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Invited Review for the 100th Anniversary of the TJEM

Mass Spectrometry-Based Steroid Profiling: Meeting Unmet Clinical Needs

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Despite ongoing concerns regarding its clinical application, mass spectrometry (MS)-based steroid assay represents a promising tool in endocrine research. Recent studies indicate that monitoring the blood levels of individual sterols provides improved diagnostic insight into hyperlipidemia compared with immunoassays routinely used in clinical practice. Hypercortisolism and hyperaldosteronism can also be easily evaluated along with successful subtyping of adrenal diseases using MS-based methods, while metabolic signatures of sex steroids provide experimental evidence of abnormal puberty and male infertility. Many MS-based biological and clinical studies are based on liquid chromatography-mass spectrometry (LC-MS) coupled to electrospray ionization and tandem MS scan modes. However, gas chromatography-mass spectrometry (GC-MS) provides better chromatographic separation. Improved chromatographic resolution enables large-scale steroid profiling to allow a bird-eye view and increase the chances of identifying potent biomarkers in endocrine research. In addition to the technical advantages of MS-based assays over immunoassays, minimizing the sample amounts with acceptable analytical sensitivity and standardization of surrogate materials provides cutting-edge tools for precision and personalized medicine.

Keywords: adrenal gland; cholesterol; clinical application; mass spectrometry; steroids
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Introduction

Immunoaffinity techniques are easy to use in clinical practice with higher analytical detectability. However, the lack of specificity for steroids of similar chemical structure and standardization across assays for accurate quantification of individual steroid hormones limits their applicability in precision or personalized medicine (Lee et al. 2006; Hsing et al. 2007; Middle 2007; Wood et al. 2008; Taylor et al. 2015). Mass spectrometry (MS) is currently recognized as a promising tool in omics-based clinical studies, and is preferred over antibody-based radioimmunoassay (RIA) and enzyme-linked immunoassay (ELISA) in steroid analysis (Faupel-Badger et al. 2010; Taylor et al. 2015). The Endocrine Society announced that biological levels of sex steroids measured using MS-based assays are important endpoints in clinical study and practice (Handelsman and Wartofsky 2013).

Several clinical studies mainly focus on the biochemical mechanisms of specific biomolecules based on the known relevance of receptor expression in disease behavior, such as glucocorticoids in Cushing's syndrome and estrogens in both breast cancer and osteoporosis (Jordan et al. 2001; Moreira et al. 2018; Page-Wilson et al. 2019). However, metabolic changes involving other steroids such as cholesterols and androgens in pathophysiology and subtyping of Cushing's syndrome have been demonstrated (Barbetta et al. 2001; Arnaldi et al. 2010). Breast cancer and osteoporosis are also closely associated with cholesterol oxidation (Nelson et al. 2011; Kloudova et al. 2017). The use of cutting-edge MS-based steroid profiling enables the assessment of metabolic signatures of steroid hormones in diseases, and this birds-eye view of complex steroid cascades may be used to identify novel steroid metabolic pathways (Choi and Chung 2015; Keevil 2016; Rege et al. 2018).

In general, MS-based metabolite profiling yields multiple data sets derived from complex biological specimens. A single biomarker may lack sensitivity and specificity for predictive/prognostic detection of disease as well as thera-

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peutic evaluation, which is not adequate to improve patient management (Shin et al. 2013; Xia et al. 2013). To enhance and ensure integrated understanding of the steroid metabolome and its relationship with other disease pathways, the whole metabolism along with precursors and metabolites related to the steroids of interest should be monitored collectively, together with interpretation of their metabolic consequences. Such extensive information could be useful in determining metabolic signatures for precision medicine.

This review presents the current status of MS-based steroid profiling techniques applied to both individual and whole steroid metabolomes with a focus on biomarker discovery, clinical application, and technical advances. To address the unmet clinical needs, the role of high-throughput analysis with less-invasive and reduced sampling procedure, improved analytical sensitivity for pathological confirmation, evaluating local concentrations using both snap-frozen and paraffin-embedded tissues, and standardization with surrogate materials are also discussed.

