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Title:

"Pathophysiological and diagnostic evaluation of andrological and endo-gynaecological conditions through innovative analytical methods investigating androgen milieu"

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Introduction:

Steroids:

Steroids are four-ringed organic molecules which act as endocrine hormones and as functional components of cell membranes. Steroids are primarily produced in the adrenal cortex and the gonads. Other secondary body sites that also are involved in steroid biosynthesis and metabolism are, among the others, the adipose tissue, epidermis, liver, prostate and the brain (Porcu et al., 2016)(Schiffer et al., 2019).

Their function is mediated through their interaction with intracellular receptors, and their physiological activities are relevant throughout the adult lifespan. In fact, they control a broad variety of processes including sodium, potassium and water balance, blood volume and pressure, module inflammation and responses to illnesses and stress, development and maintain of sexual characteristics and sexual function (Miller et al., 2011).

The pathway to produce steroids starts from cholesterol. Cholesterol sources can be both endogenous and exogenous. The exogenous uptake starts from the diet; in fact, after emulsification by bile acids, the enterocytes of the small intestine are able to absorb cholesterol and package it into chylomicrons that can travel through the bloodstream up to the cells. Endogenous sources are represented by a de novo biosynthesis, derived from acetate in the endoplasmic reticulum (Rittenberg & Bloch, 1945), or an uptake from a different source. In fact, the hydrolysis of cholesterol esters stored in lipid droplets, cholesterol esters from LDL and HDL cholesterol, and free cholesterol residing in the plasma membranes, where it guarantees their structure and functions (Azhar et al., 2003). The adrenal cortex and gonads can biosynthesize cholesterol de novo under the regulation of tropic hormones, using plasma lipoproteins as the predominant source of cholesterol for steroid biosynthesis (Kraemer, 2007). Furthermore, cholesterol, through an enzymatic pathway that is common between the aforementioned production sites, it is able to be further modified to produce site-specific steroid hormones.

Steroid biosynthesis:

Steroidogenesis is a procedure that aims to produce de novo steroids. The steroid pathway (figure 1) to produce different steroid hormones is highly conserved between species and shared among the endocrine glands; however, different hormone productions characterize the different sites. This variability happens due to the expression of distinct converting enzymes (Payne & Hales, 2004).

Two major classes of enzymes are involved in this biosynthetic process, namely the cytochrome P450 (CYP) and hydroxysteroid dehydrogenase (HSD). CYP are heme-containing enzymes which utilize NADPH as an electron donor. Considering steroidogenesis, hydroxylation, which is the introduction of a hydroxyl group (–OH) into an organic compound, and C–C bond cleavage are considered the most relevant reactions (Guengerich & Yoshimoto, 2018). These CYP enzymes can be divided into two groups based on their intracellular location and mode of electron transfer:

- CYP type I enzymes: located within the inner mitochondrial membrane, they are dependent on ferredoxin and ferredoxin reductase for the delivery of their electrons from NADPH. Ferredoxin reductase is a flavoprotein that oxidizes NADPH and transfers electrons to a small iron-sulfur protein, named ferredoxin, which acts as a mobile electron carrier;
- CYP type II enzymes: located in the endoplasmatic reticule, they are dependent on CYPs which acts as oxidoreductases for electron delivery. These oxidoreductases contain a flavin adenine dinucleotide and mononucleotide that allow the enzyme to oxidize NADPH and reduce the CYP enzyme in a stepwise manner (Lin et al., 1993; Zöllner et al., 2008).

The other main functional class of enzymes involved in steroidogenesis are the HSD; they are also dependent on NADPH and NADP as co-factors (Chatuphonprasert et al., 2018). HSDs belong to the short-chain alcohol dehydrogenase reductase enzyme superfamily and are subdivided into the short chain dehydrogenases and the aldo-keto reductases (AKR). HSD enzymes catalyze the conversion of a hydroxysteroid to its corresponding ketosteroid counterpart and vice versa, and in doing so, regulate their binding to steroid receptors (Penning et al., 2019). Even though the majority of these reactions are reversible, a prominent directionality is normally observed. In fact, the two HSD3B isoforms, HSD3B1, expressed in the placenta and peripheral tissue, and HSD3B2, located in the adrenal and gonadal glands, catalyze an irreversible reaction. They convert the hydroxy-group (-OH) on carbon 3 of a steroid metabolite to a keto group (C=O), also named dehydrogenation: e.g to convert pregnenolone into progesterone, 17α-Hydroxypregnenolone into 17α-hydroxyprogesterone (17OHP), Dehydroepiandrosterone (DHEA) into androstenedione, androstenediol to testosterone and Androstadienol to androstadienone; furthermore, they isomerize the double bond between the carbon atoms in C5 and C6 to C4 and C5; this reaction is also named isomerization from Δ5 to Δ4, and namely irreversible (Luu The Van et al., 1991; Thomas et al., 1988).

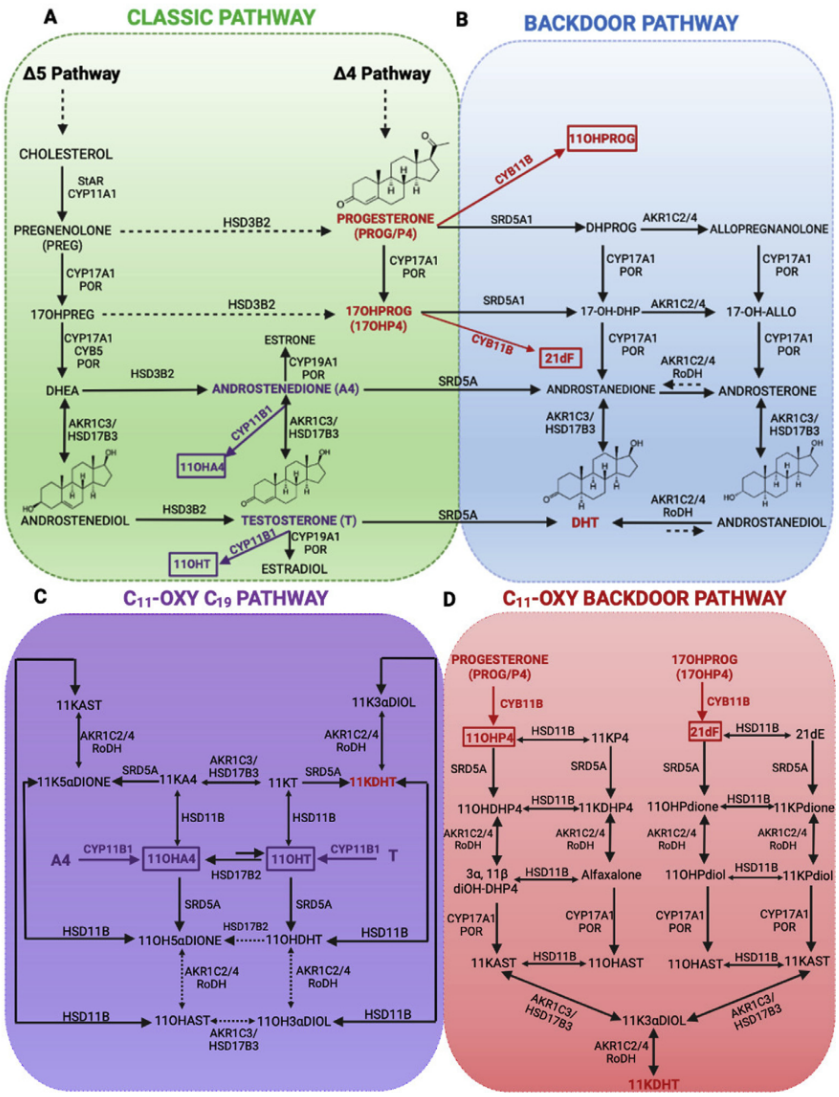
The main steroid production sites are the adrenal glands and the gonadal glands.
Adrenal glands are paired glands located in the abdomen. Their etymology derives from their anatomical position, which is above the kidneys (*ad-renal*). These glands are divided in an outer area, the cortex and an inner one, the medulla. The cortex is responsible for steroid hormones production, and it is further separated into three different zones; each of these presents different enzymes and therefore produces distinct hormones (Miller, 2017). The outermost area is called zona glomerulosa, which expresses the hydroxylase enzyme CYP11B2, also called aldosterone synthase, to produce aldosterone. The centrally located zona fasciculata specifically expresses a closely related homologous, the CYP11B1 enzyme, also called 11β-hydroxylase, to produce the glucocorticoid steroid cortisol. The innermost zona reticularis produces the adrenal androgens DHEA, DHEA-sulphate (DHEAS), androstenedione, and testosterone via the enzymes 3βHSD3, which catalyze a dehydrogenation, AKR1C3/17betaHSD which acts as a dehydrogenase and induce an isomerization, and Sulfotransferase 2A1, which adds a free sulphate (Miller & Auchus, 2011).

Through the biosynthetic pathway, other various enzymes modify chemical groups and side chains across the four-ringed structure, giving each steroid intermediate or final product a proper specificity. Mutations in the genes encoding key biosynthetic enzymes, such as 21α-Hydroxylase or 17α-Hydroxylase, can cause common endocrine conditions such as congenital adrenal hyperplasia (Miller, 2018).

The other two main sites of steroid production are the male and female gonads, testicles and ovary, respectively. Testicles are paired organs located in the scrotum, outside the body where they maintain a temperature of 34°C to optimally work. The main steroid that is produced and secreted is testosterone; the biosynthetic machinery belongs to specialized testicular cells that are located in the interstitium of the testicles, called Leydig cells. Differently from the cells in the zona reticularis, which are regulated at a hypothalamic-pituitary level by CRH and ACTH, Leydig cells are sensitive to GnRH-LH stimulus. This is coupled with the abundant expression of 3βHSD2 and 17βHSD3 to catalyze the various steroidogenic reactions (figure 2). This results in a high production of DHEA, which is not sulphated and can be therefore metabolized to Testosterone (Flück et al., 2003)(Miller and Auchus, 2013).

On the other hand, ovarian steroidogenesis presents greater complexity. Ovaries are paired organs located in the pelvis. The enzymatic machinery is divided between two different cell types, theca and granulosa cells which surround the follicle during oogenesis of the ovarian cycle. The main products of this pathway, in fact, are estradiol and progesterone which are produced at different magnitudes during each phase of the ovarian cycle (figure 2). Steroidogenesis begins in the theca cells under LH control, where cholesterol is converted into pregnenolone and subsequently into androgens (androstenedione and testosterone). These androgens diffuse into the granulosa cells, which, under FSH stimulation, express aromatase (CYP19A1) to convert androstenedione into estrone and estradiol. Granulosa cells lack the enzymes CYP11A1 and CYP17A1 required for direct androgen production. Additionally, a small proportion of androstenedione in the theca cells is converted to testosterone by AKR1C3/17βHSD5 (Miller & Auchus, 2011; Voutilainen et al., 1986).

Figure 1. Steroid pathways



Physiological activities:

Considering their hydrophobic nature, steroids cannot circulate freely in the bloodstream to reach the target organs. They need to be bound to carrier plasma proteins, such as Sex Hormone Binding Globulin (SHBG) and albumin; when they reach target cells, they can diffuse across cell membranes, due to their similar polarization, to reach the cytoplasm and therefore activate specific intracellular hormone receptors (Rosner et al., 2010).

Among their main activities, steroids are widely recognized to be involved in development, growth, reproduction and homeostasis. The most renowned steroids with their activities are enlisted in Table 1. They include the adrenal steroids cortisol and aldosterone, the adrenal androgen precursors 17OHP, DHEA and DHEAS, and the reproductive steroids estradiol, testosterone, and dihydrotestosterone (DHT). While the steroid production pathway reaches the final step, a large number of intracellular steroid intermediates, some of which are now thought to potentially have physiological roles in abnormal states or in complex genetic

diseases. In fact, over the last years, research is shedding a light to 11-hydroxy and 11-ketosteroids that seem to have a more important role than once thought, in particular in androgen related conditions such as polycystic ovary syndrome (PCOS) (Turcu et al., 2020).
Once crossed the cell membrane steroids bind to, and therefore activate, nuclear receptors. Each steroid hormone can activate a very restricted number of receptors, often only one specific nuclear receptor protein. Nuclear receptors are intracellular protein derived and ligand-dependent transcription factors that exert their effects by binding directly to specific sites near genes in genomic DNA (Alexander et al., 2019). All nuclear receptors share a common structure consisting of a N-terminal domain, a DNA binding domain with a two zinc fingers motif, a hinge region to grant flexibility, ligand binding domain, which is hydrophobic and is suitable for steroids and a C-terminal domain that is the most variable zone, deepening on the specific nuclear receptors. In fact, the nuclear receptors are classified into two subgroups:

- The first, which contains the steroid receptors, such as androgen (AR), glucocorticoid, mineralocorticoid, estrogen and progesterone receptors, is located in the cytosol; when it is inactive it is linked to heat shock proteins. When they bind a steroid ligand, they translocate into the nucleus to regulate gene expression (Kumar et al., 2006).
- The type II nuclear receptors, such as thyroid hormone receptors, are located in the nucleus and are already bound to specific sequences of the DNA, ready to be activated (Gronemeyer et al., 2004).

Traditionally, steroids have been thought to exert their actions by binding to classic nuclear receptors located within cytoplasm, which then translocate into the nucleus and regulate gene transcription over a period of hours to days. Indeed, many steroid nuclear receptors and several novel steroid receptors have been found at the plasma membrane, where they are considered to engage with signaling proteins (Foradori et al., 2008). The impact of steroids on membranes of nervous system cells has also led to suggestions that ‘neurosteroids’ may need to be regarded more as neuromodulators, or transmitters in addition to hormones (Rudolph et al., 2016).

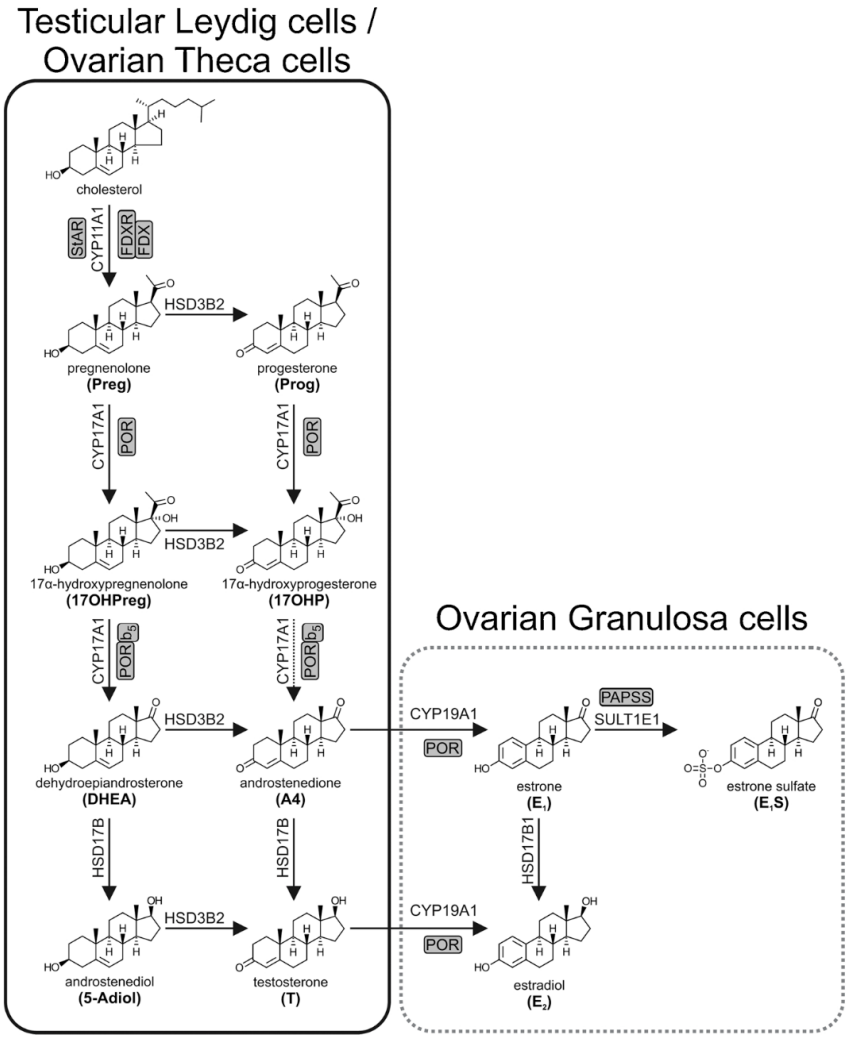


Figure 2. Testicular and ovarian steroidogenesis.

