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Steroid Biomarkers in Human Adrenal Disease

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Abstract

Adrenal steroidogenesis is a robust process, involving a series of enzymatic reactions that facilitate conversion of cholesterol into biologically active steroid hormones under the stimulation of angiotensin II, adrenocorticotropic hormone and other regulators. The biosynthesis of mineralocorticoids, glucocorticoids, and adrenal-derived androgens occur in separate adrenocortical zones as a result of the segregated expression of steroidogenic enzymes and cofactors. This mini review provides the principles of adrenal steroidogenesis, including the classic and under-appreciated 11-oxygenated androgen pathways. Several adrenal diseases result from dysregulated adrenal steroid synthesis. Herein, we review growing evidence that adrenal diseases exhibit characteristic modifications from normal adrenal steroid pathways that provide opportunities for the discovery of biomarker steroids that would improve diagnosis and monitoring of adrenal disorders.

Keywords

adrenal; steroids; adrenal disease; androgens; biomarkers

1. Introduction to the human adrenal gland and its steroid pathways

The human adrenal gland is a multifaceted endocrine organ. It consists of an inner medulla and an outer cortex that have different embryological origins - neural crest cells for the medulla and mesoderm for the cortex. The adult adrenal cortex produces three distinct classes of steroid hormones, including mineralocorticoids, glucocorticoids and androgens (Fig. 1). The cytoarchitecture of the adrenal cortex was first described by Arnold in 1866, who classified the three layers as zona glomerulosa (ZG), zona fasciculata (ZF) and zona reticularis (ZR) [1]. The relevant functional characterization of these zones has defined their specific roles in adrenal steroidogenesis: the ZG synthesizes the mineralocorticoid

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aldosterone, the ZF produces the glucocorticoid cortisol [2, 3], whereas the human ZR produces adrenal-derived androgens and precursors including dehydroepiandrostenone sulfate (DHEA-S) [4–7]. While angiotensin II is the major agonist for the ZG [8], adrenocorticotropic hormone (ACTH) is the primary stimulus for the ZF and ZR [9].

The key enzymes involved in adrenal steroid production are either cytochrome P450 enzymes (CYPs) or hydroxysteroid dehydrogenases (HSDs) (Fig. 1). Even though some of the steroidogenic enzymes and cofactors/proteins are present in all the cortical zones [ex: cytochrome P450 cholesterol side-chain cleavage (CYP11A1) and steroidogenic acute regulatory protein (StAR)], the zone-specific production of steroids results from the differential expression of CYP/HSD enzymes and cofactors [7, 10]. The ZG-specific presence of aldosterone synthase (CYP11B2) [11] and absence of 17α-hydroxylase/17,20-lyase (CYP17A1) [12] enables the production of aldosterone. The 17α-hydroxylase activity of CYP17A1 facilitates the synthesis of cortisol in the ZF [12]. The biosynthesis of DHEA-S in the ZR is contingent on the presence of only three steroidogenic enzymes, namely CYP11A1, CYP17A1 and steroid sulfotransferase (SULT2A1) [10]. Furthermore, ZR expression of cytochrome b_5 (CYB5A), an allosteric regulator of CYP17A1 that enhances its 17, 20-lyase activity, is required for robust production of DHEA-S [12]. Another distinctive ZR feature that allows its production of DHEA-S is the lack of 3β-hydroxysteroid dehydrogenase type 2 (HSD3B2), which contrasts to high expression in the ZG and ZF where it is needed for both aldosterone and cortisol synthesis [12, 13] (Fig. 1). The relative lack of ZR HSD3B2 expression decreases its enzymatic competition with CYP17A1 for pregnenolone (Preg) and 17α-hydroxypregnenolone (17OHPreg), thus increasing the flux of substrate toward DHEA-S. Historically, there have been reports that the adrenal also produces low but significant amounts of bioactive androgens including testosterone (T) [14–17]. Recently, we demonstrated an intra-adrenal pathway for androstenedione (A4) conversion to T via type 5 17β-hydroxysteroid dehydrogenase (AKR1C3), which has its highest cortex expression in the ZR [6, 10, 12, 18]. In addition, we recently characterized the adrenal synthesis of 11-oxygenated androgens and their precursors [6, 19]. These steroid derivatives of A4 and T include: 11β-hydroxyandrostenedione (11OHA4), 11β-hydroxytestosterone (11OHT), 11-ketoandrostenedione (11KA4) and 11-ketotestosterone (11KT). These steroids are most likely of adrenal origin because 11β-hydroxylase (CYP11B1), an enzyme mainly expressed in adrenocortical cells, readily converts T and 11OHA to 11OHT and 11OHA4, respectively [20, 21] (Fig. 1). 11OHT and 11OHA4 can in turn be oxidized to their respective keto-derivatives, 11KT and 11KA4 in peripheral tissues expressing 11β-hydroxysteroid dehydrogenase type 2 (HSD11B2) [6, 21–23] (Fig. 1). Furthermore, 11KA4 is a much better substrate for AKR1C3 than A4, leading ultimately to efficient production of 11KT [24]. Unlike the conventional adrenal steroids DHEA and DHEA-S, 11OHT and 11KT effectively activate the androgen receptor [6, 25, 26].

