients may be misclassified as normo-androgenemic if A4 is not measured (Knochenhauer et al., 1998). Measurement of T alone may therefore not be sufficient to categorize patients as normoandrogenemic or hyperandrogenemic. A recent study in a large cohort of

PCOS patients has shown that increased A4 is a more sensitive marker of PCOS-related androgen excess and that the type and severity of androgen excess determines the extent of metabolic risk in PCOS (O'Reilly et al., 2014). This finding has been recognized as field changing (Conway et al., 2014; Keevil, 2014) and was recently confirmed by another large study (Pasquali et al., 2016).

Previous studies have considered the 11-oxygenated adrenal steroid, 11OHA4, as a potential marker for adrenal androgen production in PCOS, though results were inconclusive (Carmina, 1992; Holownia et al., 1992; Hudson et al., 1990; Owen et al., 1992; Stanczyk et al., 1991). For example, studies have shown that 11OHA4 levels are more sensitive to adrenal stimulation and suppression than DHEAS (Stanczyk et al., 1991) and that the ratio of A4 to 11OHA4 may be a good marker for adrenal hyperandrogenism (Carmina, 1992), while others have found that although 11OHA4 is elevated in the majority of PCOS woman, it is not a reliable marker for this condition (Owen et al., 1992). It is, however, worth noting that many of these studies relied on immuno-based assays, which are not accurate and have been surpassed by the use of modern mass spectrometry based assays (Bloem et al., 2015).

To date, and to the best of our knowledge, other 11-oxygenated steroids have never been considered in the context of PCOS. Given that 11OHA4 is clearly elevated in PCOS (Carmina, 1992; Carmina et al., 1986; Stanczyk et al., 1991), and that 11OHA4 is a precursor to active 11-oxygenated androgens (K.-H. Storbeck et al., 2013), the peripheral metabolism of 11OHA4 and other adrenal 11-oxygenated steroids may contribute to the symptoms associated with androgen excess. Since 11KDHT has been found to be as potent as DHT (Pretorius et al., 2016) and peripheral 5 α -reductase activity is increased in PCOS patients (Stewart et al., 1990), the reduction of 11KT to 11KDHT may be significant.

Furthermore, 11-oxygenated steroids could potentially serve as biomarkers of PCOS. Indeed, recent advances in analytical techniques now make it possible to simultaneously and accurately measure multiple steroids in a single sample, thereby significantly expanding current diagnostic capabilities (Bloem et al., 2015). Including a panel of C19 steroids such as T, A4, DHEA, DHEAS, DHT, 11OHA4, 11KA4,

11OHT, 11KT and 11KDHT could therefore potentially improve diagnostics of PCOS and may result in clearer differentiation of subtypes in this common endocrine disorder.

7. Conclusion

The identification of 11-oxygenated steroids with androgenic activity, especially the recent characterisation of 11KT and 11KDHT, challenges the paradigm that T and DHT are the only potent natural androgens and thus have significant implications for our understanding of androgen related diseases. Furthermore, recent advances in technology allowing for the accurate quantification of physiologically relevant concentrations of these steroids may be useful for diagnostic and prognostic purposes. Panels of canonical C19 steroids and the 11-oxygenated steroids may allow for better improved diagnosis and classification of androgen related diseases. Taken together, 11OHA4 and its 11-oxygenated metabolites, should no longer be ignored when considering androgen related diseases.

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Table 1. Concentrations of C19 steroids in adrenal vein samples (AVS) and serum from healthy controls and 21OHD patients.