Steroid metabolism

Once produced, steroids go into the bloodstream to reach the target organs and to exert their action into the cells. It has to be considered that active steroids are continually modified by other enzymes, particularly in the liver, to produce less active steroid metabolites and foster their excretion. This also makes the molecule more hydrophilic, therefore easier to be metabolized and being excreted by kidneys and in the bile.

These reactions are addressed as phase 1 and phase 2 metabolism; while the former add or reveal functional groups of the steroid molecule, the latter are conjugation reactions that increase polarity, which also restrict the diffusion across the membranes, and water solubility. Classically, phase 1 and 2 reactions are known to be sequential, but more recent findings are making this concept evolving. In fact, Testosterone can be directly conjugated at its 17β-hydroxy group and corticosteroids through a 21-sulfation (Schiffer et al., 2019). These reactions are mainly performed in the liver, but also peripheral organs are able to play a role into these conversions. This is to preserve the tissue specific needs, a concept also known as “intracrinology”.

Phase 1 steroid metabolism

Phase 1 reactions are: reduction of the 3-keto-Δ4 motif, the interconversion of hydroxy- and keto-groups by HSDs/oxoreductases and additional hydroxylations by CYPs.

Steroid A-ring reduction

The inactivation of gluco- and mineralocorticoids occurs through the reduction of ring A in the four-ring structure characteristic of steroid molecules. This process also facilitates the peripheral activation or inactivation of androgens. Two sequential reductions are required: first, the reduction of the Δ^4 double bond (C=C), followed by the reduction of the 3-keto group (C=O) to a hydroxyl group (-OH). These steps generate a 3α/β-hydroxy-5α/β-H motif, which is a common feature of excreted metabolites. Importantly, the 5α or 5β-reduction is irreversible, and the stereochemistry of this reduction plays a significant role in regulating the biological activity of androgens (Wilson, 2001).

The first step in this metabolic pathway can involve either 5α- or 5β-reduction. In the case of 5α-reduction, the reaction is catalyzed by steroid 5α-reductase (SRD5A) enzymes, which include three main isozymes. SRD5A1 is primarily expressed in the liver and peripheral tissues, whereas SRD5A2 is predominantly found in male reproductive and genital tissues (Penning et al., 2019). SRD5A3, although it exhibits minor steroid 5α-reductase activity, plays an essential role in N-linked protein glycosylation. Steroid 5β-reduction, on the other hand, is catalyzed by aldo-keto-reductase family member AKR1D1, which is primarily expressed in the liver. AKR1D1 is the only human enzyme capable of catalyzing the 5β-reduction of 3-keto-Δ^4 steroids and bile acids (Chen & Penning, 2014).

Due to their differential tissue expression patterns, SRD5A isoforms, which are widely expressed in peripheral tissues including the liver, contribute to a global view of metabolism, whereas AKR1D1 expression, which is restricted to the liver, provides insights primarily into hepatic reduction. Furthermore, the relative abundance of 5α- and 5β-reduced metabolites varies across different structural classes of steroids.

The second step in the reduction of the A-ring involves the conversion of the 3-keto group to a hydroxyl group. This reaction is catalyzed by members of the AKR family, specifically AKR1C1, AKR1C2, AKR1C3, and AKR1C4. Among these, AKR1C4 is considered a liver-specific enzyme that works in conjunction with AKR1D1 to yield 5β,3α-metabolites. The other isozymes are expressed in peripheral tissues in a tissue-specific manner (Jin & Penning, 2007).

Hydroxysteroid dehydrogenation and reduction

The interconversion of hydroxyl (-OH) and keto (C=O) groups (Fig. 3) at positions 11, 17, and 20 plays a significant role in regulating steroid activity through their interaction with specific receptors. These reactions are catalyzed by enzymes belonging to the short-chain dehydrogenase/reductase (SDR) superfamily and the AKR superfamily.

11β-Hydroxysteroid dehydrogenases

Two isoforms of HSD11B play a key role in regulating glucocorticoid inactivation and reactivation by catalyzing the interconversion of 11β-hydroxy- and 11-ketosteroids. Thereby, they modulate systemic and tissue-specific glucocorticoid action, regulation of mineralocorticoid and 11-oxygenated androgen activity (Tomlinson et al., 2004).

HSD11B1 is primarily a reductive enzyme that catalyzes the activation of cortisone to cortisol in tissues like the liver, brain, kidneys, and white adipose tissue, amplifying glucocorticoid signaling. Conversely, HSD11B2 functions exclusively as an oxidative enzyme, inactivating cortisol to cortisone and preventing cortisol from activating mineralocorticoid receptors.. Both isoforms are also involved in the metabolism of 11-oxygenated androgens and also act on 7-oxygenated C19 steroids, the former using a whereby HSD11B1 functions as an epimerase interconverting the 7α- and 7β-hydroxy-stereoisomers via a 7-keto-intermediate, while the latter oxidizes the 7α-hydroxy-stereoisomer intermediate (Chapman et al., 2013).

17β-Hydroxysteroid dehydrogenases

Enzymes from the SDR and AKR superfamilies regulate the activity of androgens and estrogens by interconverting active 17β-hydroxy- and inactive keto-forms. Therefore, excreted metabolites are predominantly in the keto-form. To date, 14 human enzymes with 17β-HSD/oxoreductase activities have been identified (Lukacik et al., 2006). Generally, these enzymes are multifunctional and often have overlapping substrate specificities and expression patterns, allowing for redundant enzymes to cover in case of deficiency of another enzyme. However, certain enzymes have been identified as milestones for specific androgen and estrogen metabolic reactions (Marchais-Oberwinkler et al., 2011).

20-Reduction

Both glucocorticoids and progesterone undergo 20-reduction, resulting in the formation of α- or β-stereoisomers before excretion. Importantly, the 20α-reduction of progesterone, primarily catalyzed by AKR1C1, effectively terminates its progestogenic activity. For glucocorticoids, 20-reduction is mainly observed in their downstream tetrahydro metabolites.

Cytochrome P450-catalyzed steroid oxidations

In addition to the well-characterized steroidogenic CYP enzymes, hepatic xenobiotic-metabolizing CYPs can modify steroid hormones, producing a wide variety of minor metabolites. These enzymes perform reactions such as hydroxylation, further oxidations, and even C-C bond cleavages. Unlike steroidogenic CYPs, hepatic CYPs are less selective in their substrate binding, accepting a broad range of steroids with low stereo- and regioselectivity. Consequently, multiple CYP enzymes can contribute to the same reaction, and variations in their expression levels make it challenging to pinpoint which enzyme predominantly drives a specific process. In total, at least 17 hepatic CYP enzymes are potentially involved in steroid metabolism.

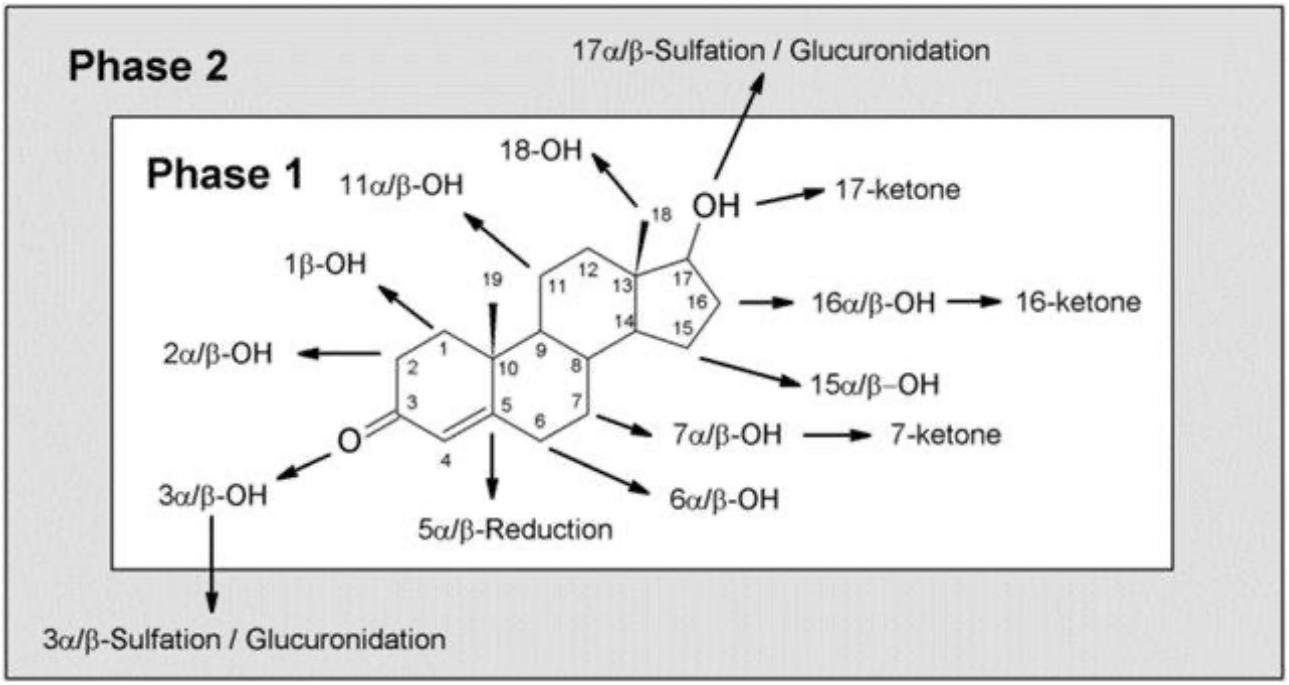
Hydroxylation

Among the phase I reactions that metabolize steroids, hydroxylation is one of the commonest. Hydroxylation aims to add a -OH group to an active C of the steroid. It induces an inactivation of the steroid and add a further functional site for phase 2 metabolism. The HSDs are also a large and important family of enzymes involved in steroid biosynthesis and inactivation.
Among Δ4 steroids, the hydroxylation of cortisol at the 6β position by CYP3A4 is well-known.
In Δ5 steroids like DHEA, hydroxylation typically occurs at the 16α and 7α positions in adults, while in neonates, hydroxylation at positions like 16β, 21, 18, and 15 is more common (Niwa et al., 2015).

C-C bond cleavage

CYP can also catalyze the cleavage of carbon-carbon (C-C) bonds in multi-step oxidative reactions. Examples include the side-chain cleavage of cholesterol by CYP11A1, a critical step in steroidogenesis and the 17,20-lyase activity of CYP17A1, which converts C21 steroids into C19 steroids. Hepatic CYPs use similar mechanisms to cleave 17α,21-dihydroxypregnanes.

Figure 3. Graphical schematization of phase I and II reactions.



Phase 2 steroid metabolism

In the liver, two main phase II reactions happen: sulphation and glucuronidation (Figure 3). These reactions aim to make steroids more hydrophilic and polar, guaranteeing an easier excretion in the bile and in the urine. In order to happen, these reactions need first to activate the functional group and secondly to transfer it from the donor to a hydroxy group of the

involved steroid. While the conjugated steroids are thought to be biologically inactive, this concept has not proven to be always confirmed (Schiffer et al., 2019).
It has also to be considered that sulfation is reversible, and sulphated steroids can be further hydrolysed to free steroids by sulfatases while glucuronidation is irreversible, apart from some gut bacteria activity (Mueller et al., 2015). Other minor phase II reactions are represented by methylation, cysteine or glutathione conjugation and fatty acids esterification.

Steroid sulphation and desulphation

The steroid sulfotransferases (SULT) enzymes are a large family of enzymes that transfer a sulphate molecule to the steroid ring from a donor molecule called 3'-phosphoadenine-5'-phosphosulphate. Their activities are pivotal in regulating the activity of Δ5 steroids and estrogens; in fact, important SULT enzymes include SULT2A1, which converts DHEA to DHEAS in the adrenal cortex, and SULT1E1, which sulphonates and inactivates estradiol for excretion (Falany, 1997).
Sulfation occurs in two steps: (1) activation of the inert sulphate anion to 3'-phospho-adenosine-5'-phosphosulfate (PAPS), that is a sulphate donor, cathalized by PAPS synthase, and (2) transfer of the sulphate group by SULTs on hydroxy or amino groups. Five cytoplasmic SULTs are involved in steroid sulfation in humans: SULT1A1, SULT2E1, SULT2A1, SULT2B1a and SULT2B1b. These enzymes have overlapping substrates (Vítků & Hampl, 2023).
Sulphation can also happen twice in a molecule. While bis-sulphates in steroid metabolomes were identified in the 1960s, renewed interest arose recently with advanced methods (Lightning et al., 2021). Importantly, sulphation is reversible, as steroid sulphatases regenerate unconjugated bioactive steroids; they are expressed ubiquitously, and can be upregulated in steroid-dependent cancers, making it a potential drug target (Wang et al., 2024).

Steroid Glucuronidation

Glucuronidation plays a significant role in Δ4 steroid metabolism. UDP-glucuronic acid serves as the activated donor molecule for this process. The glucuronic acid is coupled to a steroid hydroxy group, preserving stereochemistry in the respective position (Mackenzie et al., 1992). UGT-glucuronosyltransferase enzymes of the UGT1A and UGT2B subfamilies, are expressed in the liver and extrahepatic tissues, and catalyse this process. A-ring-reduced steroid metabolites are primarily excreted as 3-glucuronides. Additionally, linked di-glucuronides and bis-glucuronides may form (Argikar, 2012).

Minor Phase II reactions:

O-methylation of catechols represents the only reaction which reduces water solubility and is the grounding reaction for phase 2 estrogen metabolism and is catalysed by catechol-O-methyltransferase (COMT). COMT methylates 2- and 4-hydroxyestrogens, forming methylated derivatives. S-adenosylmethionine represents the donor molecule composed by methionine and ATP. This enzyme works mainly in the liver, brain, kidney adrenal glands and lungs (Bastos et al., 2017).

New discovered pathways that are included in phase 2 reactions are the cysteine conjugates of androgens, cortisol and progesterone, both in urine and plasma. A dehydrogenation is followed by a glutathione S-conjugation and subsequent extracellular degradation to a cysteine (Fabregat et al., 2014).
Fatty acid esterification of pregnenolone, DHEA and androstenedione has also been described. Acyl-CoA acts as a donor to acylate steroids from HDL cholesterol which has plasma lecithin:cholesterol acyl transferase; furthermore, this activity can be exerted also in peripheral tissues (Hochberg et al., 1991).

Steroid excretion

Urinary excretion:

Eighty percent of steroids conjugates are excreted in the urine, while the rest is excreted in the bile. Excretion of glucuronides is usually faster than the sulphates, mostly due to the irreversibility of glucuronidation. When the conjugated steroids reach the nephron from the bloodstream, an organic anion symport is used to transport the molecules. In addition, a carrier and an electrochemical gradient are used to concentrate steroids in the lumen. Excreted unconjugated steroids are low, accounting for the 5-10% of the total urine steroid pool. Some enzymatic activations also happen in the urinary tract such as 20b HSD and 17,20 lyase (Eriksson & Gustafsson, 1972).