A Brief Introduction to MS Analysis

A major advantage of MS-based steroid profiling compared with immunoassay is the simultaneous analysis of a single sample via a multiplexed assay, which is enabled by the combination of chromatographic separation methods, such as gas chromatography (GC) and liquid chromatography (LC), prior to MS detection (Table 1). This approach not only yields the levels of individual steroids, but also indicates the metabolic ratios corresponding to the related enzyme activities (Moon et al. 2009, 2013; Choi and Chung 2015; Kim et al. 2016). Recent advances in MS detection techniques have led to the isolation of targeted steroids from matrix interference using tandem mass spectrometry (MS/MS; Wudy and Choi 2016), and to separate steroid isomers by ion mobility-MS (Ahonen et al. 2013). However, multi-component analysis for accurate quantification can be achieved using chromatographic separation, due to similarities in chemical structure and the existence of hundreds of steroids in the body.

The GC and LC methods primarily differ based on the physical state of the mobile phase used with gas and liquid, respectively. In GC, the resulting sample dissolved in liquid after dilution or purification is moved very fast by an inert gas (known as carrier gas), such as helium or argon, and separation is achieved by a complex mechanism based on the differences in boiling points of the analytes and the molecular interactions through the capillary column coated with stationary phase inside. In contrast, the LC mobile phase travels down a solid support (the stationary phase) packed in a column, and the sample is separated based on size, charge, binding affinity, and/or hydrophobicity.

Steroid compounds are mostly soluble in organic solvents, but are not very volatile due to higher polarity as well as lipophilicity. The relatively polar steroids, grouped as corticosteroids, estrogens, progestagens, and bile acids, are preferably analyzed by LC-MS, while few steroids are detected with good analytical sensitivity in GC-MS (Fig. 1). Despite the advantages of LC-MS in steroid analysis, a GC-MS is more valuable and powerful due to the better chromatographic resolution achieved by the higher flow rate than in LC-MS. GC-MS has therefore been widely used in steroid analysis for over 40 years with chemical

Table 1. Comparison of mass spectrometry and immunoaffinity-based assays.

	MS-based assay	Immuno-assay
General aspects		
Detection mechanism	Measuring the mass-to-charge ratio of molecule	Binding affinity between an antibody and an antigen
Quantitative analysis	Yes	Yes
Molecular identification	Yes (based on molecular mass calculation)	No
Analytical sensitivity	Excellent (with chemical derivatization)	Excellent
Batch-to-batch reproducibility	Excellent	Good (not in trace analysis)
Multiplexing	Excellent	Poor
High-throughput	Excellent	Poor
Minimum sampling amounts required for steroid analysis*		
Progesterone	100 μL for all steroids in a sampling	30 μL
17-hydroxyprogesterone		50 μL
Cortisol		20 μL
Aldosterone		100 μL
DHEA sulfate		20 μL
Androstenedione		50 μL
Testosterone		20 μL
17β-Estradiol		50 μL

*Sampling amounts are based on the analytical protocol of different commercially available products.
MS, mass spectrometry; DHEA, dehydroepiandrosterone.