2. Diseases that Disrupt Adrenal Steroidogenic Pathways

2.1. Disorders causing Adrenal Androgen Excess

2.1.1. Congenital Adrenal Hyperplasia—Congenital adrenal hyperplasia (CAH) is a term used collectively for a group of autosomal recessive genetic defects in cortisol

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biosynthesis [27]. 21-hydroxylase (CYP21A2) deficiency (21OHD) is the most common form of CAH, accounting for more than 90% of all CAH cases [28]. Based on the presence or absence of cortisol insufficiency, diagnosis of 21OHD is routinely classified into classic and nonclassic forms, respectively [29]. Worldwide, the incidence of classic 21OHD is 1:16,000 live births [30, 31]; whereas nonclassic 21OHD is much more frequent, having an occurrence of up to 1:200 in Caucasians and Ashkenazi Jews [32]. The combined prevalence for classic and nonclassic 21OHD makes it one of the more common genetic diseases. The classic forms of 21OHD are divided into two groups: “salt wasting” and “simple virilizing”, depending on whether or not mineralocorticoid synthesis is sufficiently defective to cause hypotensive crises in newborns. Although subclassification of 21OHD is generally helpful, the spectrum of the disease severity actually depends on the underlying CYP21A2 mutations, the resulting residual activity of the enzyme, and poorly understood factors such as modifier genes.

CYP21A2 is a steroid-metabolizing enzyme required for the synthesis of both aldosterone and cortisol (Fig. 1). The decrease in circulating cortisol biosynthesis in 21OHD releases negative feedback on the hypothalamus and the pituitary gland. This leads to a steep augmentation in the secretion of the corticotropin-releasing hormone (CRH) and ACTH, which in turn causes hyperplasia of the adrenal cortex. An enzyme block at the CYP21A2 step in the steroidogenic pathway results in a build-up of the upstream substrates, particularly 17α-hydroxyprogesterone (17OHP4). Elevated serum 17OHP4 has been traditionally used as a biomarker of 21OHD and for diagnosis and treatment monitoring [31, 33] (Table 1). Serum 17OHP4 testing has several drawbacks that include: 1) false-positive results are commonplace in premature and sick newborns [34, 35]; 2) false-negative results have been reported in newborn screening [36] and are more prevalent in girls [37], particularly when mothers were prenatally exposed to glucocorticoids [38]; 3) dynamic confirmatory testing under cosyntropin stimulation is mandatory for most cases [39]; 4) even after cosyntropin stimulation, some 17OHP4 values cannot distinguish between nonclassic 21OHD and the carrier state. In order to overcome the pitfalls of 17OHP4 testing 21OHD, researchers began searching for additional steroid biomarkers. Milewicz et al first proposed the utilization of 21-deoxycortisol (21dF) concurrently with 17OHP4 after ACTH stimulation for improved diagnosis of 21OHD [40]. They hypothesized that since 21dF is an 11β-hydroxylated product of 17OHP4 and is produced solely by the adrenal cortex, its serum levels would be elevated in tandem with and more consistently than 17OHP4 in a 21OHD patient. Later studies by Costa-Barbosa et al suggested the analysis of (21dF +17OHP4)/cortisol ratio to improve the positive predictive value of newborn screening and diagnosis of the carrier state [41, 42]. Our recent study also indicated 21dF as a valuable discriminatory steroid between treated 21OHD patients and age- and sex-matched controls [43]. In addition to 17OHP4 and 21dF, we also demonstrated a significant increase in two other upstream steroids: 16α-hydroxyprogesterone (16OHP4) and 11β-hydroxyprogesterone (11OHP4) [43] (Table 1).