Bile excretion

Biliary excretion, and subsequent faecal excretion, is related to the quota that gut microbiota is able to participate in the metabolism of the respective steroid. In fact, excretion with faeces is low due to the high resorption rate of steroids in the gut. Steroid conjugates are mostly excreted in the bile, in particular bis-sulphates liver derived steroids. With more specificity, in the bile we can usually find 17-deoxysteroids, such as mineralocorticoids, as opposed to 17-hydroxy C21 steroids like cortisol. The steroids that are not reabsorbed are excreted in the feces. This quota comprehends unconjugated (mostly), mono- and bis-sulphated steroids. Gut microbiota expresses a hydrolase activity for conjugated steroids and this could play a role also in steroid reabsorption (W. Taylor, 1971).
Estrogens are particularly not excreted in feces, because they are highly excreted in the bile but also highly reabsorbed (CANTAROW et al., 1942).

Salivary excretion

Unconjugated steroids passively diffuse over the membrane of acinar cells and the ratio that we can find highly reflects the serum concentrations. This is the rationale behind the use of salivary sampling for steroid assessments. However, steroid metabolism can happen also in the salivary glands, such as the presence of HSD11B2 in the parotid gland. While free steroids spread in the saliva independently from the salivary flow rate, the conjugated steroids are highly flow rate dependent (Lewis, 2006).

Overview of the main steroids produced in humans:

Corticosteroids:

Cortisol is a C21 steroid that acts as a crucial mediator of the systemic stress response and plays significant roles in various physiological contexts throughout fetal and adult life (Sapolsky et al., 2000). Cortisol levels in the bloodstream follow a circadian rhythm, peaking in the morning and declining in the evening. Its release from the adrenal glands is tightly regulated by direct negative feedback to the hypothalamus and anterior pituitary, inhibiting CRH and ACTH, respectively.

The zona fasciculata in the adrenal gland is the predisposed site to cortisol production. Starting from pregnenolone, the CYP17A1 and HSD3B2 convert this substrate to 17OHP. CYP21A2 is requested to subsequently convert it into 11-deoxycortisol and therefore CYP11B1 converts it into cortisol (Alemany et al., 2022).

In the brain, cortisol is central to the stress response. Stressors like trauma, infection, fear, or pain activate the hypothalamus-pituitary-adrenal axis to release cortisol (Sapolsky et al., 2000). Elevated cortisol serves two primary roles:

1. Preparing the body for a response to the stressor.
2. Modulating downstream processes to manage the stress response effectively.

The final result is an augmented gluconeogenesis, increased heart rate, and enhancing neural awareness and acuity, and suppressive effects on the immune system, with the reduction of anti-inflammatory responses. Considering the pivotal effects on the immune system, and the development of synthetic glucocorticoids like dexamethasone and prednisolone over 50 years ago has made them cornerstone treatments for numerous inflammatory and autoimmune conditions (Barnes, 1998).

As catabolic hormones, glucocorticoids play a central role in metabolic regulation. They promote protein breakdown to release amino acids for hepatic gluconeogenesis, glycogen breakdown, and the mobilization of fatty acids from adipose tissue. However, elevated cortisol levels, whether due to endogenous overproduction (as in Cushing's syndrome) or synthetic glucocorticoid use, can result in excess hepatic glucose output, leading to hyperglycemia and diabetes. Chronic high cortisol levels can result in systemic effects characteristic of Cushing's disease, including adipose redistribution, hyperglycemia, type-2 diabetes, hypertension, muscle wasting, and osteoporosis.

These effects are mediated through the widespread expression of the glucocorticoid receptor, in contrast to the more restricted expression of the mineralocorticoid receptor (Lu & Cidlowski, 2006).

Mineralocorticoids:

Enzyme expression in the zona glomerulosa of the adrenal glands is tailored to produce the C21 mineralocorticoid, aldosterone. The main role of the aldosterone is the regulation of solute and fluid homeostasis, primarily in the kidney and colon (Rogerson & Fuller, 2000). In the collecting duct of the kidney, aldosterone promotes the reabsorption of sodium, excretion of

potassium, water uptake, and therefore increased fluid volume, thereby maintaining systemic blood pressure.

Synthesis and release of aldosterone from the adrenal gland is tightly regulated by the renin-angiotensin system. The renin-secreting juxtaglomerular cells of the kidney sense volume, and low volume (with low sodium) stimulates secretion of renin, a protease enzyme, that cleaves circulating angiotensinogen to release angiotensin peptides, thereby stimulating the adrenal zona glomerulosa to synthesize and secrete aldosterone (Hattangady et al., 2012). Elevated plasma potassium is also able to directly stimulate aldosterone release from adrenal glomerulosa cells.

The synthesis starts from the conversion of cholesterol to pregnenolone (CYP11A1 mediated) and subsequent production of the Δ4 steroid progesterone. This represents the substrate to produce 11-deoxycorticosterone by CYP21A2. Zona glomerulosa cells are enriched with these two enzymes and lack CYP17A1 (Coulter, 2005; Suzuki et al., 2000); this ensures that all the biosynthetic process is directed to aldosterone production. Two isoforms of CYP11B are expressed in the zona glomerulosa, both with the ability to catalyse the 11β-hydroxylation of 11-deoxycorticosterone yielding corticosterone. CYP11B2, which is also known as aldosterone synthase, additionally exhibits 18-hydroxylase and 18-methyl oxidase activity, which are required to convert corticosterone to aldosterone via the 18-hydroxycorticosterone intermediate (Kawamoto et al., 1990).

The actions of aldosterone in collecting duct cells (and other epithelia) are mediated by the mineralocorticoid receptor, a member of the nuclear receptor superfamily. This receptor, however, is also activated by the related steroid cortisol, which normally circulates at much higher concentrations in the bloodstream. However, inappropriate activation by cortisol is prevented by the presence of the enzyme 11βHSD2, which converts cortisol to inactive cortisone, thereby preventing its activation (Funder et al., 1988) . Very high levels of cortisol, such as in conditions like Cushing's disease, can overwhelm 11βHSD2 and cause inappropriate hypertension. Aldosterone may also have other functional roles in tissues such as the brain, where the receptor is expressed in neural centres controlling thirst and appetite, but the mechanisms involved are not well understood (Geerling & Loewy, 2009).

Table 1. List of the important physiological endogenous steroid hormones and synthetic steroid hormone agonists.

Steroid	**Endogenous (E) or Synthetic (S)**	**Cell Receptor**	**Major Functional Roles in Development or Clinical Importance**
Cortisol/Corticosterone	E	GR, MR	Fetal organ development,

			metabolism, anti-stress and anti-immune responses
Aldosterone	E	MR	Postnatal renal function, fluid homeostasis and blood pressure control
Estradiol	E	ERα, ERβ	Puberty, female reproduction
Testosterone/Dihydrotestosterone	E	AR	Puberty, male reproduction
Progesterone	E	PR	Maintenance of pregnancy
DHEA/DHEAS	E	AR	Adrenal androgen precursor
17-Hydroxyprogesterone (17OHP)	E	PR, AR (indirectly)	Precursor in cortisol and androgen synthesis; diagnostic marker for CAH

Abbreviations:
DHEA: dihydroepiandrosterone; DHEAS: dihydroepiandrosterone-sulphate; GR: Glucocorticoid Receptor; MR: Mineralocorticoid Receptor; ER: Estrogen Receptor; AR: Androgen Receptor; PR: Progesterone Receptor; CAH: Congenital Adrenal Hyperplasia

Sexual Steroids:
Production of sexual steroids in adult gonads is regulated by the hypothalamus-pituitary-gonadal axis, which involves GnRH, LH, and FSH and includes balanced feed-forward and feedback loops. This system is sex-specific, with the testis or ovary serving as the target organs. Unlike the regulation of androgen production in the HPG axis, the mechanisms controlling adrenal androgen production are not fully understood, although ACTH is a key co-regulator (Sze & Brunton, 2020).

Estrogens:
The group of estrogen steroids comprises estrone (E1), estradiol (E2), estriol (E3), and estetrol (E4). Among these, E2 is the most abundant estrogen. Estrogens' multiple functions include stimulation of utero-placental blood flow, endometrial growth and differentiation, contraction of the myometrium, and proliferation of the mammary epithelium (Mesiano, 2001; Yu et al., 2022). They exert their effect via different types of receptors, the nuclear estrogen receptors (ERs) α and β as well as membrane-associated receptors. The synthesis of estrogens is

controlled by diverse endogenous hormones such as E2, cortisol, calcitriol, CRH, hCG, insulin, and leptin (Hamilton et al., 2014).

The ovary can produce steroids following two biosynthetic pathways: de novo or utilizing circulating DHEA and androstenedione of adrenal origin. In the first case, ovarian steroid can be described by the "two-cell, two-gonadotrophin" model (Figure 2). Two different cell types - granulosa cells of the follicle and the surrounding theca cells - perform distinct reactions due to specific enzyme expression. In addition, each cell type is differentially regulated by two pituitary hormones: FSH acting on granulosa cells and LH regulating both theca and granulosa cells (Hillier et al., 1994).

Steroidogenesis starts in the theca cells under LH control, leading to the production of pregnenolone and progesterone. These cells also produce androgens (androstenedione and testosterone), as they express the enzymes CYP17A1 and HSD3B2. However, theca cells lack aromatase (CYP19A1) and therefore cannot synthesize estrogens. The androgens produced in the theca cells diffuse into the granulosa cells, where, under FSH stimulation, they are converted into E1 and E2 by CYP19A1. A smaller fraction of androstenedione may also be converted into testosterone by AKR1C3/17βHSD in the granulosa cells (Cui et al., 2013).

While the ovary is capable of de-novo steroidogenesis, studies have shown that it also efficiently makes use of DHEA of adrenal origin for the production of androgens and estrogens. Ovarian theca cells seem to present the enzymatic pathway for the appropriate functioning of the backdoor pathway, which produces DHT bypassing DHEA, androstenedione, and testosterone. Ovarian steroidogenesis commences with puberty when the onset of hypothalamic-pituitary-gonadal signalling leads to an increase in FSH and LH, which in turn regulate steroidogenic activity. While ovarian steroidogenesis demonstrates a menstrual cycle-dependent profile in premenopausal women, the absence of ovarian follicles in the postmenopausal ovary significantly reduces the production of estrogens. The contribution of postmenopausal ovaries to circulating levels of active androgens is, however, controversial (Davison & Davis, 2003).

Progesterone:

Progesterone is the major and most relevant progestagen. In humans, the ovarian corpus luteum secretes progesterone from the luteal phase of the ovarian cycle until week 8 of gestation, thereafter, the placenta completely takes over the production (Malassiné et al., 2003). Progesterone exerts many functions. Briefly, it is an intermediate in the production of other steroid hormones (Figure 2) and a neurosteroid involved in brain function. Progesterone is crucial for a successful pregnancy as it supports blastocyst implantation, maintains pregnancy, and prepares the mammary glands for lactation (Di Renzo et al., 2012; Miller & Auchus, 2011).

The action of progesterone on target tissues is mediated by two progesterone receptor isoforms (A and B) that work as ligand-activated transcription factors. Under physiological conditions, the withdrawal of progesterone receptor-mediated signalling triggers menstruation and parturition (Patel et al., 2015).

After mitochondrial import, cholesterol is hydroxylated at two positions (C-20 and C-22), and the cholesterol side chain is cleaved off by P450 side-chain cleaving enzyme (CYP11A1). CYP11A1 is a rate-limiting enzyme of steroidogenesis and converts cholesterol to pregnenolone, the precursor of all other steroid hormones (Slominski et al., 2015).

Focus on Androgens:

In adult males, testosterone is the most abundant serum androgen, with approximately 90-95% produced by Leydig cells in the testes and the remaining 5% synthesized in the zona reticularis of the adrenal glands2. Total serum testosterone concentrations in men are typically around 12-30 nmol/L at the age of 30, but these levels decline annually at a rate of approximately 1.6% starting at 35 years of age (Snyder et al., 2018). In selective tissues, testosterone can be converted to 5α-DHT, the most potent endogenous androgen, which has 5-10 times greater affinity for the AR compared to testosterone.

In women, total serum testosterone levels are about 10 to 15 times lower than in men of the same age, ranging between 0.4-2.1 nmol/L. This is due to the androgen production profile in women being equally divided between the adrenal glands and ovaries, but with a greater emphasis on producing testosterone precursors (Sarkar, 2019). The adrenal glands primarily synthesize inactive androgen precursors, including DHEA, DHEAS, androstenedione, and 11β-hydroxyandrostenedione (Rege et al., 2013). These precursors are often referred to as weak androgens due to their low affinity for the AR (Naamneh Elzenaty et al., 2022).

These precursors are transported to target tissues, where they are activated enzymatically through intracrine mechanisms involving multiple pathways. In menstruating women, the adrenal cortex predominantly produces DHEA and DHEAS, while theca cells in the ovaries are more focused on producing androstenedione which is subsequently converted to testosterone and E2. Only small amounts of these androgens are released into circulation. After menopause, with the cessation of ovarian steroidogenesis, circulating androstenedione levels are halved, and total testosterone levels decrease by approximately 25% (Burger, 1999).

The gonads and the adrenal glands contribute to androgen production both pre- and postnatally, in a developmentally specific manner. Originating from the urogenital ridge during embryogenesis, they share genetic and steroidogenic features. In postnatal life, androgen production in the zona reticularis begins only around ages 6-8 during adrenarche, when the enzymatic machinery is reoriented to primarily produce DHEA and DHEAS (Auchus, 2011). Adrenal C19 steroid production peaks during the third and fourth decades of life in both men and women, after which it declines significantly (Labrie et al., 1997b). Notably, 80% of circulating DHEA in postmenopausal women is adrenal in origin, with the remainder produced by the ovaries (Labrie et al., 2011). In fetal development, the testes form early and produce high levels of testosterone during the first and second trimesters, critical for the masculinization of the male fetus. After birth, testosterone levels drop sharply but rise again temporarily during minipuberty (days 30-100 of life), primarily in male newborns; then they remain low until puberty (Becker & Hesse, 2020). In contrast, the fetal ovary is steroidogenically inactive during the prenatal and neonatal periods, initiating steroid production only at puberty (Itriyeva, 2022). While the classic androgen biosynthesis pathway is well-understood, recent

research has uncovered alternative pathways and novel active androgens, further expanding our understanding of androgen biology.

Androgen biosynthesis by the classic pathway

All steroid hormones are produced from cholesterol through a cascade of enzymes encoded by genes common to all steroid-producing organs (Figure 1). The Δ5 pathway leads to androgens production, both in the zona reticularis of the adrenal and in the gonadal glands. This is considered the "classic pathway".

Cholesterol is transported inside the mitochondria to serve as the substrate for the first step of steroidogenesis; in particular, the steroidogenic acute regulatory protein (StAR) shuttles it to the inner mitochondrial membrane. Here, cholesterol is converted to pregnenolone by the side-chain cleavage system composed of CYP11A1, ferredoxin, and ferredoxin-reductase (Galano et al., 2022). CYP17A1 is subsequently responsible for the 17-alpha hydroxylation into 17α-hydroxypregnenolone and CYP17A1 also exerts a 17,20-lyase activity to produce C19 steroids from C21 precursors.

In the zona reticularis of the adrenal gland, due to the low activity of hydroxysteroid dehydrogenase type 2 (HSD3B2), the Δ5 pathway predominates, leading to abundant production of DHEA, which is mostly sulphated via a phase II reaction (SULT2A1), making DHEAS the most abundant circulating C19 androgen. In the adrenal glands, DHEA can further be converted to androstenedione by HSD3B2 and be transformed to testosterone by the low adrenal levels of AKR1C3/17β-HSD5. A minor fraction of DHEA is conversely converted into androstenediol by AKR1C3. Due to the low activity of HSD3B2, DHEA and androstenediol primarily serve as precursors for testosterone production (Burger, 2002).