Steroid	AVS¹ (nmol/L)	Controls² (nmol/L)	21OHD² (nmol/L)
DHEAS	3827 ± 1317	3793.4 (1585.1-5066.5)	508.7 (213.0-1745.2)
DHEA	125 ± 56.9	6.0 (4.1-11.0)	1.0 (0.55-2.9)
A4	79.0 ± 46.9	1.5 (0.77-2.2)	5.4 (2.5-13.6)
T	0.78 ± 0.26	0.90 (0.42-10.7)	2.8 (1.3-5.6)
11OHA4	157 ± 96.2	3.9 (2.3-5.1)	11.6 (6.2-26.2)
11KA4	0.99 ± 0.33	1.0 (0.67-1.4)	3.2 (1.9-4.8)
11OHT	0.48 ± 0.17	0.49 (0.30-0.69)	1.9 (0.69-3.4)
11KT	0.39 ± 0.09	1.7 (0.96-2.6)	5.7 (3.5-12.1)

¹Data from Rege et al 2013. Expressed as mean ± SEM (n=7)

²Data from Turcu et al 2016. Expressed as median (interquartile range) (n=38 for both controls and 21OHD)

Table 2. Androgenicity of C19 steroids

Steroid	% induction at 1nM¹	Potency² (nM)	Potency³ (nM)	Efficacy³ (%)
A4	NM	87	-	-
T	60.9	0.52	19.6	96.21
DHT	100	-	3.00	99.14
11OHA4	2.3	*	-	-
11KA4	3.4	469	-	-
11OHT	30.0	2.3	-	-
11KT	62.1	2.7	15.8	107.59
11KDHT	96.2	-	1.35	113.84

¹Data from Storbeck et al. 2013. All values relative to that of DHT which was set at 100%

²Data from Rege et al. 2013. Induction was relative to that of a basal control.

³Data from Pretorius et al. 2016. Efficacies relative to that of the synthetic AR agonist mibolerone which was set at 100%.