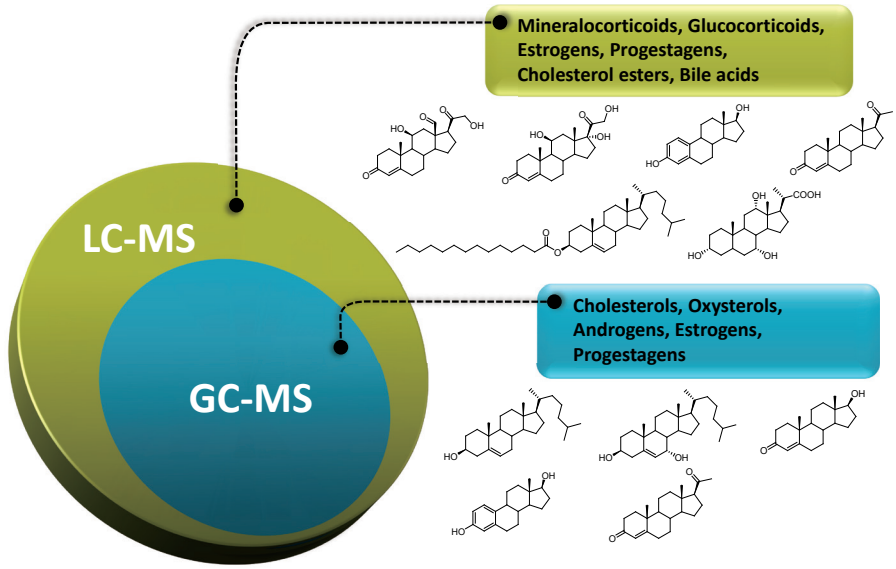

Fig. 1. Application of gas chromatography and liquid chromatography coupled to mass spectrometry in steroid analysis. GC-MS, gas chromatography-mass spectrometry; LC-MS, liquid chromatography-mass spectrometry.

modification methods (Choi and Chung 2015; Shackleton et al. 2018). Chemical modification of the polar functional groups in a steroid backbone facilitates its vaporization during GC separation, as well as increases the analytical sensitivity of LC-MS analysis.

Steroidomics in Biomarker Studies

Cholesterol homeostasis

As the primary precursor of steroid hormones in all animal cells, cholesterol is an essential cell membrane component and is biosynthesized in 37 metabolic steps via mevalonate pathway, also known as the HMG-CoA (3-hydroxy-3-methyl-glutaryl coenzyme A) reductase pathway. The abnormal metabolism of cholesterol may lead to various metabolic disorders in all ages (Raffaï and Weisgraber 2003; Porter and Herman 2011). The association of systolic and diastolic blood pressure with low-density lipoprotein (LDL)-bound cholesterol was reported recently (Zhang et al. 2019), although the biochemical roles of cholesterol in cardiovascular disease remain controversial (de Lorgeril et al. 2010; Abbasi 2019). Moreover, the lipid profiles of total cholesterol and the ratio of LDL/high-density lipoprotein (HDL) cholesterol may be poor predictors of cholesterol biosynthesis (Seo and Choi 2015). The metabolic ratios of lanosterol and lathosterol, which are the upstream precursors of cholesterol biosynthesis (Fig. 2), correlated positively with the up-regulation of cholesterol biosynthesis rather than total and LDL-cholesterol levels (Son et al. 2015). The serum levels of lathosterol alone may be an indicator of whole-body cholesterol biosynthesis in humans (Kempen et al. 1988).

Both biosynthesis and the efflux/influx of lipoprotein-bound cholesterols regulate the intracellular levels of cholesterol, and an understanding of cholesterol homeostasis can be used to elucidate the pathophysiological mechanisms affected by altered cholesterol levels. The MS-based metabolic signatures of cholesterols serve to quantify free cholesterol and its precursors/metabolites as well as dietary plant sterols, which indicate cholesterol biosynthesis and absorption (Kempen et al. 1988; Son et al. 2014, 2015; Dayspring et al. 2015).The levels of biologically-active free cholesterol strongly correlated with total- and LDL-cholesterol levels, but not with HDL-cholesterol. In addition to 7α-hydroxylation of cholesterol, which is the major pathway of cholesterol catabolism in the body (Hahn et al. 1995; Pullinger et al. 2002), the cholesterol homeostasis can also be maintained by other oxysterols. Furthermore, excessive dietary cholesterol can also suppress cholesterol biosynthesis in the liver (Accad and Farese 1998). Among the different products of cytochrome P450-mediated hydroxylation of different carbons in the cholesterol structure (Fig. 2), the 27-hydroxylation product, 27-oxysterol, mediates the reverse transfer of cholesterol to the liver (Björkhem et al. 1994), while 4β-hydroxylation catalyzed by CYP3A4 may indicate a slow elimination of cholesterol (Bodin et al. 2002).