Due to the high levels of CYP11B, the adrenal glands give rise to 11-oxygenated C19 steroids by converting androstenedione and testosterone to their 11β-hydroxyl derivatives, with 11OH-androstenedione being the major product due to the high abundance of androstenedione. Both 11OH-androstenedione and 11β-hydroxytestosterone (11OHT) may be converted to their 11-keto counterparts mainly peripherally, due to higher concentration of 11β-hydroxysteroid dehydrogenase type 2 (HSD11B2) compared to the adrenal glands (Pretorius et al., 2017; Turcu et al., 2016). Adrenal vein sampling in healthy women has demonstrated that the adrenal gland secretes DHEAS (low micromolar range), DHEA, 11OH-androstenedione, and androstenedione (medium nanomolar ranges), followed by 5-diol, 11-ketoandrostenedione, testosterone, 11OHT, 11-ketotestosterone (11KT), and DHT (low nanomolar ranges) (Rege et al., 2013). The adrenal glands thus secrete only very low amounts of active androgens, but mainly androgen precursors that are activated in peripheral tissues.

Like in the zona reticularis, the Leydig cells in the testes follow the Δ5 pathway due to the co-expression of CYP17A1 and CYB5A (Figure 1,2). However, in Leydig cells, subsequent metabolism is directed at testosterone biosynthesis due to the absence of sulfation reactions by SULT2A1 and by the highly expressed HSD3B2 and HSD17B3. DHEA is then converted to testosterone via androstenedione or androstenediol through the action of HSD3B2 and HSD17B3, respectively, with HSD17B3 playing a key role in testicular androgen biosynthesis.

AKR1C3/17β-HSD5 expression has been reported in Leydig cells and may also contribute to testosterone production (Zirkin & Papadopoulos, 2018). Testicular steroid output is predominantly testosterone, with lower levels of androstenedione and DHEA also being released into circulation. Androgen and androgen precursor production by the ovary follows a similar route to that of the testes (Figure 2).

Novel androgens and biosynthesis pathways

Recent research has expanded the understanding of androgen biosynthesis beyond the classic pathways of testosterone and DHT production, identifying alternative biochemical routes and novel bioactive androgens (Miller & Auchus, 2019). Previously regarded as inactive intermediates destined for excretion, these metabolites are now recognized as significant contributors to androgen activity. Disorders like congenital adrenal hyperplasia (CAH) have served as models to uncover these novel pathways and steroids, such as the "backdoor" pathway, which bypasses the conventional routes to produce DHT. This pathway involves key steps mediated by enzymes like SRD5A1 and AKR1C2/4 and has been linked to genetic mutations in humans (Homma et al., 2006).

The "backdoor" pathway begins with 17OHP, leading to the production of DHT via intermediates like androsterone and androstanediol. During fetal development, progesterone can also serve as a starting point. While androsterone and androstanediol are androgenic, their binding affinities to the AR are significantly weaker than testosterone (Homma et al., 2006).

A subject that is growing in prominence is the 11-oxygenated androgens, long recognized but only recently gaining attention for their relevance. The adrenal cortex produces substantial amounts of 11β-hydroxyandrostenedione, an androgen precursor for 11-ketosteroids. These transformations involve enzymes such as CYP11B1 in the adrenal glands and HSD11B2 in the kidneys (Homma et al., 2006). Unlike classic androgens, 11-oxy androgens predominantly exert their effects in peripheral tissues like adipose tissue, where 11KT and 11KDHT significantly contribute to androgenic activity (Pretorius et al., 2017)

These findings highlight the complexity of androgen biosynthesis and its role in androgen excess disorders, offering potential avenues for further research and therapeutic targeting.

Androgen action:

Androgens exert their effects binding to the AR and activating a vast range of different biological processes (Sutinen et al., 2017). Accordingly, the AR is differentially expressed in many tissues, other than inner and outer genitalia, including bones, muscles, the brain and nervous system, the cardiovascular and immune system (Chang et al., 2013).

In humans, the AR gene is located on the X chromosome at position Xq11-12. As previously stated, the AR is a member of the class I nuclear receptor transcription factor family, subdivided in five functional regions. It can be regulated by genomic or post-translational modifications.

Not only testosterone and DHT bind the AR, but also the so called “precursors” and even 11-oxygenated steroids are renowned to bind the same receptor, even if with different potency (Naamneh Elzenaty et al., 2022). Once androgens are bound to the AR, their effect may be at the genomic, slower-DNA related, or non-genomic level (Flück & Pandey, 2014).

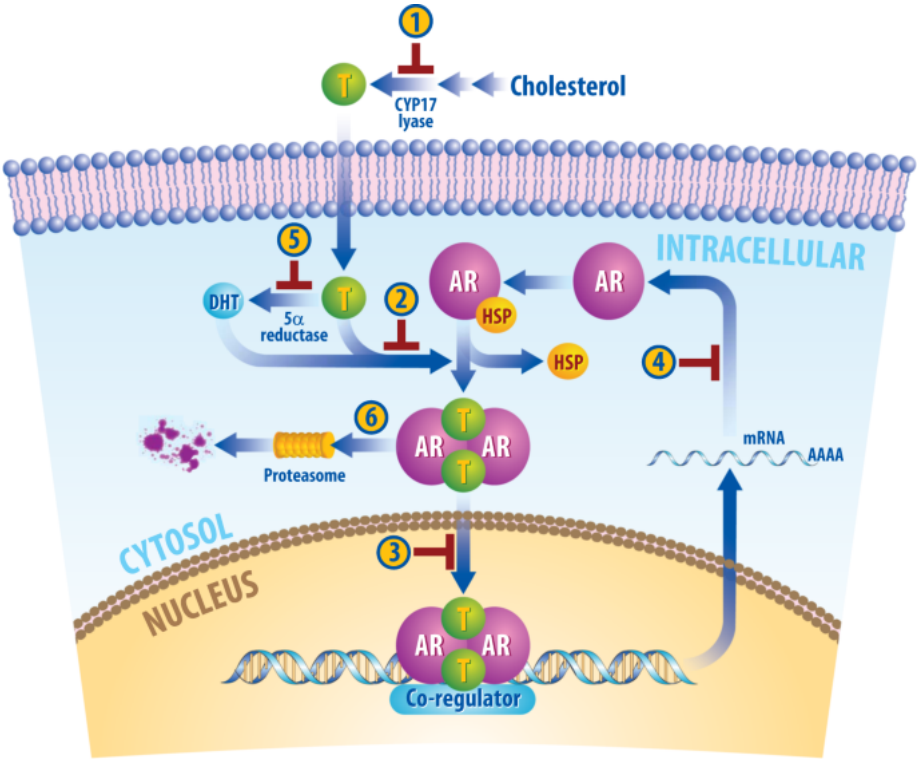


Figure 4. Androgen receptor function

In fact, AR is located in the cytoplasm bound to a complex of different heat shock proteins (HSPs), chaperones, and co-chaperones (Chaturvedi & Dehm, 2019), Figure 4. As soon as the androgen metabolite is bound, AR changes its conformation and dissociates from the chaperones. Once the AR is activated, it can act either at the genomic or non-genomic level (Chaturvedi et al., 2019).

In the former case, the complex transits through the nuclear pores into the nucleus. In the nucleus, the complex binds as a dimer to androgen response elements present in promoters of target genes. Upon binding of an AR dimer, several co-regulators join the binding complex to promote the transcription of target genes (Chaturvedi & Dehm, 2019). In the latter case, AR signaling in the cell occurs quickly. In this case the timespan is within seconds or minutes and usually targets plasma membrane proteins or receptors, which activate intracellular kinase cascades that enhance cell proliferation and survival, such as MAPK/ERK signaling (Liao et al., 2013).

ANDROGEN METABOLISM:

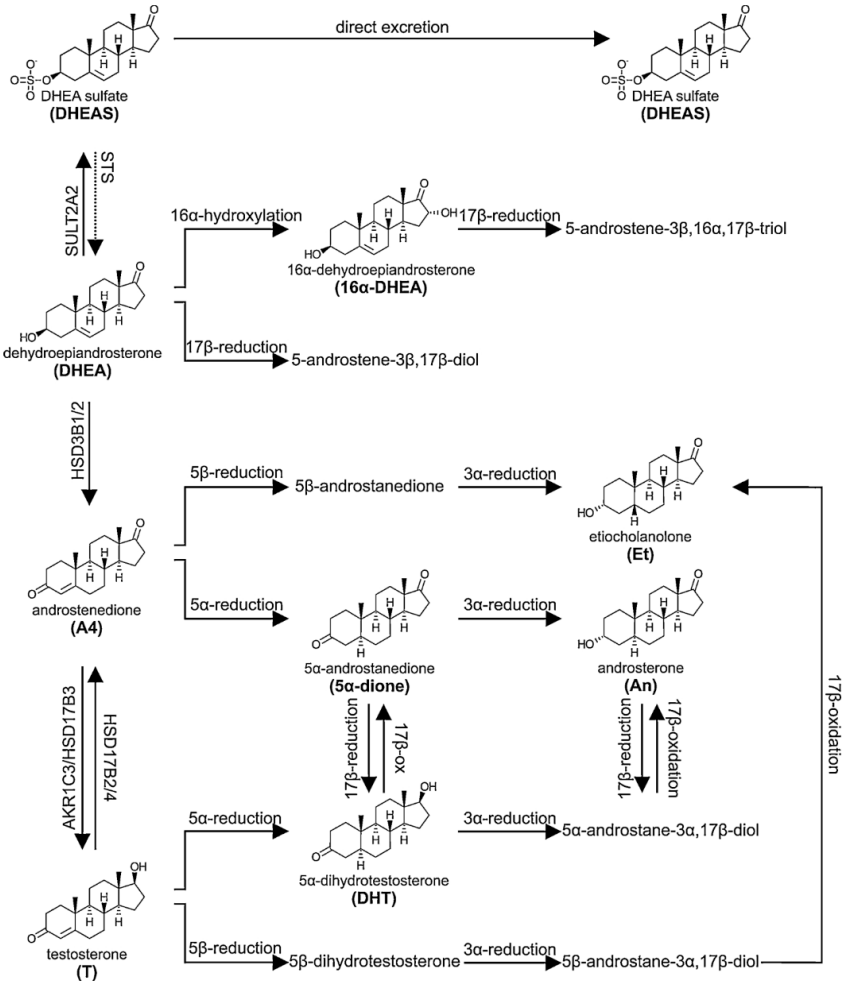


Figure 5. Partial scheme of androgen metabolism

Considering androgen metabolism (figure 5), the most important step that leads to activation and inactivation of androgenic steroids is the 5α-/5β-reduction of the Δ4 steroid moiety common to all androgen precursors. The SRD5A1-2 exert the 5α-reduction within target tissues, but not every molecule goes through this mechanism; in fact, the quota that is able to escape it undergoes a 5α- or 5β-reduction within the liver (Traish, 2012). DHT is produced as a consequence of a 5α-reduction of testosterone, while 5β-reduction catalyzed by AKR1D1 acts mainly as an inactivation step. In fact, 5β-DHT, as opposed to 5αDHT, binds poorly or does not bind AR (Chen & Penning, 2014).

In any case, following 5α or 5β-reduction, androgen metabolites are subject to reduction of the 3-keto group on 3α-stereoselectivity. Importantly, 3α,5α-reduced metabolites can potentially be converted back to the 3-keto metabolite by oxidative 3α-HSDs such as in the alternative DHT biosynthesis pathway. The majority of 5α/β-3α-metabolites of testosterone and DHT, which contain a 17β-hydroxy, are converted to 17-ketosteroids by the action of the oxidative 17β-HSDs, namely HSD17B2 and HSD17B4. As a result, androgen metabolites are excreted with a 17-keto/17β-hydroxy ratio of approximately 10:1 (Schiffer et al., 2019).

Therefore, the primary urine androgen metabolites are androsterone and etiocholanolone (Figure 4). While androstenedione and testosterone can be metabolized to either androsterone or etiocholanolone, DHT is further 3α-reduced and thus only reflected in the androsterone fraction. In fact, both androsterone and etiocholanolone are subject to glucuronidation at the 3-position. This phase 2 metabolism can occur in the liver or within peripheral target tissues. The glucuronidation of C19 steroids is catalyzed by three members of the UGT2B subfamily, namely: UGT2B7, UGT2B15, and UGT2B17 with different selectivity based on the substrate specificity for the 5α/β-stereoisomers. UGT2B17 has the highest activity of all UGTs towards androstenedione, testosterone, and DHT (Gauthier-Landry et al., 2015).
While Δ5 steroids like DHEA and pregnenolone are excreted almost exclusively as sulfates, sulfation of other C19 steroid metabolites is considered a minor phase 2 reaction. SULT2A2 targets 3α- and 17β-hydroxyl groups and has been shown to sulfate androstanediol, testosterone, and DHT. Hydroxy groups in positions 16β, 17α/β, and 18 are also important targets for sulfation of C19 steroids (Mueller et al., 2015).
In the urine, we can further find, as major androgen precursor metabolites, DHEA and 16α-hydroxy-DHEA (figure 5). Circulating DHEA is readily 16α-hydroxylated by CYP3A4/7 in the liver. The abundant conjugated androgen precursor, DHEAS, is water-soluble due to sulphate molecule and is largely excreted in an unmodified form, represented by the urinary DHEA fraction following deconjugation (Callies et al., 2000).
Talking about the 11-oxygenated androgen precursor, 11OHandrostenedione presents a clear metabolism; in fact, it undergoes sequential 5α- and 3α-reduction, yielding 11β-OHAndrostenedione, which is readily quantifiable in urine. 11β-OHAndrostenedione can also derive from cortisol metabolism, but only by a small percentage (5–10%). The metabolism of the active 11-oxygenated androgen, such as 11KT, has yet to be fully elucidated. Studies investigating the potential conjugation of 11-oxygenated steroids suggest that glucuronidation is less efficient compared to the classic androgens (Pretorius et al., 2017).

THE CONCEPT OF INTRACRINOLOGY

The steroid serum concentrations depend mainly on the production rate of the adrenal glands and gonads. It has to be considered that peripheral tissues, such as adipose tissue, bone, nervous system can play a role in concentrating steroids. It depends on their serum levels, their link to SHBG or albumin and the cellular influx and efflux, in particular for conjugated steroids which require active transports (Laurent et al., 2016).
Active androgens are subsequently inactivated enzymatically prior to being released from the cell for excretion. This concept of hormone action is termed "intracrinology" and is distinct from the classical concept of "endocrinology," where a designated gland secretes active hormones into circulation, exerting direct effects on receptors in target tissues (Schiffer et al., 2018).
In addition to the classical C19 steroids (DHEAS, DHEA, testosterone and androstenedione), recent advances in mass spectrometry-based analytical methodology have allowed to identify and quantify non-classical androgen metabolites (Bloem et al., 2015). For example, comparing

serum of healthy premenopausal women and premenopausal women with PCOS, the 11-oxygenated androgens were the predominant C19 steroids in women with PCOS; in particular, the circulating levels of the active androgen 11KT were 3–4 times higher than those of T in healthy premenopausal women (O'Reilly et al., 2017). Similar results were recently observed by another study, which quantified androgen levels in patients with 21-hydroxylase deficiency (Turcu et al., 2017).

Although one could easily presume that most C19 steroids in circulation are produced by steroidogenic tissues, androgen metabolites released from peripheral tissue make a significant contribution to the circulating pool of C19 steroids and thus highlight the importance of local androgen activation and metabolism by intracrine mechanisms.

Furthermore, the major fractions of androgen metabolites are conjugated to facilitate their excretion. Indeed, a study aimed to quantify androgen metabolites and their sulfo- and glucoconjugates in serum; it showed that testosterone and DHT circulate predominantly in their unconjugated form, while their metabolites androsterone, epiandrosterone, and 3α-adiol were mostly conjugated (Zang et al., 2017). It has previously been suggested that the sum of the circulating metabolites androstenedione-glucuronide and 3α/β-adiol-glucuronide should be used as estimates of active androgens produced in peripheral tissue (Labrie et al., 1997).