Along with an elevation in serum 17OHP4, another central hallmark of 21OHD is androgen excess, which prompts the diagnosis at birth in females [28, 44]. Females with classic 21OHD are born with ambiguous genitalia [28], while adult women with nonclassic 21OHD might present with hirsutism, irregular menses, and infertility [44]. In both males and

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females, the excess adrenal androgen production can lead to premature pubarche, rapid somatic growth, advanced bone age, and subfertility [45, 46]. The excess 17OHP4 resulting from CYP21A2 deficiency is diverted through the pathways left accessible, to form potent androgens, such as T (Table 1). CYP17A1 mediates the conversion of 17OHPreg to DHEA (5 pathway) and of 17OHP4 to A4 (4 pathway). The catalytic efficiency of the human 17,20-lyase, however, is ~100 times greater for the 5 reaction, as compared with the 4 reaction [47], in part explaining the enormous 17OHP4 accumulation in 21OHD. In patients with 21OHD, significant A4 synthesis might still occur via the 4 pathway due to very high intra-adrenal 17OHP4. Curiously, the routinely measured androgens, DHEA, DHEA-S, A4 and T do not show a reliable correlation with the clinical evidence of androgen excess in 21OHD patients, particularly women and children [48, 49]. With that in mind, we hypothesized that the 21OHD adrenal must produce other unrecognized androgens to account for this paradox. Indeed, detailed characterization of the androgens and androgen precursors in classic 21OHD demonstrated that the four 11-oxygenated steroids, 11OHA4, 11KA4, 11OHT, and 11KT, along with A4 and T (Table 1) are significantly higher in both male and female patients with classic 21OHD compared to age-matched controls. The increased androgen release may reflect a disrupted adrenal zonation with intermingled expression of both HSD3B2 and CYB5A that was recently observed in a 21OHD adrenal [19, 50]. In women and children with classic 21OHD, 11KT is the dominant bioactive androgen in the circulation, while among men with well controlled disease, testis-derived T is the dominant circulating androgen [19, 50]. Of note, DHEA-S is paradoxically low in patients with 21OHD, including those in poor control despite chronic ACTH stimulation and elevation of other androgens [19, 51] (Table 1). In contrast, our studies indicated that patients with classic 21OHD had increased pregnenolone sulfate (Preg-S) and 17OH-Preg sulfate levels compared to sex- and age-matched controls [19, 50] (Table 1).

In summary, several advances have been made in the identification of novel steroids and steroid panels in classic and non-classic CAH. These include elevated circulating steroid pathway intermediates that may improve diagnosis and monitoring as well as novel bioactive androgens that appear to contribute significantly to the phenotypic properties seen in these diseases. Larger prospective studies are now underway to validate the utility of these markers and to optimize parameters for diagnosis, assessment of disease control, and treatment monitoring.

2.1.2. Premature Adrenarche—The developmental biology of the human adrenal cortex extends well into postnatal life and represents one of the most complex ontogeny schemes of human endocrine physiology. The fetal adrenal gland synthesizes abundant DHEA-S and little cortisol. The fetal zone regresses at birth, and the DHEA-S production in the neonatal adrenal abruptly stops. Adrenarche refers to the onset of adrenal production of DHEA and DHEA-S that reflects the expansion of the ZR that appears to start around 5–6 years (y) of age in both boys and girls [12, 52]. This slow rise in androgenic steroids and their precursors manifests clinically as pre-pubertal initiation of axillary hair, pubic hair, and acquisition of adult body odor. The adrenarche-associated increase in DHEA and DHEA-S has been attributed to the segregated expression of HSD3B2 in the ZF, and of CYB5A and SULT2A1 in the developing ZR (Fig. 1) [53–56]. While DHEA-S is an appropriate marker

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for the progression of adrenarche, it is not an active androgen, and it requires peripheral conversion to more potent androgens to mediate the phenotypic manifestations of adrenarche. Normal adrenarche is a gradual process that precedes and is independent of puberty. Premature adrenarche (PremA) is defined as an early rise in adrenal androgen production resulting in the appearance of pubic or axillary hair before age 8 y in girls and 9 y in boys. PremA is reported to occur in 1.5% of Caucasian and as high as 9.5% in African-American girls. A number of studies suggest that PremA could be a harbinger of adolescent and adult diseases, most notably increased insulin resistance and cardiovascular risk. Girls with PremA have a higher susceptibility for developing polycystic ovary syndrome (PCOS) later in life, which is present in 5–10% of women of reproductive age. In addition, obesity has been associated with increased risk of PremA, suggesting an association with the early onset in adrenal androgens seen in PremA.