Plant sterols can contribute to the maintenance of cholesterol homeostasis, and their levels in the blood reflect cholesterol absorption (Miettinen et al. 1990; Seo and Choi 2015). Sitosterolemia, which is caused by gene mutations in the ATP-binding cassette subfamily G5 or G8 (*ABCG5* or *ABCG8*), results in the increased intestinal absorption of plant sterols, leading to severe hypercholesterolemia and intertriginous xanthomas (Berge et al. 2000). It is one of the rare diseases in hyperlipidemia characterized by excessive reduction in whole-body cholesterol biosynthesis (Miettinen 1980). However, the immunoassays used in clinical practice cannot distinguish the increased levels of

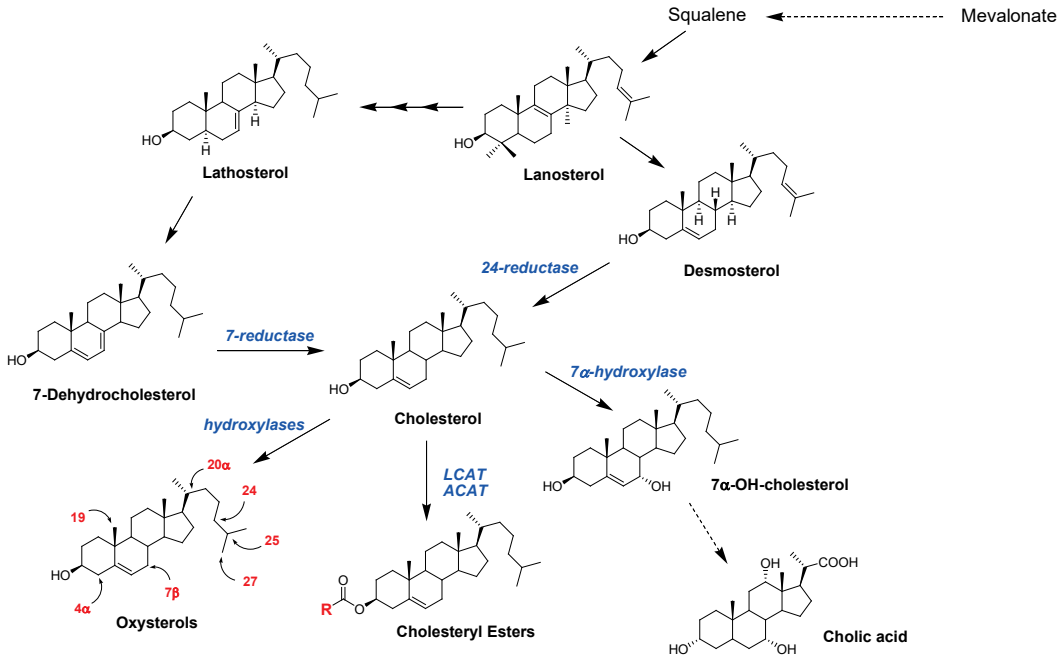

Fig. 2. Cholesterol biosynthesis and other related metabolic pathways.
LCAT, lecithin-cholesterol acyltransferase; ACAT, acyl CoA:cholesterol acyltransferase.

sitosterol, which is the most abundant plant sterol, from cholesterol due to their structural similarity. The MS-based assay can be used to identify patients with extremely higher levels of blood sitosterol in routine screening of steroid profiles, which can also be used to ensure normal range of patient's LDL-cholesterol level (Park et al. 2014).

Adrenal corticosteroids

Pregnenolone is a metabolic intermediate synthesized from cholesterol, via two other intermediates, 22*β*-hydroxycholesterol and 20*α*, 22*β*-dihydroxycholesterol, catalyzed by cholesterol side-chain cleavage enzyme (cytochrome P450scc) located in the mitochondria. It is sequentially converted into steroid hormones in the adrenal glands and gonads (Fig. 3), regulated by anterior pituitary tropic hormones, such as adrenocorticotropic hormone (ACTH), follicle-stimulating hormone, and luteinizing hormone. The outer cortex of adrenal glands is subdivided into three layers that produce specific steroid metabolomes: zona glomerulosa secreting mineralocorticoids, zona fasciculate synthesizing glucocorticoids, and zona reticularis producing adrenal androgens.