Principles of intracrine androgen steroid metabolism

After cellular influx, an androgen precursor steroid is enzymatically activated by cell-specific enzymes and pathways before exerting its effect via the AR. Intracrinology is defined by the following principles:

1. The receptor (AR in the case of androgens) and hormone precursor-metabolizing enzymes are co-expressed in the same cell.
2. An inactive hormone precursor (e.g., DHEAS, DHEA, androstenedione, 11OH-androstenedione) is taken up from circulation.
3. Each cell determines the amount of the active hormone (e.g., testosterone, DHT, 11KT, 11KDHT) produced intracellularly via a specific set of enzymes.
4. The hormone is enzymatically inactivated in the same cell prior to efflux.
5. The cells release no significant number of active hormones, preventing a systemic excess (Labrie, 1991; Labrie et al., 1997).

Dysregulation of intracrine pathways can result from (1) alterations in expression, leading to tissue-specific effects, or (2) mutations/polymorphisms in involved enzymes, leading to systemic effects.

The enzymes involved in intracrine metabolism are the ones involved in phase I and phase II reactions. Additionally, C19 steroids can serve as substrates for A-ring aromatization, yielding estrogens. HSD3B enzymes introduce the 3-keto-Δ4 motif into precursor androgens (e.g., DHEA and 5-diol), while reductive 17βHSD enzymes convert the 17-keto group common in androgen precursors to a 17β-hydroxyl group. These two motifs (3-keto-Δ4 and 17β-hydroxyl) are shared by all active androgens.

Unlike the adrenal and gonadal isoform HSD3B2—mutations of which cause a congenital adrenal hyperplasia variant—mutations in HSD3B1, almost exclusively expressed in peripheral tissues and the placenta, are not known. AKR1C3 (HSD17B5) is suggested to significantly contribute to peripheral androstenedione activation by conversion to testosterone (Miller & Auchus, 2011). Specific AKR1C3 polymorphisms are linked to PCOS and elevated testosterone levels in women (Ju et al., 2015) and are overexpressed in prostate cancer, potentially promoting tumor progression through increased intratumoral androgen levels (Fung et al., 2006).

Enzymes with 5α-reductase activity reduce the Δ4-double bond. In the presence of 3-keto and 17β-hydroxyl groups, this reduction leads to maximum androgen potential, as seen with DHT and 11KDHT (Pretorius et al., 2016). Systemic upregulation of 5α-reductase activity is observed in women with PCOS, enhancing glucocorticoid clearance and androgen activation in peripheral tissues (Fassnacht et al., 2003), and is linked to metabolic dysfunction (Sanchez-Garrido & Tena-Sempere, 2020).

SRD5A2, encoding steroid 5α-reductase type 2, is expressed in male reproductive tissues, and its disruption impairs local DHT formation, leading to disordered sex development in 46,XY individuals (Okeigwe & Kuohung, 2014). The sequence of androgen activation is substrate-specific, as SRD5A enzymes can also reduce the Δ4-double bond of 17-keto steroids prior to 17β-reduction.

Quantification of increased panels of steroids, including conjugated metabolites, is needed to provide further insight into intracrine metabolism and its role in endocrine disorders. It should be noted that all androgen concentrations reflect their circulating levels, but this is not necessarily indicative of the steroid concentration available for cellular uptake (Laurent et al., 2016). The perfection of a method involving mass spectrometry may help in better defining peripheral androgen activities.

CLINICAL CONDITIONS OF INTEREST

The effects of androgen deficiency and excess on sexual development and function are most effectively studied in patients with rare monogenic variants in genes associated with androgen biosynthesis and action. In contrast, acquired disorders of androgen deficiency and excess do not typically result in differences of sex development (DSD) but can still significantly impact pubertal development, fertility, and overall health.

In the context of andrology and endo-gynaecological conditions, part of them could be possibly related to disruption of steroidogenesis, or alteration in androgens, but it is not clearly ascertained from the routine blood test.

Idiopathic infertility

In andrology, some men suffer from an infertility condition, which is defined as the lack of a pregnancy after 12 months of unprotected sexual intercourses (Agarwal et al., 2021). These men can present semen samples with altered sperm parameters, that can be below the 5th percentiles of normality, in relation to the WHO sperm reference parameters (*WHO Laboratory Manual for the Examination and Processing of Human Semen*, n.d.); they derive from studies who analyzed sperm parameters of men who were considered fertile because they reached a pregnancy after not more than 12 months of sexual intercourses. Genetic, epigenetic or endocrine-disruptor chemicals have been called into question in this regard, without definitive conclusions (Bracke et al., 2018). In particular, when no cause is identified, these men are defined as idiopathic, and no definitive conclusions have been drawn. However, a potential role of androgen metabolism disruption could be hypothesized.

Some studies have already tried to deepen the knowledge in this way, in particular analyzing the sperm of men with infertility with a steroidomic approach. For example, a study analyzed different steroids using different immunoassays profiled semen and serum of healthy men and infertile men. No particular differences in concentration were underlined in the assessments, even including 7OH-DHEA, a metabolite with potential anti-inflammatory activities exerted by disruption of glucocorticoid receptors (Hampl et al., 2003).

A recent study compared an extended steroid profile in semen samples of healthy men using a mass spectrometry approach. They succeeded in analyzing both classic androgens, gluco- and mineralocorticoids and their metabolites (Olesti et al., 2020).

No current data are available in regard to an extended serum steroid panel using mass spectrometry in the infertility setting.

Polycystic ovary syndrome

Of interest, some androgenic conditions in women, such as PCOS, or hirsutism, present a frank manifestation of higher androgen activity, but not always a laboratory explanation of these conditions has been found. Therefore, they can be labelled as idiopathic.

PCOS is considered an endocrinological and metabolic condition that affects up to 15% of women in their fertile age. Clinically heterogeneous by nature, PCOS is defined by a combination of signs and symptoms of androgen excess and ovarian dysfunction in the absence of other specific diagnoses (Escobar-Morreale, 2018). To be affected by the syndrome, women have to present oligomenorrhea, biochemical hyperandrogenism or clinical hyperandrogenism, defined as the presence of acne, alopecia and/or hirsutism. Furthermore, the burden on their health is pivotal due to potential risk on general health, linked to obesity, diabetes and cardiovascular diseases.

Clinical and/or biochemical evidence of androgen excess is common but not universal in women with PCOS. The ovaries are considered the primary source of androgen excess in PCOS. Among the androgens, androstenedione has been suggested as a superior marker of hyperandrogenism and metabolic risk compared to testosterone (Lerchbaum et al., 2014). However, it has to be taken into account the adrenal and the peripheral role in androgen excess.

Studies over time have shown higher levels of 11-oxy circulating androgens. In fact, serum levels of 11OHA4, 11KA4, 11OHT, and 11KT are significantly higher in women with PCOS than in healthy controls. Specifically, O'Reilly and colleagues recruited 114 women with PCOS and assessed serum androgens using mass spectrometry. They observed not only the expected increase in classical androgens (testosterone, androstenedione, dehydroepiandrosterone) but also an elevation of 11-oxygenated derivatives (11-ketoandrostenedione, 11beta-hydroxytestosterone, 11-ketotestosterone) in both serum and urine (O'Reilly et al; 2017). This suggests that androgen excess in PCOS arises from multiple sources, including the ovaries, non-gonadal tissues such as the adrenal glands, peripheral tissues, and intermediary metabolic processes. These findings imply that the underlying defect in PCOS causes a dysregulation of androgen production across various steroidogenic pathways.

Gender incongruence

Among these conditions, we must also enumerate gender incongruence (GI). GI is defined as a condition of marked and sustained clinical incongruence between the experienced gender and the sex assigned at birth (Coleman et al., 2022). To date, biological causes of gender incongruence are yet to be definitively found.

Some biological theories have tried to understand whether there is present or not a biological reason for this condition. They started from different brain development during fetal life or during early life. Some studies have also investigated hormonal milieu in these people, with no definite findings. This is because no sex hormonal metabolism disruption has been highlighted in these people and also because gender identity is considered a more complex condition which involves psychological, social and cultural aspects (Polderman et al., 2018).

Not many studies have investigated hormones with a steroidomic approach in this population. A study evaluated by Savkovic and colleagues evaluated 23 transgender men undergoing gender-affirming hormone therapy and compared them with cisgender men, both eugonadal and hypogonadal. Differences were identified in the ratio of certain androgen metabolites in

urine (e.g., testosterone/epitestosterone and 5alpha,3alpha-androstanediol/5beta,3alpha-androstanediol) (Savkovic et al., 2018).

EXTENDED STEROID ANALYSIS USING MASS SPECTROMETRY

Mass spectrometry - general considerations:

The need in the endocrinological setting for a more sensitive and specific assessment of steroids in different media, such as serum, urine, saliva etc. From the 1960s the immunometric methods have taken the scene in the context of steroid evaluation, and still widely worldwide (Macklin et al., 2020). They are based on the recognition of the molecule of interest by a certain antibody used. These methods have numerous advantages, relating to the automation of the technique and the consequent enormous productivity. However, their use brings along some downsides: problems of specificity and sensitivity, especially when analysing complex biological samples, the limited number of hormones that can be measured in the same session and the difficult performance in complex scenarios. In particular, when steroid levels into the sample are quite low, for example when measuring E2 in male or children blood samples, the specificity of the immunoassays has been called into question. A cross reaction with similar compounds and subsequently an overestimation of the results can happen; this can be problematic both from a diagnostic and therapeutic point of view (Stanczyk et al., 2018). Furthermore, the biological matrix can cross-react with antibodies and lead to inaccurate measurements (Závada et al., 1978). Other significant drawbacks are represented by lack of standardization of immunoassay methods, due to the wide distribution and the different kits that are used due to the differences between antibodies and kit formulations from various suppliers.

To overcome these problems, immunoassays have been trying to evolve, through different approaches. The Endocrine Society, however, felt the urge to publish a position statement back in 2013, and to assemble the “Sex Steroid Assays Reporting Task Force” recommending that clinical steroid biochemistry should be supported by mass spectrometry (MS) and in particular for research purposes (Handelsman & Wartofsky, 2013).

MS traces its origins back to the 1930s; it is based on the separation and selection of molecules and ions. Over the years, advances were made using gas chromatographer (GC-MS), able to detect serum and urine steroid profiles, including androgens and cortisol derived metabolites (Ryhage, 1964). Over the years, the GC-MS method improved through the quadrupole mass analyser, HPLC and more reliability of sample extraction. The subsequent use of Liquid chromatography–tandem mass spectrometry (LC–MS/MS) made the method much simpler to use routinely due to easier sample preparation (A. E. Taylor et al., 2015).

Mass spectrometer basic principles:

The mass spectrometer function is based on which medium is used to run the analysis. In particular, if GC/MS is used, the specimen is vaporised in the GC injector. The analytes in the

sample are in the gaseous form and travel through a heated column to the detector. These analytes are energized as ions and based on their mass and charge reach the detector at different times. This method of analysis was developed over 40 years ago by a group of scientists, such as Shackleton & Snodgrass and Sjövall (Shackleton & Snodgrass, 1974) (Sjövall, 1975). GC–MS has been largely superseded in the past 10 years by LC–MS/MS due to improvements in HPLC systems and column design as well as the introduction of tandem approach. The use of liquid chromatography permits the separation of a mixture of liquid analytes into separate analytes fractions; the analytes are retained by a column packed with stationary phase. The retention happens due to different variables:

- Polarity
- Mass
- Charge

Therefore, the single analyte is identified unequivocally, free from any interference. In the case of a gas spectrometer, the chromatography column requests enough room to be held, because it is usually longer than 10m. The use of a liquid chromatography column, paired with two quadrupole mass analyzers, allows it to be usually shorter than 20 cm. The use of 4 quadrupoles, namely a squared filter which create an electric field that allows the transmission of only ions with a specific mass and charge, allows to firstly select the ion, then the newly formed ions are selected and therefore gain specificity with low running times (about 10 minutes, compared to 30 minutes of GC-MS method).

To maintain reliability over time, it is noteworthy to underline to go through a full validation of the method that can be continuously monitored. Validation of a LC–MS/MS assay requires investigation into a number of parameters such as selectivity, sensitivity, stability, reproducibility and matrix effects, which should be completed during method development for each steroid (Honour, 2011).

How a Mass Spectrometer Works

To properly assess in the same run a defined panel of steroids, the method has to go through different steps (figure 6).

1. Ionization

The sample to be analyzed is introduced into the mass spectrometer and converted into charged ions. This process is achieved using different ionization techniques, such as Electrospray Ionization (ESI), suitable for liquid compounds, or Matrix-Assisted Laser Desorption Ionization (MALDI), commonly used for biomolecules.

2. Acceleration

The generated ions are accelerated using an electric field, propelling them toward the analysis system. The energy gained is proportional to their charge, ensuring that ions with the same mass-to-charge ratio (m/z) achieve uniform velocities.

3. Separation

The accelerated ions enter a mass analyzer, where they are separated based on their mass-to-charge ratio (m/z). This is achieved through interaction with magnetic or electric fields,

which deflect lighter or more highly charged ions differently than heavier or less charged ones.

4. Detection

The separated ions are detected by a detection system, such as a time-of-flight (TOF) detector or a photomultiplier. The resulting signal is proportional to the number of ions with a specific m/z, and this data is processed to produce a mass spectrum.

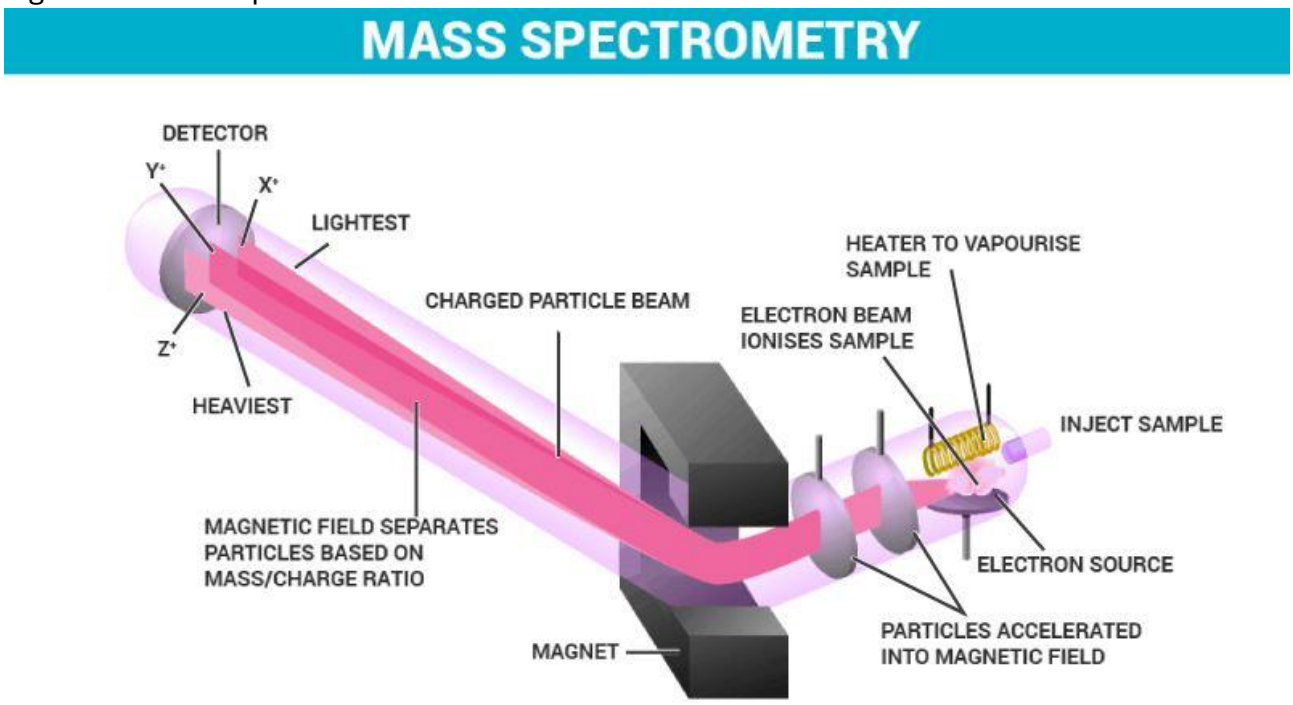
5. Mass Spectrum Interpretation

The resulting spectrum represents the relative abundance of ions as a function of their mass-to-charge ratio. This information allows for the identification of molecules in the sample, their structure, and other chemical or physical properties (Hicks et al., 2014; Schwarz et al., 2009).