The molecular origins for the vast majority of PremA cases remain unknown; however, rare genetic causes of PremA have been defined, providing insights into the role of certain steroidogenic pathways in adrenal androgen synthesis. One such cause of premature pubarche and/or hyperandrogenemia is apparent cortisone reductase deficiency due to decreased HSD11B1 (11β-hydroxysteroid dehydrogenase type 1) activity [57, 58]. Although genetic studies demonstrated a normal sequence of the *HSD11B1* gene in most affected individuals, inactivating mutations have been found in the *H6PDH* (hexose-6-phosphate dehydrogenase) gene [59, 60]. Inactivating mutations in *H6PDH* disrupts HSD11B1 oxo-reductase activity resulting in decreased peripheral conversion of cortisone to cortisol. The impact of decreased cortisol regeneration is a mild rise in ACTH, and augmentation in adrenal steroid production that includes androgen androgens. Another rare genetic cause of PremA is mutations in 3′-phosphoadenosine 5′-phosphosulfate (PAPS) synthase type 2 (PAPSS2) [61]. The sulfonation of steroids by SULT2A1 requires PAPS, a sulfate donor, and synthesis of PAPS requires the enzymes PAPSS1 or PAPSS2 [62, 63]. On examination of an 8-year-old girl with early pubic and axillary hair, Noordam et al concluded that the cause of hyperandrogenism was a set of compound heterozygous mutations in *PAPSS2* [61]. As a result of impaired DHEA sulfonation, the unconjugated DHEA pool in this subject was available for conversion into bioactive androgens, such as T.

The age-related patterns in the production of other potent androgens and their precursors have been investigated in PremA with varying results. Historically, children with PremA have been shown to exhibit higher serum concentrations of androgen precursors, such as DHEA, DHEA-S and A4 (Table 1), as well as their urinary metabolites [64–70]. It should be noted that these steroids are poor androgen receptor agonists and hence would not be the direct androgen receptor activators that cause the clinical manifestations of PremA. Until now, T has been the measure of choice as a marker of androgen excess in pre-pubertal children. Unfortunately, the contribution of T to the progression of normal adrenarche and PremA has been controversial. Although many studies have shown that T levels do not rise during early adrenarche [71–73], several other analyses have suggested that T does increase and can therefore contribute to the progression of PremA [64, 67, 68, 72, 74–76]. We recently characterized alterations in the childhood circulating steroid biome, including adrenal-specific 11-oxyandrogens in girls with PremA and an age-matched reference population [26]. Our analysis indicated that PremA is characterized by broad changes in the

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steroid biome, including early elevations in circulating concentrations of adrenal-derived steroid sulfates, T, and all the 11-oxyandrogens (Table 1). Another highlight of this study was the observation that children have 3-fold higher circulating levels of 11KT than T. Given that 11KT has an androgenic potency approaching that of T and is the dominant circulating androgen in girls during adrenarche and PremA, we suggested that 11KT is an important mediator of the precocious androgenic manifestations of PremA [26].

These recent findings support the need to expand studies of 11-oxyandrogens in normal physiology and diseases of androgen excess. A specific role for this novel adrenal androgen pathway in both normal and PremA seems likely, and measurements of these steroids might help to differentiate precocious puberty from PremA. Whether or not an early elevation in adrenal production of these steroids is an indicator of a higher risk for adult-onset disease remains to be investigated.

2.1.3. Polycystic Ovary Syndrome (PCOS)—PCOS is a common ovarian disorder, affecting 5–10% of women of reproductive age. PCOS is a complex medical condition that manifests as oligomenorrhea, hyperandrogenism, with or without polycystic ovaries. The probable link between the development of PCOS and its phenotypic symptoms is thought to involve genetic and epigenetic mechanisms [77–79]. More importantly, PCOS patients are at an increased risk of developing metabolic anomalies, including obesity, insulin resistance, type 2 diabetes mellitus and cardiovascular disease.

Androgen excess is a hallmark of PCOS, but the exact origin of hyperandrogenemia remains in question [80]. While the ovary has traditionally been considered the primary source of androgens in PCOS women, elevated adrenal androgens, especially DHEA, DHEA-S and A4 (Table 1), have been documented in a subset of patients with PCOS [81–83]. Thus, along with the ovary, the adrenal gland has been acknowledged as a source of hyperandrogenism in PCOS for the past two decades [84]. Indeed, the few studies that examine ovarian and adrenal vein sampling in PCOS women have shown that the adrenal, ovary or both can show elevated T secretion compared to controls [14, 15, 17]. While some PCOS patients do not decrease androgen production after suppression of the pituitary-gonadal axis, a subset of other PCOS patients decrease circulating androgens following glucocorticoid suppression of the pituitary-adrenal axis. This also suggests that both the adrenal and the ovary can contribute to androgen excess in this disorder [85, 86]. Nonetheless, the mechanisms causing both ovarian and adrenal androgen excess in PCOS remain ambiguous. Some researchers have speculated a generalized adrenocortical hyper-reactivity to ACTH in patients with PCOS, after observing an exaggerated response of adrenal androgens and cortisol to cosyntropin stimulation in this population [87, 88].