* No measurable response

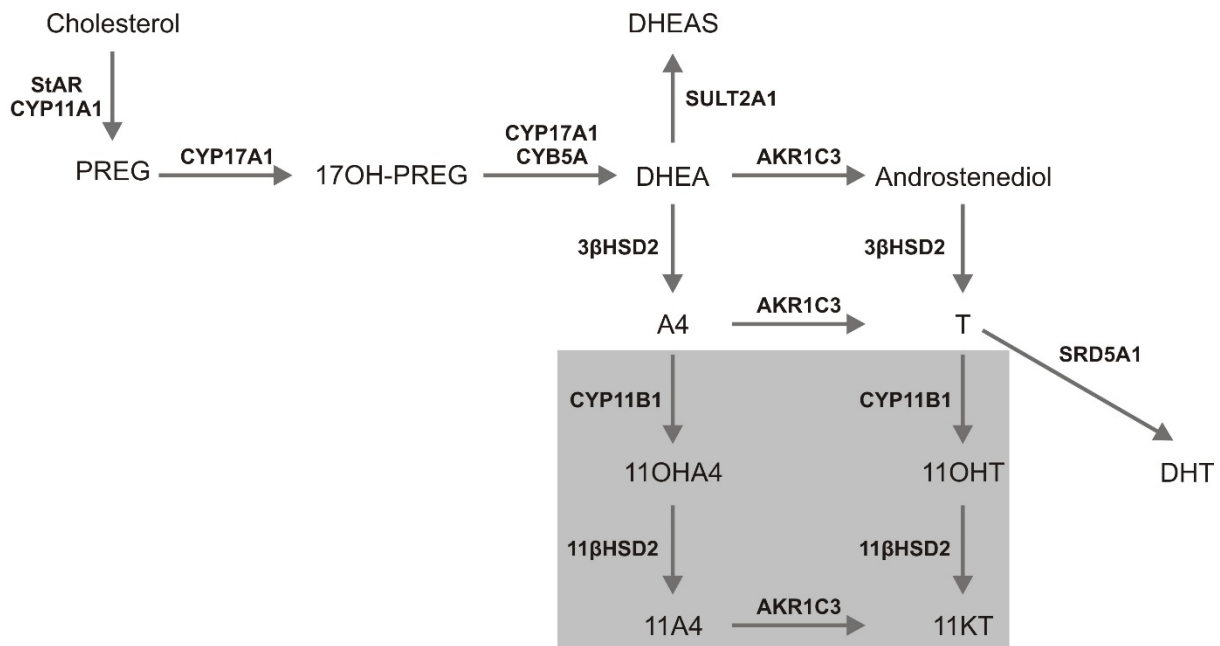


Fig 1. C19 steroid biosynthesis in the adrenal. The biosynthesis of 11-oxygenated steroids are shown in the grey box. 3βHSD2, 3β-hydroxysteroid dehydrogenase type 2; 11KA4, 11-ketoandrostenedione; 11KT, 11-ketotestosterone; 11OHA4, 11β-hydroxyandrostenedione; 11OHT, 11β-hydroxytestosterone; 17OH-PREG, 17α-hydroxypregnenolone; 17OH-PROG, 17α-hydroxyprogesterone; A4, androstenedione; AKR1C3, aldo-keto reductase 1C3; CYB5A, cytochrome b₅; CYP11A1, cytochrome P450 cholesterol side chain cleavage; CYP11B1, cytochrome P450 11β-hydroxylase; CYP17A1, cytochrome P450 17α-hydroxylase/17,-20-lyase; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulphate; DHT, 5α-dihydrotestosterone; PREG, pregnenolone; SRD5A1, steroid 5α-reductase type 1; StAR, steroidogenic acute regulatory protein; T, testosterone.