Pitfalls and Strengths of Mass Spectrometry

MS is a powerful analytical technique with notable strengths, including high sensitivity, specificity, and the ability to analyze complex mixtures. Its capability to distinguish isobaric compounds, quantify analytes across a broad dynamic range, and provide structural information makes it invaluable in fields like proteomics, metabolomics, and pharmaceutical research. However, MS also presents challenges. Sample preparation can be time-consuming and prone to contamination, especially in highly sensitive applications. Matrix effects, where co-eluting compounds interfere with ionization, can reduce accuracy. Additionally, the cost and complexity of instrumentation, along with the need for skilled operators, limit accessibility. Despite these pitfalls, the continued advancement of ionization techniques, mass analyzers, and software has made MS increasingly versatile and reliable for both qualitative and quantitative analyses (Clarke, 2017).

Figure 6. A mass spectrometer



Rationale:

Clinical laboratories traditionally assess “classic” androgens trying to investigate or diagnose a specific condition. The quantification of steroid panels is now gaining widespread traction due to advances in technology, further driven by the potentiality linked to the use of mass spectrometers. Even if biochemical pathways for the biosynthesis and metabolism of steroid hormones are now mostly well defined, a gulf still exists with regard to the application of this knowledge into the clinical practice, due to lack of a broad picture of steroid metabolites concentrations. Furthermore, some conditions still lack a clear pathophysiological characterization, in particular the ones which are conceivably androgen related but still referred to as “idiopathic”.

Therefore, the aim of this study was to extensively evaluate, via the use of liquid mass spectrometry with an “extended targeted” steroidomic approach, blood samples collected from people with clinical conditions of interest, potentially related to androgen metabolism. This may help in better identifying them from a physiologic and pathophysiological point of view.

Methodology:

This is a cross-sectional observational study. People were selected during routine clinical activity at the Division of Endocrinology, Diabetes and Metabolism at the AOU Città della Salute e della Scienza, Turin (director Prof. E. Ghigo) from November 2022 to November 2024. To be enrolled, individuals had to be at least 18 years old, not taking any drug potentially disrupting steroid metabolism and be characterized by one of the following conditions:

- Male idiopathic infertility (defined as the failure to achieve a clinical pregnancy after 12 months of regular, unprotected sexual intercourse, without any organic or genetic cause in the male partner, with sperm parameters above the 5th centile, excluding a female cause)
- Gender incongruence (a marked and persistent incongruence between the perceived gender identity and the gender assigned at birth)
- Polycystic ovary syndrome (defined by the presence oligomenorrhea, clinical or biochemical hyperandrogenism and polycystic ovaries)
- Congenital adrenal hyperplasia (group of genetic disorders characterised by disrupted adrenal steroidogenesis).

Individuals with gender incongruence (defined also as transgender) were assessed before starting any gender affirming hormone therapy (GAHT). Assigned female at birth (AFAB) individuals were asked to assess hormones in the early follicular phase, within 5 days from the start of the menses. Proper blood samples between 8 and 10 AM after an overnight fasting were obtained from recruited patients. Samples were immediately sent to the laboratory after the collection and centrifuged at 1500 g for 10 min. Following 15 min of stabilization at room temperature, serum aliquot was transferred in screw cap 5 mL polypropylene tubes (Sarstedt, Nümbrecht, Germany) that were stored at -80°C until the day of analysis.

In the first part of the study, we collected the history of each patient enrolled.

We included anthropometric evaluation that included age, weight (kg), height (m), body mass index (BMI) kg/m^2, smoking habit. Then we assessed the clinical history, regarding the number and typology of comorbidities affected, number of medications taken, any concurrent genetic conditions. People who reported conceivable further endocrinological conditions that could alter steroidogenesis were excluded.

Eventually we reported ultrasound evaluations of testicles volume in assigned male at birth (AMAB) people and ovarian morphology in AFAB individuals, both by transabdominal or transvaginal ultrasound, based on the preference of the patient.

Subsequently, a steroidomic target panel was evaluated for each of the aforementioned clinical conditions, in order to obtain a comprehensive panel that could widely characterize these conditions. To obtain this panel, a LC-MS/MS approach was used to contemporary analyse free steroids, comprehensive of phase I metabolites, and phase II metabolites of androgen related analytes. The panel obtained for each condition was analyzed within the same sex assigned at birth group.

In the second part of the analysis, a comparison was carried out between the phase II metabolites obtained by the investigated serum samples and the ones obtained by healthy

volunteers, defined as the ones without any clinical conditions and not taking any drug (Ponzetto et al., 2023). To obtain this, they were recruited by the local Blood Bank of the same University hospital in Turin, Italy. In fact, to be a blood donor, the volunteers had to meet the following inclusion criteria:

- Be between 18 and 65 years of age;
- be healthy (not suffering or having a clinical history with diabetes mellitus, cardiovascular diseases, neurological disease, neoplasms, autoimmune diseases).
- Not taking any medication
- body weight no less than 50 kg;
- systolic blood pressure should not exceed 180 mmHg and the diastolic blood pressure not above 100 mmHg;
- hemoglobin should not be less than 12.5 g/dL in women and 13.5 g/dL in men.

Forty men (mean age 47.7 ± 14.1 years) and 40 women (mean age 38.9 ± 14.9 years) were recruited.

The study protocol was approved by the ethics committee of the current University Hospital (Protocol n. 488,789).

STEROIDOMIC ANALYSIS BY LC-MS/MS

Chemicals and reagents

Steroid reference materials of 49 free steroids, 11 glucuro-conjugated and 16 sulpho-conjugated steroid metabolites, as well as 30 isotopically labeled internal standards (IS) were purchased from different suppliers, which are detailed in Table 2.

Table 2 - Details of purchased reference material

COMPOUND	SUPPLIER	CODE
11β-Hydroxyandrostenedione	Steraloids	A6630-000
11β-Hydroxytestosterone	Steraloids	A5760-000
11-Dehydrocorticosterone	Steraloids	Q3690-000
11-Dehydrotetrahydrocorticosterone	TRC	T293165
11-Deoxycorticosterone	Steraloids	Q3460-000
11-Deoxycortisol	Steraloids	Q1610-000
11-Ketoandrostenedione	Steraloids	A7250-000
11-Ketodihydrotestosterone	Steraloids	A2375-000
11-Ketotestosterone	Steraloids	A6720-000
16α-Hydroxyandrostenedione	Steraloids	A6680-000
16α-Hydroxyprogesterone	Steraloids	Q3330-000
16α-Hydroxytestosterone	Steraloids	A5800-000
17α,20α-Dihydroxyprogesterone	Steraloids	Q1820-000

17α-Hydroxyprogesterone	Steraloids	Q3360-000
17-Hydroxypregnenolone	Steraloids	Q4710-000
18-Hydroxycorticosterone	Merck	H-106-1ML
18-Hydroxycortisol	Steraloids	Q3754-000
20α-Cortolone	Steraloids	P9150-000
20α-Dihydrocorticosterone	Steraloids	Q3990-000
20α-Dihydrocortisone	Steraloids	Q3930-000
20α-Dihydroprogesterone	Steraloids	Q3600-000
20β-Cortol	Steraloids	P8590-000
20β-Cortolone	Steraloids	P9200-000
20β-Dihydrocorticosterone	Steraloids	Q4020-000
21-Deoxycortisol	Steraloids	Q1520-000
2β-hydroxytestosterone	Steraloids	A5625-000
3α,5β-Tetrahydrocortisone	Steraloids	P9550-000
5α-Androstanedione	Steraloids	A1630-000
5α-Dihydrocortisol	Steraloids	P5250-000
5α-Dihydroprogesterone	Steraloids	P2750-000
5-Androstenediol	Steraloids	A7830-000
5β-Dihydroprogesterone	Steraloids	P7150-000
7α-Hydroxydehydroepiandrosterone	Steraloids	A7880-000
Allopregnanolone	Steraloids	P3800-000
Allotetrahydro-11-dehydrocorticosterone	Steraloids	P2200-000
Allotetrahydrocortisol	Steraloids	P4800-000
Androstenedione	Steraloids	A6030-000
Corticosterone	Steraloids	Q1550-000
Cortisol	Steraloids	Q3880-000
Cortisone	Steraloids	Q2500-000
Dehydroepiandrosterone	Steraloids	A8500-000
Dihydrotestosterone	Steraloids	A2570-000
Epitestosterone	Steraloids	A6900-000
Estrone	Steraloids	E2300-000
Pregnanetriol	Steraloids	P9450-000
Pregnanolone	Steraloids	P8150-000
Pregnenolone	Steraloids	Q5500-000
Progesterone	Steraloids	Q2600-000
Testosterone	Steraloids	A6950-000
17-Hydroxypregnanolone Glucuronide	Steraloids	P6589-000
5α-androstan-3α,17β-diol 17-Glucuronide	Steraloids	A1207-050
5α-androstan-3α,17β-diol 3-Glucuronide	Steraloids	A1206-800
5β-androstan-3α,17β-diol 17-Glucuronide	LAD	n/a
5β-androstan-3α,17β-diol 3-Glucuronide	LAD	n/a
Androsterone Glucuronide	Steraloids	A2430-000
Cortisol 21-Glucuronide	Steraloids	Q3890-000

Etiocholanolone Glucuronide	Steraloids	A3625-000
Pregnanediol Glucuronide	Steraloids	P6040-000
Testosterone Glucuronide	Steraloids	A6980-000
Tetrahydro-11-dehydrocorticosterone 21-Glucuronide	Steraloids	P6257-000
16α-Hydroxydehydroepiandrosterone Sulphate	Steraloids	A7923-000
17α,20β-Dihydroxyprogesterone Sulphate	Steraloids	Q1890-000
17-hydroxypregnenolone 3-Sulphate	Steraloids	Q4765-000
5-Androstenediol 3-Sulphate	Steraloids	A7878-000
Allopregnanolone Sulphate	Steraloids	P3817-000
Androsterone Sulphate	Steraloids	A2460-000
Dehydroandrosterone Sulphate	Steraloids	A8470-000
Dehydroepiandrosterone Sulphate	Steraloids	A8530-000
Epiallopregnanolone Sulphate	Steraloids	P3865-000
Epiandrosterone Sulphate	Steraloids	A2534-000
Epipregnanolone Sulphate	Steraloids	P8200-000
Epitestosterone Sulphate	Steraloids	A6940-000
Etiocholanolone Sulphate	Steraloids	A3640-000
Pregnanolone Sulphate	Steraloids	P8168-000
Pregnenolone Sulphate	Steraloids	Q5545-000
Testosterone Sulphate	Steraloids	A7010-000
11β-Hydroxyandrostenedione-d4	Merck	903485
11β-Hydroxytestosterone-d4	Merck	925276
11-Deoxycorticosterone-d8	Steraloids	Q3460-020
11-Deoxycortisol-d5	Merck	710784
17-Hydroxyprgnenolone d3	Steraloids	Q4710-020
17-Hydroxyprogesterone-d8	Steraloids	Q3360-020
21-Deoxycortisol-d8	Merck	D-076-1ML
6β-Hydroxytestosterone-d3	Merck	T-034-1ML
Allopregnanolone-d4	Steraloids	P3800-025
Androstenedione-d7	Steraloids	A6030-124
Corticosterone-d8	Steraloids	Q1550-030
Cortisol-d4	LGC	TRC-C696302
Cortisone-d8	LGC	TRC-C695502
Dehydroepiandrosterone-d5	Merck	D-064-1ML
Dihydrotestosterone-d3	Steraloids	A2570-020
Epitestosterone-d5	Steraloids	A6900-030
Estriol-d3	Steraloids	E2600-015
Estrone-d4	Steraloids	E2300-010
Pregnenolone-d4	Steraloids	Q5500-024
Progesterone-d9	Steraloids	Q2600-014
Testosterone-d3	Steraloids	A6950-020
5α-androstan-3α,17β-diol 17-Glucuronide-d4	LAD	n/a
5β-androstan-3α,17β-diol 17-Glucuronide-d3	LAD	n/a

Androsterone Glucuronide-d4	Merck	A-099-1ML
Etiocholanolone Glucuronide-d5	LAD	NMIAS020
Testosterone Glucuronide-d3	LAD	NMIAS036
Androsterone Sulphate-d4	LGC	NMIAD587
Dehydroepiandrosterone Sulphate-d5	Merck	D-066-1ML
Epitestosterone Sulphate-d3	LGC	NMIAD591
Testosterone Sulphate-d3	LAD	NMIAD506

LAD: Swiss Laboratory for Doping Analyses; n/a: not available

UHPLC/MS grade methanol (MeOH) was purchased from Carlo Erba Reagents (Cornaredo, Italy), acetonitrile (ACN), dichloromethane (DCM) and isopropanol (IPA) from VWR International (Fontanay-sous-Bois, France), ultra-pure water (18.2 MΩ x cm) was obtained with the Smart2pure® system (Thermo Scientific, Waltham, MA, USA), ammonium fluoride (NH4F) for MS was supplied by Merck KGaA (Darmstadt, Germany), while saline solution (NaCl 0.9 %) was acquired from B. Braun Melsungen AG (Melsungen, Germany. Charcoal Stripped Human Serum was purchased from DivBioScience (Ulvenhout, The Netherlands).
Stock solutions of all analytes and IS were prepared at a concentration of 1 mg/mL in MeOH and stored in 1.5 mL amber glass vials at -80°C. For each compound, intermediate solutions at appropriate concentrations (1 mg/mL, 100 µg/mL, 10 µg/mL, 1 µg/mL, 100 ng/mL, 10 ng/mL) were prepared by means of consecutive dilutions of stock solutions and stored in 10 mL glass tubes at -20°C. Starting from the appropriate intermediate solution, separate series of 7 calibration solutions were prepared for free steroids and conjugated steroid metabolites. The calibration solutions for free steroids were containing all free steroids standards at concentrations of 0.1, 0.5, 1.0, 2.5, 10, 50, 150 ng/mL, while the calibration solutions for conjugated metabolites were containing all metabolites standards at concentrations of 0.1, 0.5, 1.0, 5.0, 25, 100 ng/mL and an additional calibration solution was prepared for DHEA-S, Epiandrosterone-S and Androsterone-S containing the standards at concentration of 1500, 300 and 300 ng/mL, respectively. For the preparation of the different calibration samples, 20 µL of the appropriate calibration solution were spiked in depleted serum prior to sample preparation. A mixture containing all IS of free steroid (IS mix Free) and a mixture containing all IS fo conjugated steroid metabolites (IS mix GlucSulf) were prepared spiking intermediate solutions of appropriate IS in MeOH to reach two final solutions with all IS at a fixed concentration of 100 ng/mL. IS mix Free and IS mix GlucSulf were then stored in 10 mL glass tubes at -20°C and used for the extraction procedures.