Researchers have tried to identify biomarkers that could help pinpoint the glandular source of excess androgens in PCOS, and 11OHA4 has been considered a candidate biomarker for PCOS adrenal dysregulation. Studies from the late 1980s and early 1990s demonstrated that 11OHA4 is elevated in some [89–91] but not all [92, 93] patients with PCOS. A 2017 study comprising of 114 women with PCOS and 49 healthy controls quantified the conventional and 11-oxyandrogens in peripheral serum, as well as their metabolites in 24-h urine collections using LC-MS/MS and GC-MS, respectively. [94]. Along with serum 11OHA4,

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11KA4, 11OHT, and 11KT (Table 1), the 11-oxyandrogen urine metabolite 11β-hydroxyandrosterone was significantly higher in patients with PCOS than in controls. Interestingly, the authors demonstrated that serum 11OHA4 and 11KA4, but not 11OHT or 11KT, correlated significantly, albeit weakly, with metabolic risk markers such as BMI, insulin, and HOMA-IR. A smaller study from Japan recently confirmed elevations in 11-oxyandrogens in some PCOS patients and suggested that BMI associated with elevated 11-oxyandrogens [95]. A follow-up study by the Arlt group defined an intra-adipose mechanism of androgen precursor activation via AKR1C3 that drives lipid accumulation and insulin resistance in PCOS [96]. Taken together, these studies suggest that there might be dysregulated synthesis of adrenal-derived 11-oxyandrogens and precursors in certain forms of PCOS and might also explain the clinical manifestations associated with androgen excess in women with normal serum T.

2.2. Adrenocortical Carcinoma

Adrenocortical Carcinoma (ACC) is a rare disease with an incidence of 1 per million per year and a dismal prognosis, with a 5-year survival rate of <30% [97]. Early detection of these tumors is imperative, as the primary curative approach is early surgical resection [98]. Once tumor cells extend beyond the adrenal capsule, cure rates drop drastically, and metastatic involvement of distant organs is not curable. Therefore, distinguishing ACC from benign adrenocortical adenomas (ACA) is important to provide appropriate adrenal clinical care. Both ACC and ACA originate from steroidogenic cells and can overproduce steroid hormones, leading to clinical syndromes, such as Cushing syndrome, virilization or primary aldosteronism. Paradoxically, overt hormone excess is found in only 60% of ACC and a small fraction of ACA [97]. Currently, the differential ACC vs ACA diagnosis is primarily based on imaging, which can be subjective and poses some risk due to radiation exposure. Growing evidence suggests that urine and/or serum-based steroid biomarker analysis might provide an additional diagnostic tool that is less expensive and virtually risk-free (Table 1).

Several steroid intermediates have long been a useful tool in the diagnosis of ACC. The first study of urinary steroid metabolites by Touchstone and colleagues (1954) demonstrated the exceptionally high levels of tetrahydro-11-deoxycortisol (THS), the major 11-deoxycortisol (11dF) metabolite, in the urine of a patient with metastatic adrenocortical carcinoma [99]. This observation was subsequently confirmed by other small studies [100–102]. Urinary 5 steroid metabolites including 5-pregnenediol (5-PD) and 5-pregnenetriol (5-PT)—degradation products of pregnenolone (Preg) and 17OHPreg are also elevated in ACC [100, 102–104]. One of the initial studies of serum steroids in ACC by Gröndal et al demonstrated elevated levels of Preg-S in all and of 11dF in 72% of the ACC patients (Table 1) [105]. More recently, a comprehensive study from the European Network for the Study of Adrenal Tumors (ENS@T, www.ensat.org) used GC/MS to compared the excretion of 32 urinary steroid metabolites from patients with ACA (n=102) or ACC (n=45) [106]. They observed that ACC patients often had increased urinary THS, and the steroid precursor metabolites, 5-PD and 5-PT. The ENS@T group then developed a sophisticated mathematical algorithm for predicting if an adrenal tumor is an ACC from urine steroid profiling [106]. Kerkhofs et al. and Hines et al later confirmed the results of the ENS@T group, thus proving that urine

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steroid profiling is a highly specific and sensitive method to diagnose ACC pre-operatively and distinguish ACC from ACA [107, 108].