The synthesis and secretion of the major glucocorticoid in humans, cortisol (also known as hydrocortisone) is regulated by ACTH, which in turn is regulated by the hypothalamic corticotropin-releasing hormone. Abnormal metabolic functions leading to hypercortisolism and hypocortisolism can result in Cushing's syndrome and Addison's disease, respectively. Theoretically, the significantly increased cortisol levels in the body can be used to diagnose Cushing's syndrome, which is a complex procedure entailing 24-h urinary, late-night salivary and dexamethasone-suppression tests, to evaluate free cortisol concentration (Loriaux 2017; Eisenhofer et al. 2018). In addition, the differential diagnosis of the subtypes of Cushing's syndrome requires multiple steps. In contrast, the MS-based profiling methods can be conducted to define the particular subtype of Cushing's syndrome as well as other adrenal diseases in a single run (Phillipou 1982; Hines et al. 2017; Loriaux 2017; Eisenhofer et al. 2018). Compared with healthy subjects, patients with Cushing's syndrome show significantly increased serum levels of cortisol precursors, 11- and 21-deoxycortisols, while decreased levels of aldosterone were found in both ectopic and pituitary diseases. In addition to lower levels of cortisol, LC-MS-based assay showed extremely low levels of androstenedione and decreasing serum testosterone concentrations with age in Addison's disease indicating hypocortisolism (Kao et al. 2001; Methlie et al. 2013).

Primary aldosteronism, caused by adrenal hyperplasia or tumors, is the most common cause of secondary hypertension, and is characterized by an excess of the mineralocorticoid aldosterone, resulting in low renin levels. The patients also show excess glucocorticoids, with significantly increased cortisol levels, in addition to frequent glucocorticoid co-secretion (Arlt et al. 2017). The synthesis of the hybrid steroid 18-oxocortisol from cortisol is catalyzed by aldosterone synthase (CYP11B2), and its plasma levels determined via adrenal vein sampling (AVS) are significantly higher in aldosterone-producing adenoma than in both control and idiopathic hyperaldosteronism (Nakamura et al. 2011). The peripheral levels of 18-oxocortisol can also be used to discriminate unilateral adenoma from bilateral diseases in primary aldosteronism (Satoh et al. 2015),