Sample preparation

Two different sample preparations were optimized to extract from serum matrix free steroids and steroid conjugated metabolites. Supported liquid extraction (SLE) on ISOLUTE SLE+ (Biotage, Uppsala, Sweden) 400 µL 96-well plates was used to extract free steroid hormones from serum samples. For each sample, 200 µL of serum were spiked with 10 µL of the IS mix Free, diluted with 200 µL of water, and then agitated for 5 min at 600 rpm. Each well was then loaded with 400 µL of each pretreated sample and positive pressure of 3 psi was applied for 30

s to facilitate sample loading and adsorption; the elution was carried out, after a 5 min waiting period, by adding in three consecutive steps 300 µL of DCM-IPA 90:10 (v/v), for a total of 900 µL, to each well and applying a pressure of 3 psi for 1 min. Extracts collected in collection plates evaporated for approximately 15 min at 40 °C under a stream of nitrogen, and finally reconstituted with 50 µL of a MeOH-H_2O 50:50 (v/v) solution (reconstitution solvent). After 10 min of shaking at 600 rpm, 20 µL of each extract were injected into the LC-MS/MS system.
Solid phase extraction (SPE) was employed for extracting steroid conjugated metabolites from serum samples using EVOLUTE® EXPRESS ABN 30 mg (Biotage, Uppsala, Sweden) 96-well plates. Prior to sample loading, each well was conditioned with 1 mL of MeOH and then equilibrated with 1 mL of 2% H_3PO_4 solution, applying after each step a high positive pressure gradient of 1 min. Serum sample pre-treatment was carried out by spiking 20 µL of the IS mix GlucSulf in a sample aliquot of 200 µL, diluting the latter with 200 µL 2% H_3PO_4 and then agitating the sample for 5 min at 600 rpm. Each well was then loaded with 400 µL of pre-treated serum samples, and a high positive pressure gradient of 1 min was applied. A washing step with 1 mL of H_2O was performed and finally elution was performed by adding 500 µL of a MeOH-ACN 50:50 (v/v) solution to each well and applying a low positive pressure gradient of 3 min. The extracts were collected in collection plates, evaporated for approximately 30 min at 50°C under a stream of nitrogen, and finally reconstituted with 100 µL of reconstitution solvent. The plate was then shaken for 10 min at 600 rpm, and 20 µL of each extract were finally injected into LC-MS/MS for analyses.

LC-MS/MS analysis
Analyses were carried out using a Nexera X2 UHPLC system (Shimadzu, Tokyo, Japan) coupled to a Citrine Triple Quad MS/MS system (AB Sciex, Ontario, Canada). The system control and data analysis were performed by AB Sciex Analyst and MultiQuant software, respectively.
For free steroids analysis, chromatographic separation was achieved using a RRHD Zorbax Eclipse Plus C18 column (150 × 2.1 mm, 1.8 µm; Agilent, Santa Clara, CA, USA) set at 55°C. Mobile phase A was 2 mM ammonium fluoride in H_2O and mobile phase B was pure MeOH. A linear gradient from 40% to 90% B over 24 minutes was performed, followed by a washing step at 98% B for 4 min, and finally, the column was re-equilibrated for 2 min at initial conditions for a total run time of 30 min. The injected volume was 20 µL and the flow rate was set at 300 µL/min. ESI-MS/MS analysis was performed in polarity switching mode. Source temperature was maintained at 550°C, and ion spray voltage was set at 5500 and -4500 V in positive and negative ionization modes, respectively. Curtain gas pressure was set at 35 psi, nebulizer gas pressure at 45 psi, and heater gas pressure at 60 psi. One MS/MS transition was selected for each target analyte and IS. Declustering potential, entrance potential, collision energy, and cell exit potential for each MS/MS transition were optimized by infusing standard solutions of each target analyte and relative IS at 100 ng/mL in reconstitution solvent.
For steroid conjugated metabolites, chromatographic separation was achieved using the same LC column and mobile phases. The linear gradient started from 42% to 67% B over 24 minutes, followed by a washing step at 98% B for 3 min, and finally, the column was re-equilibrated for 2 min at initial conditions for a total run time of 29 min. The injected volume was 20 µL and the

flow rate was set at 300 µL/min. ESI-MS/MS analysis was performed in polarity switching mode. Source temperature was maintained at 500°C, and ion spray voltage was set at 5000 and -4000 V in positive and negative ionization modes, respectively. Curtain gas pressure was set at 35 psi, nebulizer gas pressure at 40 psi, and heater gas pressure at 55 psi. Again, only one transition was selected for each target analyte as well as IS and MS parameters were optimized by infusing standard solutions of each target analyte and relative IS at 100 ng/mL in reconstitution solvent.

Statistical analyses:

Distribution of continuous variables was checked using Shapiro Wilk testing and Q-Q plots. Non-normally distributed variables are presented as median and interquartile range [25-75] while normally distributed data are reported as a mean ± standard deviation (SD).

Comparisons between the groups were made at first visually using scatter plots to identify the distribution. Therefore, the Mann–Whitney U test was used for skewed data, Student's t-test was used for normally distributed data, while Fisher's exact test was used for categorical variables. Bonferroni post hoc corrections were applied to account for multiple comparisons whenever appropriate.

Whether a significant difference was highlighted, an ANCOVA analysis was run to adjust for covariates, after properly modifying the non-parametric variables into a logarithmic function to better fit the normal distribution.

For the comparisons between the reference ranges of the groups of interest and the control groups, Z tests were run to find any statistical difference. By standardizing the differences between the observed group medians and the reference medians (controls), Z-scores allowed us to determine whether deviations are statistically significant, accounting for variability within the control population. A deviation from the Z-score ±1.96 (corresponding to a 95% confidence interval), was used to identify significant deviations. A Welch test was used when mean and SD were available for the reference ranges.

The statistical analysis was conducted using the IBM SPSS program (IBM SPSS Statistics for Windows, Version 28.0. Armonk, NY, USA: IBM Corp). The statistical significance level was set at $p < 0.05$.

Results:

General considerations about the enrolled patients:

For this analysis, we have enrolled 130 patients with different clinical conditions: idiopathic infertility, gender incongruence, PCOS, idiopathic hirsutism, classic and non-classic adrenal hyperplasia.

For this analysis, we decided to focus mostly on gonadal related conditions and in particular male idiopathic infertility, gender incongruence, and PCOS, for a total of 85 blood samples included and analysed.

Assigned female people were all in their reproductive age, with a median of 23.75 [20.20-27.37] years, while assigned male were older than the former group 26.96 [21.17-37.47] years of age ($U = 1317.5$ $z = 2,48$. $p = 0.013$). BMI was comparable between the two groups, considered in the normal range: for assigned female BMI: 22.99 [20.59-27.60] kg/m^2 and for assigned male 22.61 [19.05-26.01] ($U = 751$. $z = -7.52$. $p = 0.472$).

Current smoking habit was reported in the 26% of AFAB and 23% of AMAB individuals, respectively.

Regarding the comorbidities, 24% of the sample reported not having a significant clinical history. The majority suffered from one clinical problem (33,7%), while the rest reported more than two health issues. Regarding the typology of health problems, AFAB people with gender incongruence reported mostly diagnosed psychiatric comorbidities (21/34=62%, Chi square test; $p<0.001$). Seventy-two percent of the sample was not taking any drugs; among the others, no one was taking any confounding hormonal therapies. The most prescribed therapies among the sample were antidepressants (14,1%, in particular SSRI) and antipsychotic drugs (6,5%), with minimal effects on CYP involved in steroidogenesis. The other reported drugs did not present significant interactions with steroidogenesis.

Testicles volumes, evaluated by ultrasonographic evaluations, were on average in the normal range values (right testicle: 12.7 ± 4.18 mL and left testicle: 12.11 ± 4.51 mL).

Regarding ultrasound of the ovaries only the 19% (9/47) showed an ovarian morphology compatible with polycystic ovaries, while the rest presented a normal or multifollicular pattern.

General considerations about the method

The final panel was able to identify 49 free steroids, including “classical” and phase I reaction metabolites and 29 sulphates and glucuronidated derivatives (the full list is available in the supplementary section).

In this study, we aimed to focus on the androgen metabolites (C21 backbone), including the related sex steroids estranes (C18 backbone) and pregnanes (C21 backbone); therefore, we did not analyze the gluco and mineralocorticoid related analytes.

Strengths of the used LC/LC tandem MS method

1. Separation of Isobaric Isomers

A significant characteristic of the method lies in its ability to separate isobaric isomers, which is particularly crucial in the analysis of steroid hormones and their metabolites, due to their similar molecular mass.

2. Sensitivity and Linearity

The method demonstrates exceptional sensitivity and linearity, critical for the accurate measurement of steroid hormones and their metabolites, which circulate at vastly different concentrations. These concentrations can range from a few picograms per milliliter (e.g., DHT in women) to micrograms per milliliter (e.g., DHEAS).

3. Comprehensive Serum Panel via LC-MS

This represents one of the first examples of a broad panel analyzed in serum using LC-MS/MS, encompassing free hormones and their phase I metabolites, as well as phase II metabolites such as glucuronides and sulfates.

General analysis of the samples

The method was able to define an extended steroidomic profile in all the studied conditions. During the analysis, a young girl who was diagnosed with PCOS showed abnormally high levels of testosterone and other androgenic metabolites, therefore her sample was considered an outlier and excluded. While analyzing the pool, one man with infertility was diagnosed with DSD 46, XX through karyotype analysis, therefore analyzed differently from the other samples obtained by born male individuals.

The analyzed free steroids are enlisted in Table 1, supplementary material. A total number of 29 analytes were considered of interest for the current study, both classical and first pass metabolites.

First, we assessed born female (n=49) and born male (n=34) samples, divided as follows: 22 from AMAB people with GI, 12 from men with infertility, 34 from AFAB people with GI and 15 from girls with PCOS. We excluded the previous girl with PCOS and the 46, XX DSD man.

First, from a general comparison, classic steroids were consistent with the assigned sex of the enrolled individuals. Testosterone was higher in born male group (443.11 [277.35-587.1] vs 5914.93 [3831.03-8372.84] pg/mL, U=0; $p<0.001$) while androstenedione was not (1660.44 [1084.18-2386.6] vs 990.12 [767.27-1526.82] pg/mL, U=411; $p=0.05$). Among the free androgen metabolites, but with the same consistency shown before, DHT (129.08 [77.2-224.17] vs 475.46 [298.36-802.23] pg/mL, U=108; $p<0.001$) and 16a-OHTestosterone (43.53 [28.98-60.11] vs 60.2 [41.9-76.76] pg/mL, U=476; $p =0.008$) were higher in born male while estrone (40.95 [25.54-63.61] vs 27.37 [20.4-41.45] pg/mL, U=472; $p<0.001$) was more present in the other group (Table 3).

Considering the second phase metabolites, Table 2 (supplementary material) enlists glucuronidated and sulfated metabolites that were assessed in the collected samples. Due to technical adjustments, only 77 out of 85 (n=45 born female and 32 born male) were considered adequate for the analysis.

The method was able to investigate a total of 26 derivatives but for the study, we retained a total of 24 metabolites, excluding the gluco- and mineralocorticoids analytes. We compared the distributions between assigned male and female individuals in our cohort. Also, the androgen derived metabolites were concordantly higher in the born male group, such as 5aab-diol 17-Glucoronate, 5bab-diol 17-Glucoronate, 5xab-diol 3-Glucoronate, Androsterone Glucoronate, Testosterone Glucoronate, Epitestosterone Sulphate, Testosterone Sulphate and 5-Androstenediol Sulphate in the assigned male group ($p<0.05$). The method properly detected the sex specific differences in the analyzed metabolites (Table 4).

We have enlisted the LLOQ (Lower Limit of Quantification) of the method used with the LC MS/MS approach, referring to the minimum concentration of an analyte that can be quantified with acceptable precision and accuracy. The LLOQ ensures that results are reliable even at very low concentrations. The report can be found in the Supplementary Material section, Table 3 and 4.

Comparisons between the investigated groups

In the second part of the study, a comparison was performed between the different groups.

First, we revised the profile shown by transgender people, and we did not outline a specific pattern among the first and second phase metabolites. Subsequently, their clinical history was revised and people with conceivable steroid related diseases, such as AFAB individuals with signs of hyperandrogenism and AMAB people with history of drug abuse were excluded. Referring to them as healthy people without any known androgen related disruption, we considered them as reference groups.

For this analysis, the total number of available samples for this analysis were 75. Among the investigated people we assessed: 28 AFAB and 21 AMAB with gender incongruence, 15 girls with PCOS and 11 men with infertility. The statistical comparisons between groups were carried out using Mann-Whitney test with the statistical significance corrected for multiple comparisons using post-hoc tests whether necessary.

First, an initial evaluation was made by visualizing the interplay of the data using box and whisker plots and scatter plots; this helped to choose the metabolites that could be most probably different between the different assigned sex at birth groups.

Between assigned male at birth individuals and men with idiopathic infertility (Table 5), the latter showed higher levels of the steroid precursor pregnenolone (1743.94 [1167.41-2498.15] vs 4872.77 [2741.26-12327.4], $U=174$; $p<0.001$). Among the metabolites, the infertility group showed higher levels of pregnanetriol (1977.69 [1316.58-3210.58] vs 4268.56 [2419.44-5726.48], $U=174$; $p=0.014$), allopregnanolone (221.14 [198.18-1085.45] vs 3000.18 [636.62-5829.24], $U=110$; $p=0.006$) and 5-dihydroprogesterone (45.37 [29.19-98.24] vs 173.76 [111.37-597.1], $U=124$; $p<0.001$). On the other hand, lower levels of 7a,hydroxydehydroepiandrosterone in the infertility group (189.91 [121.97-292.96] vs 66.72 [22.87-98.99], $U=15$;$p=0.001$) were assessed.

The abovementioned evaluations were confirmed even after controlling the comparisons for age and BMI (pregnenolone p<0.001, R^2adj: 0.424; pregnanetriol p<0.025, R^2adj: 0.225; allopregnanolone p=0.01, R^2adj: 0.384, 5-dihydroprogesterone p=0.006, R^2adj: 0.40; 7a,hydroxydehydroepiandrosterone, p=0.020, R^2adj: 0.288).
Furthermore, we compared these findings also with the assigned female groups and the evidence from literature, and the emerged trend was confirmed for the infertility group (Attal-Khémis et al., 1998).
In regard to the AFAB group who did not report any sign of androgen related disease (Table 6), we compared them with girls with PCOS. Only testosterone showed to be higher in PCOS group (373.32 [175.18-513.28] vs 585.54 [421.26-695.62], U=319; p=0.002), also when controlling for age (p=0.009, R^2adj:0.201), but not for BMI (p=0.052, R^2adj:0.117) and for age and BMI (p=0.059, R^2adj:0.130).

The same approach was repeated for second phase analytes.
Men with idiopathic infertility showed lower levels of DHEAS (2770.52 [1850-3462.12] vs 1624.07 [1333.58-2331.01] ng/mL; U=49, p=0.016), androsterone sulphate (764.29 [459.26-1483.77] vs 328.08 [273-482.94] pg/mL; U=40, p=0.005) and epiandrosterone sulphate (79.73 ± 43.62 vs 46.02 ± 15.55 pg/mL; t=3.05, P=0.005), Table 7.
After correction with age and BMI as covariates, DHEAS (p<0.001, R^2adj=0.415) and androsterone sulphate (P=0.014, R^2adj=0.255) retained this significance; epiandrosterone sulphate, however, showed to be still significant when adjusted for age (p=0.045, R^2adj: 0.146) but not when controlling also for BMI (p=0.226). It has to be considered that DHEAS is renowned to be age dependent and its levels lower with aging (Guazzo et al., 1996).
A slight difference in DHT derivatives was shown in PCOS women, Table 8; in fact, they showed higher levels of 5bab-diol-17glucuronide (0.66 [0.40-1.32] vs 1.86 [0.67-5.39] ng/mL, U=242, p=0.029) and 5xab-diol-3glucuronide (0.84 [0.54-1.54] vs 1.08 [0.87-2.20] ng/ml; U=237, p=0.043). After adjusting variables using the ANCOVA analysis, this finding was still significant controlling for BMI and age (5bab-diol-17glucuronide, p=0.044, R^2adj=0.157 and 5xab-diol-17glucuronide: p=0.046 R^2adj=0.154).