The comprehensive LC-MS/MS analysis of serum steroids can also be used to identify ACC steroid biomarkers and allow rapid and frequent monitoring of disease progression and response to therapy. We recently evaluated a panel of 15 steroids by LC-MS/MS in random serum samples from patients with ACC and ACA along with age- and sex-matched controls. 11dF and 17OH-Preg sulfate were significantly augmented in patients with ACC as compared to those with ACA and controls (Table 1) [109]. Recent studies by Taylor et al and Schweitzer et al also demonstrated that elevated serum/plasma 11dF is a consistent biomarker for ACC [110, 111]. The serum and urinary steroid findings collectively suggest decreased ACC expression of HSD3B2 and/or CYP11B1 when compared to ACA [112]. The above steroid metabolomics studies, thus, indicate that increased concentrations of steroid synthetic pathway intermediates allow improved discrimination of ACC from ACA. The non-invasive nature of serum biomarker analyses might prove to be useful and practical in clinical care for patients with adrenal lesions.

2.3. Primary Aldosteronism

Primary aldosteronism (PA) comprises two main subtypes: unilateral aldosteronism, mainly caused by aldosterone-producing adenomas (APA) and bilateral adrenal hyperplasia (BHA). Histologic studies have shown that some APAs display a ZG-like histology, with small, compact cells, while other APAs are composed of large, lipid-rich cells, similar to those seen in ZF [113, 114]. The latter group of APAs appear to be the source of 18-hydroxycortisol (18OHF) and 18-oxocortisol (18oxoF), which are structurally related to both aldosterone and cortisol, and often referred to as “hybrid steroids” [115, 116] (Table 1). These two steroids are produced by CYP11B2 using cortisol as a substrate, although some 18OHF can be produced by CYP11B1 [117, 118]. In normal adrenal tissue, expression of CYP11B2 is limited to the ZG, while CYP17A1 and CYP11B1 are selectively expressed in the ZF and ZR, and therefore the production of 18OHF and 18oxoF in normal subjects is very low. Numerous studies have found higher 18OHF and 18oxoF in PA patients compared to patients with essential hypertension. More specifically, within the PA population, levels of these hybrid steroids were much higher in subjects with APAs compared to BHA [119–121]. A more recent study of 234 Japanese PA patients suggested that peripheral plasma 18oxoF and 18OHF can discriminate APAs from BHA with promising sensitivity and specificity (0.83/0.99 for 18oxoF and 0.62/0.96 for 18OHF, respectively) [122]. In contrast, a European study found significant overlap for both 18oxoF and 18OHF between APAs and BHA, with limited utility of these steroids to discriminate between the two subtypes [123]. In this same study, however, a panel incorporating 12 different steroids correctly classified 80% of PA patients as having APA or BHA. The reason for greater steroid overlap in the European study was made clear by comparing the analysis of steroids with the somatic mutations causing tumor aldosterone production.

Somatic mutations in genes encoding ions channels and pumps have been identified in APA, including: *KCNJ5* (encoding the Kir3.4 (GIRK4) potassium channel), *ATP1A1* (encoding a Na^+/K^+ ATPase alpha subunit), *ATP2B3* (encoding a Ca^{2+} ATPase), and *CACNA1D*

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(encoding a voltage-dependent L-type calcium channel) that all lead to an increase in constitutive aldosterone production [124–129]. A recent European study demonstrated that specific somatic mutations in APAs define distinct steroid profiles in adrenal vein plasma, which might reflect differences in the underlying biology of these tumors [130]. The authors showed increased concentrations of the hybrid steroids 18OHF and 18oxoF in plasma samples from APA carrying a *KCNJ5* mutation, owing to a predominantly ZF phenotype (increased expression of *CYP11B1* and *CYP17A1*) as opposed to APAs with *ATPase* or *CACNA1D* mutations that display principally a ZG phenotype. Furthermore, a 7-steroid panel, including aldosterone, 18oxoF, 18OHF, 11-deoxycorticosterone, corticosterone, cortisol, and 21dF, correctly classified 92% of APAs according to genotype (Table 1). Follow-up studies, however, showed no significant differences in concentrations of peripheral venous adrenal steroids from PA patients according to histopathologic phenotypes (solitary functional adenoma, hyperplasia, and aldosterone-producing cell clusters) [131]. With the advent of the more sensitive and specific LC-MS/MS methodology for measuring serum steroids, it appears that peripheral and adrenal vein steroid biomarkers might prove to be a major factor in not just differentiating APA from BHA, but also in subtyping the various forms of APAs. Additional prospective outcome-based studies should be able to reveal whether this technique is a better alternative to AVS and can be translated to routine clinical practice.