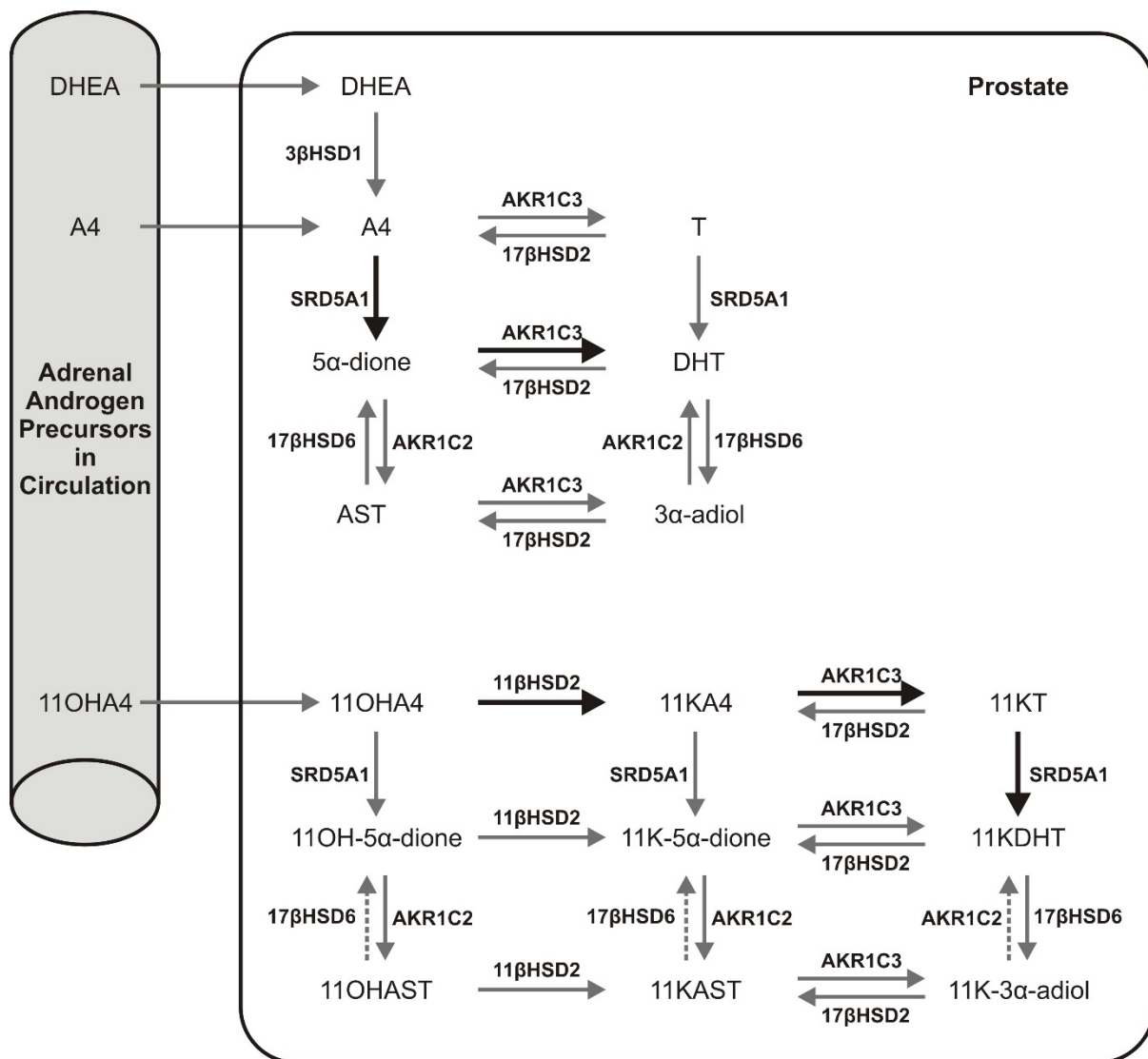


Fig 2. C19 steroid metabolism in castration resistant prostate cancer. 3α-adiol, 3α-androstanediol 3βHSD1, 3β-hydroxysteroid dehydrogenase type 1; 5α-dione, 5α-androstanedione; 11βHSD2, 11β-hydroxysteroid dehydrogenase type 2; 11K-3α-adiol, 11-keto-3α-androstanediol; 11K-5α-dione, 11-keto-5α-androstanedione; 11KA4, 11-ketoandrostenedione; 11KAST, 11-ketoandrosterone; 11KDHT, 11-keto-5α-dihydrotestosterone; 11KT, 11-ketotestosterone; 11OH-5α-dione, 11β-hydroxy-5α-androstanedione; 11OHA4, 11β-hydroxyandrostenedione; 11OHAST, 11β-hydroxyandrosterone; 11OHT, 11β-hydroxytestosterone; 17βHSD2, 17β-hydroxysteroid dehydrogenase type 2; 17βHSD6, 17β-hydroxysteroid dehydrogenase type 6; 17OH-PREG, 17α-hydroxypregnenolone; 17OH-PROG, 17α-hydroxyprogesterone; A4, androstenedione; AKR1C2, aldo-keto reductase 1C2AKR1C3, aldo-keto reductase 1C3; AST, androsterone; DHEA, dehydroepiandrosterone; DHT, 5α-dihydrotestosterone; SRD5A1, steroid 5α-reductase type 1; T, testosterone.