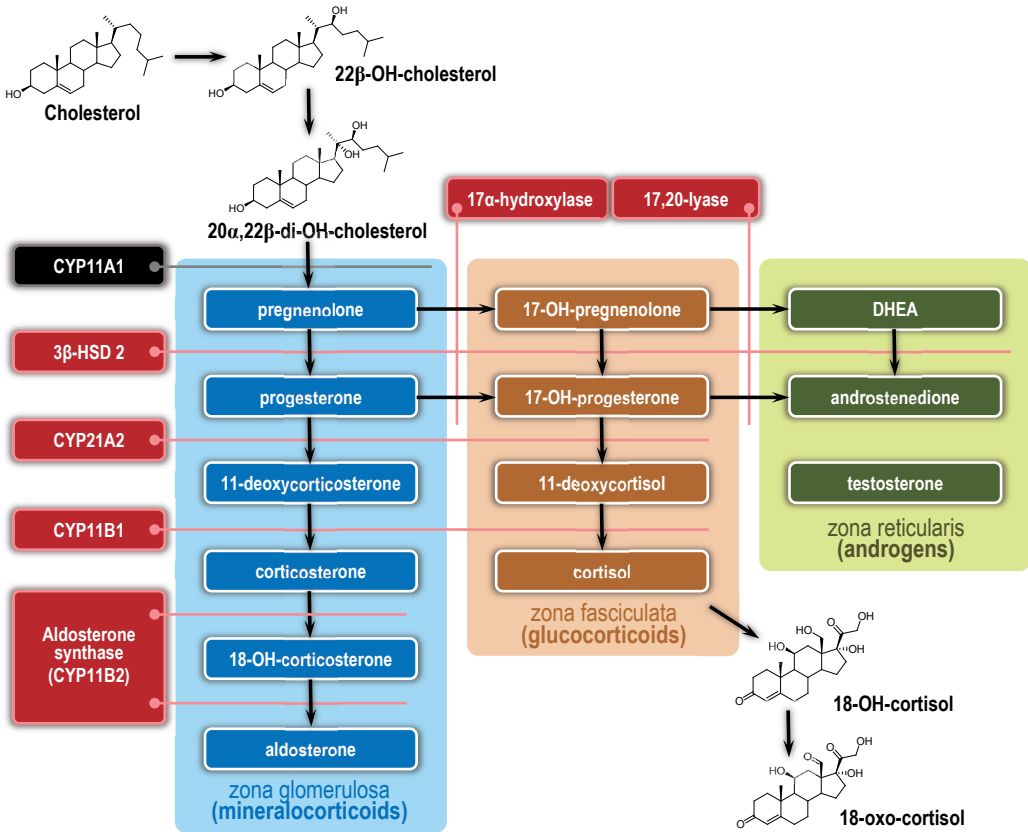

Fig. 3. Metabolism of adrenal steroids. The three different layers of adrenal cortex including zona glomerulosa, zona fasciculata, and zona reticularis synthesize and secrete mineralocorticoids, glucocorticoids, and androgens, respectively. CYP, cytochrome P450; HSD, hydroxysteroid dehydrogenase; DHEA, dehydroepiandrosterone.

which may avoid unnecessary surgery for nonfunctioning adrenocortical nodules concurrent with hyperplasia or microadenoma. Serum 18-hydroxycorticosterone, and urinary 18-hydroxycortisol and 18-oxocortisol were also identified as good diagnostic biomarkers for primary aldosteronism (Mulatero et al. 2012). The MS-based profiling combined with AVS resulted in the identification of 13 adrenal steroids, including aldosterone and cortisol, and represents a powerful clinical tool for the evaluation of patients with primary aldosteronism (Peitzsch et al. 2015).

Congenital adrenal hyperplasia (CAH) encompasses enzyme deficiencies associated with adrenal steroidogenesis resulting in impaired biosynthesis of corticosteroids and androgens. For example, elevated levels of 17-hydroxyprogesterone (17-OHP) are detected in 21-hydroxylase (CYP21A2) deficiency, which is the most common type of CAH (El-Maouche et al. 2017). Measurement of 17-OHP levels in dried blood spot (DBS) is globally used in clinical practice, but the immunoassay often generates false-positive outcome (Janzen et al. 2007) due to the presence of other steroids with similar chemical structure. Although a strong correlation was observed between serum 17-OHP levels measured by immunoassay and MS-based DBS analysis (Birkebaek et al. 2017), evaluating a single compound 17-OHP using these techniques is not confined to pediatric cases due to its production by the gonads and in other conditions, including prematurity. Therefore, a robust and selective MS-based profiling of multiple adrenal steroids is required to improve diagnostic sensitivity as well as monitor other types of deficiencies associated with 11β-hydroxylase, 17α-hydroxylase, and lipoidal CAHs (Saenger et al. 1995; Peter et al. 2008; Rossi et al. 2010; Fiet et al. 2017).

Sex steroids

Gonadal androgens, which are mainly derived from the gonads, are distinct from adrenal androgens. They include progesterone, testosterone, and 17β-estradiol representing progestagens, androgens, and estrogens, respectively. Metabolic changes in sex steroids are caused by endogenous and exogenous factors, and the homeostatic control or reproductive development may be altered by endocrine-disrupting chemicals and anabolic steroids. Their metabolic dysfunction can lead to precocious puberty in children and hypogonadism in adults (Diamanti-Kandarakis et al. 2009; Marques-Pinto and Carvalho 2013; El Osta et al. 2016). Precocious puberty is caused by abnormal hypothalamic or pituitary function in case of gonadotropin-dependent central precocious puberty and defective steroidogenesis during secondary sexual develop-