2.4. Cushing syndrome

Cushing syndrome (CS) ensues from chronic exposure to excess glucocorticoids, either derived from exogenous pharmacological doses of corticosteroids or from an endogenous adrenal overproduction of cortisol [132]. Current guidelines suggest that initial screening for CS should include measurements of 24 h urinary, evening salivary free cortisol or dexamethasone suppression testing (DST) [132, 133]. If results are convincingly positive and corroborated with clinical history and physical exam findings, further dynamic testing and imaging procedures are aimed at differentiating the cause of excess cortisol. CS can be ACTH-independent, as a result of excess cortisol production from an adrenal adenoma or from bilateral micro- or macronodular adrenal hyperplasia. Alternatively, ACTH-dependent CS can result from Cushing disease, meaning an ACTH-secreting pituitary adenoma, from ectopic ACTH secretion, or rarely, from ectopic CRH production [132, 133].

Several studies, using plasma or urinary multisteroid profiles, have suggested the release of precursor steroids that could be useful for the differential diagnosis of CS [101, 134–137] (Table 1). The first report on the application of LC-MS/MS–based multisteroid profiling in subclinical CS, published in 2015, showed that suppressed adrenal androgens had good accuracy in predicting subclinical hypercortisolism [138]. This finding was followed by two reports that were limited to a small series of patients with ACC and CS [108, 110]. The most recent large cohort report implemented a panel of 15 steroids from LC-MS/MS analysis to characterize distinct plasma steroid profiles that might assist the differential diagnosis of pituitary, ectopic, and adrenal subtypes of CS [139]. The largest increases in plasma steroids among patients with CS were observed for 11dF, 21dF, 11-deoxycorticosterone, corticosterone, and cortisol when compared to patients in whom disease was excluded (Table 1). Patients with ectopic ACTH production showed the most prominent increases, but there

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was considerable variation for other steroids according to subtype. Patients with ACTH-independent CS had the lowest concentrations of adrenal androgens, whereas those with ectopic and pituitary ACTH-dependent CS showed the lowest concentrations of aldosterone (Table 1). Although this study is a strong step towards offering a supplementary single-test alternative for subtyping CS, steroid profiling as a method for characterizing the underlying cause of CS remains still in its infancy. More confirmatory analyses are required for validation of evidence-based steroid panels for diagnosis and subtype classification of CS.

3. Conclusion

Adrenal biosynthesis of mineralocorticoids, glucocorticoids, as well as both weak and potent androgens requires the tight regulation of zone-specific steroidogenic enzyme expression via physiologic trophic hormones. Although steroid biosynthesis has been studied for the past six decades, the significance of minor and alternate pathways continues to emerge. Owing to technical improvements and expanded use of steroid mass spectrometry, our understanding of complex adrenal steroidogenic pathways in physiology and disease has progressed considerably over the last twenty years. It is likely that utilization of steroid 'metabolomics' in concert with advances in our understanding of the genetics of adrenal diseases will aid in the diagnostic workup and monitoring of patients that exhibit aberrant production of adrenal steroid hormones.

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Steroid Abbreviations:

DHEA	Dehydroepiandrosterone
DHEA-S	DHEA sulfate
A4	Androstenedione
T	Testosterone
11OHA4	11β-Hydroxyandrostenedione
11KA4	11-Ketoandrostenedione
11OHT	11β-Hydroxytestosterone
11KT	11-Ketotestosterone
Preg-S	Pregnenolone sulfate
17OHPreg-S	17α-hydroxypregnenolone sulfate
Adiol-S	Androstenediol sulfate
11dF	11-Deoxycortisol

21dF	21-Deoxycortisol
11-DOC	11-Deoxycorticosterone
Preg	Pregnenolone
17OH-Preg	17α-hydroxypregnenolone
17OHP4	17α-Hydroxyprogesterone

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Highlights

- The adult adrenal cortex produces three distinct classes of steroid hormones, including mineralocorticoids, glucocorticoids and androgens owing to the zone-specific expression of key steroidogenic enzymes.
- 21-hydroxylase deficiency, premature adrenarche and some cases of polycystic ovary syndrome are associated with excess 11-oxygenated adrenal androgen production.
- Several steroid pathway intermediates have been defined in urine and blood that are set to improve the diagnosis and monitoring of patients with Cushing syndrome and adrenocortical cancer.
- Hybrid steroids that rely on the aberrant expression of adrenal glomerulosa and fasciculata steroid metabolizing enzymes have the potential of simplifying the current workup of primary aldosteronism and Cushing syndrome patients.