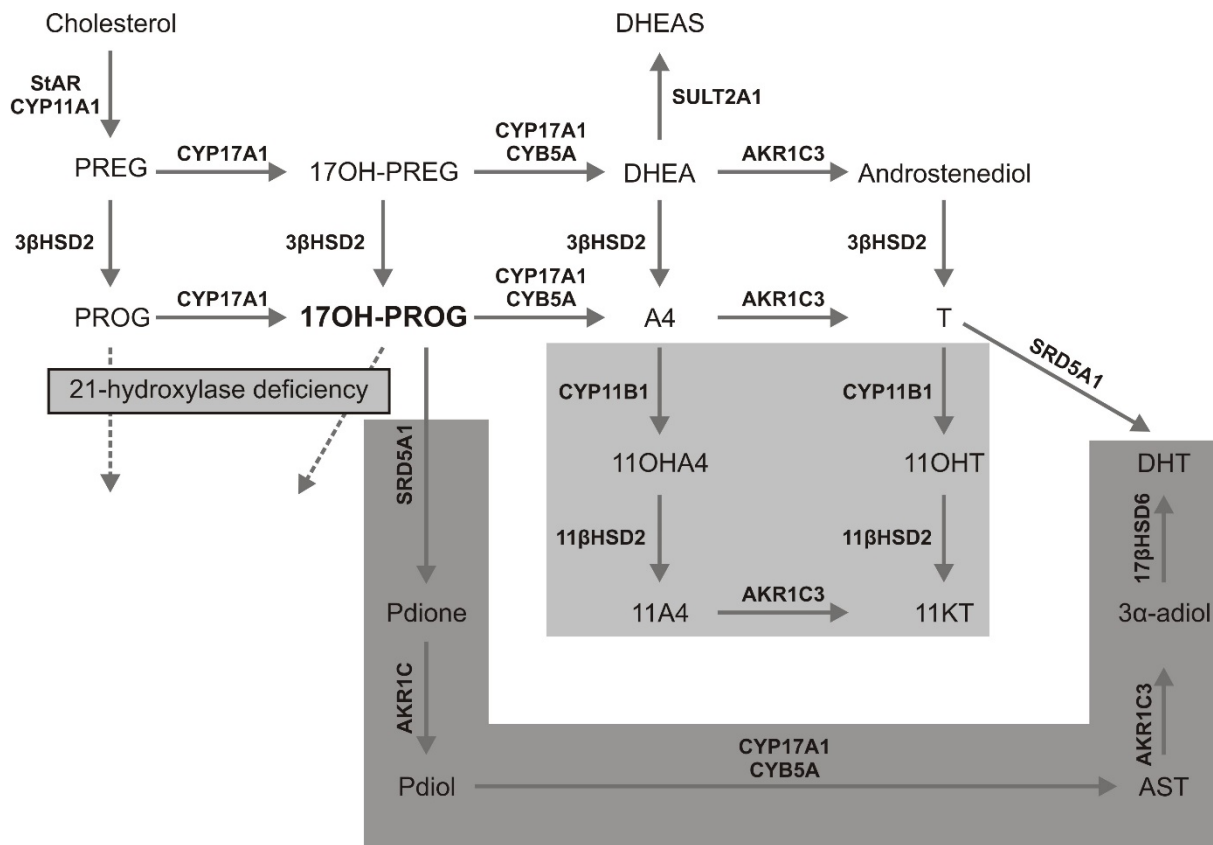


Fig 3. C19 steroid metabolism during 21-hydroxylase deficiency. The biosynthesis of 11-oxygenated steroids are shown in the grey box. The 'backdoor pathway' is shown in dark grey. 3 α -adiol, 3 α -androstenediol; 3 β HSD2, 3 β -hydroxysteroid dehydrogenase type; 11KA4, 11-ketoandrostenedione; 11KT, 11-ketotestosterone; 11OHA4, 11 β -hydroxyandrostenedione; 11OHT, 11 β -hydroxytestosterone; 17 β HSD6, 17 β -hydroxysteroid dehydrogenase type 6; 17OH-PREG, 17 α -hydroxypregnenolone; 17OH-PROG, 17 α -hydroxyprogesterone; A4, androstenedione; AKR1C, aldo-keto reductase 1C; AKR1C3, aldo-keto reductase 1C3; AST, androsterone; CYB5A, cytochrome b₅; CYP11A1, cytochrome P450 cholesterol side chain cleavage; CYP11B1, cytochrome P450 11 β -hydroxylase; CYP17A1, cytochrome P450 17 α -hydroxylase/17,-20-lyase; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulphate; DHT, 5 α -dihydrotestosterone; Pdione, 5 α -pregnane-17-ol-3,20-dione; Pdiol, 5 α -pregnane-3 α ,17 α -diol-20-one; PREG, pregnenolone; PROG, progesterone; SRD5A1, steroid 5 α -reductase type 1; StAR, steroidogenic acute regulatory protein; T, testosterone.

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