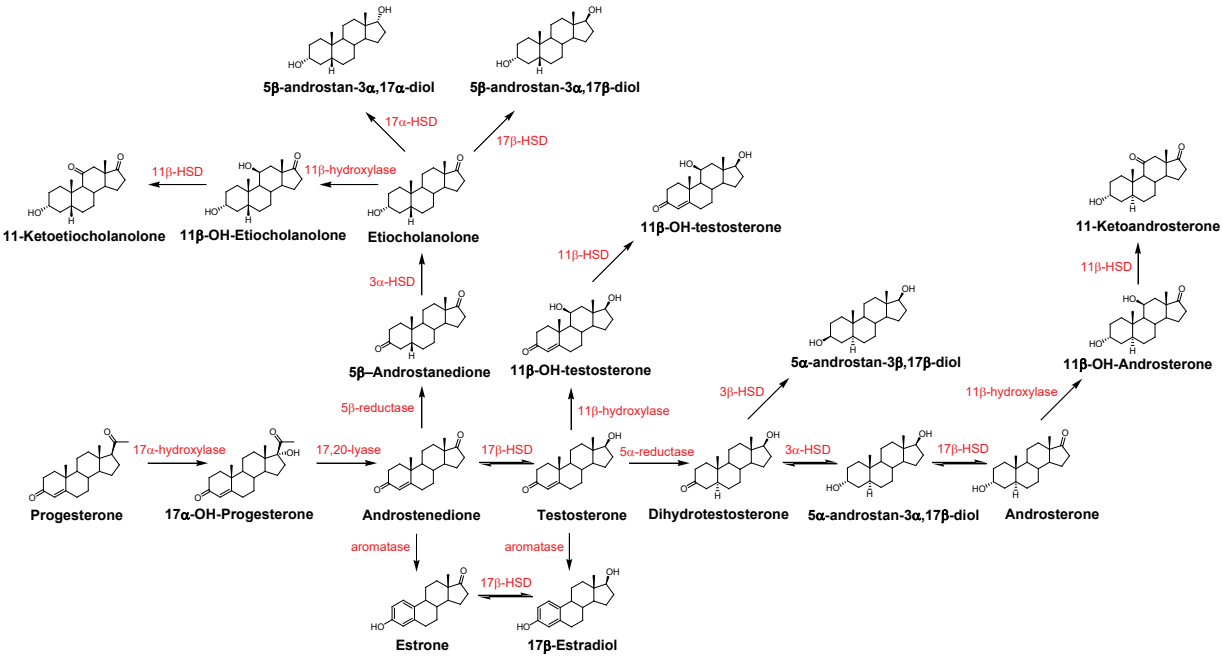

Fig. 4. Metabolic pathways of androgens and estrogens.
HSD, hydroxysteroid dehydrogenase

ment in case of peripheral precocious puberty. Androgens and estrogens play pivotal roles in pubertal onset and the prevalence of precocious puberty is significantly higher among girls. The serum levels of 17*β*-estradiol in prepubertal boys were found to be extremely low, whereas a significant increase was observed in prepubertal girls, along with higher levels of testosterone metabolites (Courant et al. 2010). Urinary 17*β*-estradiol was increased in the central precocious puberty of girls compared with that of peripheral precocious puberty and control girls (Lee et al. 2014). Interestingly, the urinary concentrations of active sex steroids, testosterone and 17-estradiol, were significantly increased among individuals with high bisphenol A levels, irrespective of both type and onset of prepuberty (Lee et al. 2014), which is still controversial (Durmaz et al. 2014; Özgen et al. 2016). Serum levels of testosterone and its oxidation products (Fig. 4), 11-hydroxytestosterone and 11-ketotestosterone, were also significantly increased in premature adrenarche compared with age-matched girls, while testosterone and 11-ketotestosterone were increased under normal onset of adrenarche (Rege et al. 2018).