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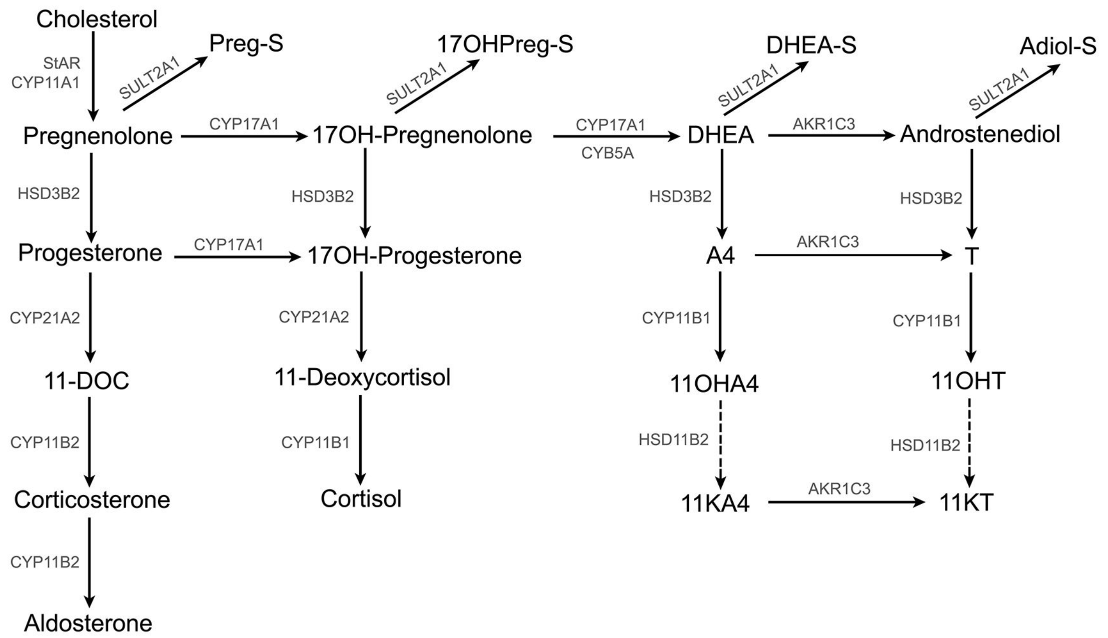

Fig. 1.
Normal Adrenal Steroidogenesis Pathway

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Table 1.

Potential Serum Steroid Biomarkers in Human Adrenal Disorders

Disease/Disorder	Discriminating Steroids	References
21-hydroxylase Deficiency	↑ 17OH-Progesterone, ↑ 16OH-Progesterone, ↑ 11OH-Progesterone, ↑ 21-Deoxycortisol, ↑ A4, ↑ 11OHA4, ↑ 11KA4, ↑ 11OHT, ↑ 11KT, ↑ Preg-S ↓ DHEA, ↓ DHEA-S	[19, 39–41, 48, 49]
Premature Adrenarche	↑ DHEA, ↑ A4, ↑ 11OHA4, ↑ 11KA4, ↑ 11OHT, ↑ 11KT, ↑ Preg-S, ↑ 17OHPreg-S, ↑ DHEA-S, ↑ Adiol-S	[26, 64–67, 70–72, 75]
Polycystic Ovary Syndrome	↑ DHEA, ↑ DHEA-S, ↑ A4, ↑ T, ↑ 11OHA4, ↑ 11KA4, ↑ 11OHT, ↑ 11KT	[80–82, 88–90, 93–95, 138]
Adrenocortical Cancer	↑ 11-Deoxycortisol, ↑ Preg-S, ↑ 17OHPreg-S	[104, 106–109]
Primary Aldosteronism	↑ 18OH-Cortisol, ↑ 18oxo-Cortisol (*KCNJ5*) ↑ Aldosterone (*ATPase*) Other discriminating steroid between mutations in *KCNJ5, ATPase and CACNA1D*: 11-Deoxycorticosterone, Corticosterone, Cortisol, 21-Deoxycortisol	[117–121, 129]
Cushing Syndrome	↑ Cortisol, ↑11-Deoxycortisol, ↑ 21-Deoxycortisol, ↑ 11-Deoxycorticosterone, ↑ Corticosterone ↓ DHEA-S, ↓ DHEA, ↓ A4 (ACTH-independent) ↓ Aldosterone (ACTH-dependent, ectopic)	[134, 136, 137]

DHEA, Dehydroepiandrosterone; DHEA-S, DHEA sulfate; A4, Androstenedione; T, Testosterone; 11OHA4, 11β-Hydroxyandrostenedione; 11KA4, 11-Ketoandrostenedione; 11OHT, 11β-Hydroxytestosterone; 11KT, 11-Ketotestosterone; Preg-S, Pregnenolone sulfate; 17OHPreg-S, 17α-hydroxypregnenolone sulfate; Adiol-S, Androstenediol sulfate.

Underlined steroids are products of aberrant steroid pathways found in adrenal disease that are only minor products of normal adrenals.