Anabolic androgenic steroids have mainly been investigated in doping tests to determine their possible abuse by athletes to enhance muscular strength and size (Strauss and Yesalis 1991). These steroids enhance muscle regeneration and function after injury (Lynch et al. 2008). However, they also show deleterious side effects, including hypertension, depression, testicular atrophy and infertility that are poorly understood. In recent years, anabolic steroid-induced hypogonadism was identified (Rahnema et al. 2014) and male infertility was mainly determined by semen analysis, which is complicated and very invasive. Testosterone contributes to the maintenance of lean mass, fat mass, strength, and sexual function, which vary widely in men (Finkelstein et al. 2013). Measuring serum testosterone levels may be an alternative strategy to monitor infertility in men; however, both seasonal and diurnal variations make it difficult to do so in clinical practice (Kempenaers et al. 2008). In addition, cross-reaction with testosterone metabolites and interference of abundant DHEA limits the use of immunoassays in monitoring testosterone (Middle 2007; Rosner and Vesper et al. 2010).The proposed MS-based analytical methods are sensitive and specific enough to differentiate eugonadal from hypogonadal men (Wang et al. 2004). Technical advances in MS-based androgen analysis are adequate for quantifying the trace levels of serum testosterone in women and children (Kushnir et al. 2006), as well as in men with chemical castration (Ko et al. 2016).

Conclusion

Despite its wide-ranging industrial and scientific applications, the MS-based assay is a relatively new clinical and laboratory technique, and is an emerging and promising tool to effectively address the healthcare needs of patients. MS-based multiplexed panels can efficiently support diagnosis and monitoring of different clinical outcomes. The LC-MS assay is preferred in routine analysis based on simple sampling procedures, such as dilution and protein precipitation, while GC-MS requires minimal purification steps. Both GC- and LC-MS are complementary because the advantages of one may offset the limitations of the other technique. Chemical derivatization can overcome potential drawbacks in the GC-MS assay, and provide better volatil-

ity and stability in GC separation, as well as enhance the ionization efficiency and MS interpretation in both quantitative and qualitative GC- and LC-MS analyses (Moon et al. 2011; Marcos and Pozo 2015; Wang et al. 2015). In particular, MS-based assay was expressed as the MVP of endocrine research (Endocrine News, March 2015); however, it can be improved to ensure superior detection via optimal sample purification and chromatographic separation methods to overcome the challenge due to structurally similar steroid hormones in the body (Moon et al. 2016; Choi 2018).

Metabolomics can be used to assess multiple metabolites in various clinical fields and offer potential biomarkers with diagnostic sensitivity and specificity. The analytical techniques used in metabolomics include non-targeted and targeted metabolite profiling approaches through qualitative and quantitative analyses, respectively. In general, non-targeted metabolite profiling increases the probability of identifying unknown biomarkers. However, most steroid hormones exist at trace levels, which are insufficient to be identified and semi-quantified. To address this issue, database-dependent metabolite profiling of 232 steroids was introduced (Jung et al. 2010), and recent advances in large-scale steroid profiling have been developed, which are not just focused on specific functional groups of steroids alone (Moon et al. 2009; Hána et al. 2019). For example, hypertensive physiology may be closely associated with adrenal and sex steroids, and not merely cholesterol metabolism (Muller et al. 2003; Suzuki et al. 2003; Walker 2007). Therefore, a large-scale overview of steroid metabolism may provide comprehensive insights to identify potential biomarkers as well as develop patient screening programs in addition to the currently used clinical steroid protocols.

MS-based analytical platforms in clinical practice are limited by the reduced sample size for automated high-throughput system, suggesting the need for improved analytical sensitivity for pathological confirmation using biopsy specimens. Surrogate materials for reproducible quantification should be further developed to provide a cutting-edge technology for precision and personalized medicine. In addition to biomarker discovery based on MS-based profiling, immunoassays and other technical advances (Hong et al. 2017; Lee et al. 2019) should be used in parallel as complementary tools for large-scale population screening in the future.

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Conflict of Interest

The author declares no conflict of interest.

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