Comparing with reference ranges of control groups
In the third step of the analysis, we compared the reference ranges obtained in our cohort with those obtained from volunteers whose blood samples were analyzed using a similar approach (for further information about the approach used, please refer to Ponzetto et al., 2023). We proceeded with the comparisons for the analytes which a reference range was available. Therefore, we used Z tests to compare AMAB people with gender incongruence and men with infertility, with control men (Table 9). Subsequently we compared AFAB people with GI and women with PCOS with reference women (Table 10).
AMAB individuals with gender incongruence exhibited significantly higher levels of the DHT derivative 5aab-diol 17- glucuronide (2.55 [1.65-2.95] vs 1.36 [0.85-2.12], Z = 4.69, p < 0.001), androsterone sulfate (887.14 ± 654.88 vs 376.4 ± 212, Z = 3.48, p = 0.002), DHEAS (2701.67

[1772.25-3382.19] vs 1450 [940-2140], Z = 5.23, p < 0.001), and epitestosterone sulfate (0.39 [0.26-0.7] vs 0.20 [0.12-0.27], Z = 6.35, p < 0.001) compared to control men.
Men with infertility exhibited significantly higher levels of epitestosterone sulfate (0.48 [0.33-0.53] vs 0.20 [0.12-0.27], Z = 7.45, p < 0.001) compared to control men.
On the other hand, AFAB individuals with gender incongruence exhibited significantly higher levels of the DHT derivate 5aab-diol 17-glucuronate (1.05 [0.62-1.61] vs 0.29 [0.14-0.51], Z = 5.23, p < 0.001) and androsterone sulfate (519.37 [347.54-754.35] vs 285.9 [174.4-478.1], Z = 3.48, p < 0.001) compared to control women. Individuals with PCOS showed higher levels of etiocholanolone glucuronide (22.25 [14.52-27.33] vs 13.1 [9.2-22.0], Z = 3.19, p = 0.001), androsterone sulfate (609.80 [467.04-817.00] vs 285.9 [174.4-478.1], Z = 4.27, p < 0.001), and DHEAS (2236.08 [1632.75-2886.83] vs 1300 [770-1660], Z = 3.58, p < 0.001) compared to control women.
AMAB and AFAB individuals with gender incongruence and men with infertility showed significantly lower levels of androsterone glucuronide and epiandrosterone sulfate compared to reference men and women. Only PCOS girls showed lower levels of androsterone glucuronate but not of epiandrosterone sulfate, (54.51 [44.58-83.13] vs 68.9 [40.4 - 123.1]; Z = -1.18, P=0.24).

Table 3. Mean and median values of free steroids considering the whole cohort of assigned female and male individuals.

Free Steroids	AFAB (Mean ± SD)	AFAB (Median [25-75])	AMAB (Mean ± SD)	AMAB (Median [25-75])
11b-Hydroxyandrostenedione (pg/mL)	855.85 ± 507.94	716.88 [450.54-1152.15]	832.72 ± 554.01	657.48 [372.98-1119.06]
11b-Hydroxytestosterone (pg/mL)	52.60 ± 33.87	43.2 [24.11-69.96]	57.56 ± 43.95	42.43 [26.58-73.79]
11-Ketoandrostenedione (pg/mL)	334.13 ± 292.02	250.1 [140.45-483.09]	323.84 ± 262.87	249.71 [168.23-371.86]
11-Ketotestosterone (pg/mL)	257.48 ± 181.09	255.12 [114.49-385.15]	251.86 ± 184.85	218.05 [71.93-417.2]
16a-Hydroxyandrostenedione (pg/mL)	88.32 ± 149.08	31.99 [20.97-91.22]	68.26 ± 88.60	30.2 [16.59-75.73]
16a-Hydroxyprogesterone (pg/mL)	93.00 ± 69.73	80.75 [46.04-110.83]	96.24 ± 57.71	90.61 [44.97-133.31]
16a-Hydroxytestosterone (pg/mL)	47.70 ± 21.28	43.53 [28.98-60.11]	64.40 ± 31.17	60.2 [41.9-76.76]
17a,20a-Dihydroxyprogesterone (pg/mL)	196.87 ± 139.73	164.84 [98.24-245.84]	399.98 ± 256.02	335.8 [218.44-518.43]
17-Hydroxypregnenolone (pg/mL)	2817.75 ± 2759.83	2284.39 [934.26-3684.19]	2062.94 ± 1574.71	1551.99 [782.36-3346.07]
17-Hydroxyprogesterone (pg/mL)	539.85 ± 505.45	392.52 [213.64-641.04]	1109.36 ± 770.52	831.67 [573.74-1569.79]
20a-Dihydroprogesterone (pg/mL)	147.67 ± 340.26	56.54 [41.52-90.68]	48.37 ± 24.26	43.6 [32.97-58.77]
2b-Hydroxytestosterone (pg/mL)	10.40 ± 4.33	9.11 [7.23-12.94]	9.44 ± 3.19	8.95 [6.94-10.86]

5a-Androstanedione (pg/mL)	102.24 ± 78.24	69.75 [45.47-140.63]	74.46 ± 44.43	72.63 [31.58-102.6]
5a-Dihydroprogesterone (pg/mL)	135.27 ± 157.04	62.55 [36.52-164.8]	175.18 ± 233.13	87.92 [44.15-184.52]
5-Androstenediol (pg/mL)	9190.02 ± 4832.85	7858.14 [5492.41-11285.75]	7732.38 ± 4307.36	6806.92 [5083.4-10804.48]
5b-Dihydroprogesterone (pg/mL)	19029.95 ± 14329.54	13579.6 [9623.89-23639.34]	14955.48 ± 8692.74	12608.6 [8886.95-21174.23]
7a-Hydroxydehydroepiandrosterone (pg/mL)	222.40 ± 158.24	168.33 [88.08-313.37]	160.32 ± 120.13	133.51 [70.3-249.23]
Allopregnanolone (pg/mL)	1199.50 ± 1729.60	596.23 [306.23-1668.74]	2319.16 ± 3917.92	975.79 [218.19-3000.18]
Androstenedione (pg/mL)	1899.55 ± 1126.24	1660.44 [1084.18-2386.6]	1191.84 ± 783.42	990.12 [767.27-1526.82]
DHEA (pg/mL)	4432.62 ± 3174.22	3047.5 [2345.79-6109.32]	3041.86 ± 2213.03	2531.86 [1727.88-4000.2]
DHT (pg/mL)	165.76 ± 125.19	129.08 [77.2-224.17]	544.99 ± 305.75	475.46 [298.36-802.23]
Epitestosterone (pg/mL)	13.50 ± 1.78	12.99 [12.03-None]	23.50 ± 13.64	20.22 [12.52-30.6]
Estrone (pg/mL)	241.04 ± 1330.09	40.95 [25.54-63.61]	32.99 ± 20.20	27.37 [20.4-41.45]
Pregnanetriol (pg/mL)	3172.50 ± 2734.82	2487.72 [1423.08-3739.33]	3235.80 ± 3118.93	2271.29 [1362.09-4226.08]
Pregnanolone (pg/mL)	1271.59 ± 1003.29	958.36 [580.05-1396.0]	1326.07 ± 736.17	1156.71 [766.5-1673.21]
Pregnenolone (pg/mL)	2311.99 ± 2619.67	1203.57 [663.16-3006.1]	4050.69 ± 4864.74	2105.19 [1454.99-4508.29]
Progesterone (pg/mL)	374.69 ± 1139.03	91.93 [69.83-148.44]	92.80 ± 51.35	74.7 [60.09-107.85]

Testosterone (pg/mL)	467.93 ± 255.29	443.11 [277.35-587.1]	6330.47 ± 3042.05	5914.93 [3831.03-8372.84]
11-Ketodihydrotestosterone (pg/mL)	1247.82 ± 1313.22	980.4 [642.99-1400.34]	1094.95 ± 812.97	916.27 [391.48-1632.69]

Table 4. Mean and median values of phase II metabolites considering the whole cohort of assigned female and male individuals.

Phase II Metabolites	AFAB (Mean ± SD)	AFAB (Median [25-75])	AMAB (Mean ± SD)	AMAB (Median [25-75])
17-Hydroxypregnanolone Gluc (ng/mL)	0.74 ± 0.87	0.43 [0.33-0.77]	0.51 ± 0.69	0.35 [0.18-0.54]
5aab-diol 17-Gluc (ng/mL)	1.24 ± 0.89	1.01 [0.57-1.63]	2.68 ± 1.36	2.52 [1.68-3.17]
5bab-diol 17-Gluc (ng/mL)	1.69 ± 2.07	0.99 [0.43-2.05]	5.80 ± 5.24	4.04 [2.44-7.35]
5xab-diol 3-Gluc (ng/mL)	1.34 ± 1.03	0.92 [0.75-1.81]	2.06 ± 0.91	1.99 [1.36-2.46]
Androsterone Gluc (ng/mL)	10.03 ± 6.46	7.34 [5.11-14.28]	12.00 ± 4.63	12.36 [7.66-14.59]
Etiocholanolone Gluc (ng/mL)	18.74 ± 9.33	16.88 [11.78-26.72]	18.69 ± 8.88	16.80 [13.72-21.51]
Pregnanediol Gluc (ng/mL)	11.29 ± 11.38	7.20 [5.52-12.06]	8.05 ± 6.92	6.21 [4.17-9.49]
Testosterone Gluc (pg/mL)	77.41 ± 24.50	68.03 [58.89-100.61]	262.98 ± 181.61	223.60 [141.92-304.10]
16a-HydroxyDHEA Sulf (ng/mL)	97.78 ± 78.24	72.87 [49.29-120.31]	89.85 ± 87.43	60.42 [34.88-108.57]
17a,20b-Dihydroxyprogesterone Sulf (ng/mL)	3.24 ± 2.81	2.55 [1.64-3.55]	4.42 ± 3.27	2.90 [1.99-6.95]
17-Hydroxypregnenolone 3-Sulf (ng/mL)	4.03 ± 2.96	3.46 [1.81-5.27]	6.83 ± 4.12	6.55 [4.25-8.71]
5-Androstenediol Sulf (ng/mL)	49.46 ± 29.55	44.00 [26.53-61.21]	72.51 ± 47.73	56.75 [40.97-88.52]
Allopregnanolone Sulf (ng/mL)	2.52 ± 5.16	1.01 [0.62-1.99]	1.27 ± 0.89	0.94 [0.67-1.78]

Androsterone Sulf (ng/mL)	689.42 ± 541.79	550.38 [368.14-741.31]	720.36 ± 587.76	561.77 [314.55-780.24]
Dehydroandrosterone Sulf (ng/mL)	9.49 ± 7.64	7.37 [4.57-11.27]	8.65 ± 7.46	6.53 [4.91-8.80]
DHEAS (ng/mL)	2069.10 ± 982.12	1908.70 [1326.78-2664.02]	2586.73 ± 1517.75	2272.26 [1632.97-3261.75]
Epiallopregnanolone Sulf (ng/mL)	2.34 ± 5.49	0.93 [0.36-2.39]	1.20 ± 1.28	0.81 [0.42-1.38]
Epiandrosterone Sulf (ng/mL)	58.22 ± 37.02	48.97 [37.96-62.19]	66.70 ± 37.81	57.78 [43.88-81.25]
Epipregnanolone Sulf (pg/mL)	229.70 ± 169.80	170.87 [120.78-254.98]	297.04 ± 426.31	163.25 [100.41-343.46]
Epitestosterone Sulf (pg/mL)	189.92 ± 153.43	156.50 [101.48-226.24]	542.63 ± 456.67	455.79 [314.11-659.79]
Etiocholanolone Sulf (ng/mL)	39.88 ± 39.88	26.27 [14.65-53.27]	27.50 ± 25.99	16.28 [5.20-52.49]
Pregnanolone Sulf (ng/mL)	0.67 ± 0.87	0.42 [0.26-0.65]	0.34 ± 0.27	0.26 [0.17-0.42]
Pregnenolone Sulf (ng/mL)	23.22 ± 15.20	18.76 [12.37-29.37]	32.56 ± 21.75	26.41 [15.08-48.02]
Testosterone Sulf (pg/mL)	113.97 ± 69.81	104.21 [60.38-142.92]	705.40 ± 760.84	364.12 [205.42-982.24]

Table 5. Mean and median values of free steroids of the assigned male groups

Free Steroids	AMAB with GI (Mean ± SD)	AMAB with GI (Median [25-75])	INFERTILITY (Mean ± SD)	INFERTILITY (Median [25-75])
11β-Hydroxyandrostenedione (pg/mL)	823.01 ± 554.66	638.72 [374.14-1261.88]	749.53 ± 469.11	738.18 [296.29-1069.26]
11β-Hydroxytestosterone (pg/mL)	62.6 ± 52.98	42.84 [25.51-92.21]	49.77 ± 24.79	41.17 [25.53-73.72]

11-Ketoandrostenedione (pg/mL)	357.08 ± 298.06	249.9 [184.63-401.01]	262.91 ± 177.3	249.02 [112.17-367.38]
11-Ketotestosterone (pg/mL)	255.94 ± 189.99	237.51 [75.02-433.22]	243.94 ± 183.39	190.45 [57.62-336.32]
16α-Hydroxyandrostenedione (pg/mL)	80.0 ± 98.8	34.21 [16.95-104.74]	31.09 ± 18.91	25.69 [14.51-53.41]
16α-Hydroxyprogesterone (pg/mL)	106.08 ± 61.88	102.45 [59.44-136.14]	78.2 ± 46.18	64.67 [35.33-119.84]
16α-Hydroxytestosterone (pg/mL)	71.27 ± 34.61	66.89 [43.07-86.12]	51.82 ± 18.99	55.74 [37.38-64.12]
17α,20α-Dihydroxyprogesterone (pg/mL)	394.98 ± 187.76	378.57 [272.52-519.22]	409.14 ± 359.44	299.53 [207.63-474.2]
17-Hydroxypregnenolone (pg/mL)	2368.96 ± 1714.17	1632.19 [807.37-3905.35]	1552.9 ± 1207.82	1345.28 [534.21-2138.83]
17-Hydroxyprogesterone (pg/mL)	1129.13 ± 704.78	1008.29 [550.54-1569.79]	1073.11 ± 911.38	682.95 [565.0-1553.37]
20α-Dihydroprogesterone (pg/mL)	48.0 ± 20.52	44.37 [33.05-57.46]	49.11 ± 31.58	40.49 [29.43-65.86]
2β-Hydroxytestosterone (pg/mL)	9.66 ± 3.48	8.98 [6.83-10.89]	8.83 ± 2.34	8.92 [7.16-9.83]
5α-Androstanedione (pg/mL)	71.83 ± 46.74	64.73 [30.46-92.16]	79.73 41.54	99.39 [38.26-113.37]
5-Androstenediol (pg/mL)	8584.41 ± 4638.03	7350.67 [6102.07-11829.11]	6170.33 ± 3237.16	5447.74 [3895.57-7966.91]
5α-Dihydroprogesterone (pg/mL)	69.96 ± 53.13	46.43 [29.33-95.41]	333.02 ± 307.26	173.76 [111.37-597.1]
5β-Dihydroprogesterone (pg/mL)	15700.89 ± 9174.26	12982.75 [8886.95-22695.98]	13588.92 ± 7928.44	11674.13 [7672.0-17788.63]
7α-Hydroxydehydroepiandrosterone (pg/mL)	199.83 ± 120.84	178.64 [113.08-262.03]	66.49 ± 41.83	66.72 [22.87-98.99]
DHEA (pg/mL)	3523.09 ± 2462.65	3005.63 [1773.43-5061.08]	2159.62 ± 1342.48	2062.68 [1136.16-2795.99]

Androstenedione (pg/mL)	1339.86 ± 842.42	1056.41 [862.98-1637.13]	920.48 ± 601.91	893.28 [374.68-1194.55]
DHT (pg/mL)	547.36 ± 302.64	475.46 [313.28-764.43]	540.65 ± 324.89	523.32 [227.57-830.07]
11-Ketodihydrotestosterone (pg/mL)	926.49 ± 647.62	763.5 [358.42-1494.65]	1403.8 ± 1010.59	1353.84 [554.45-2361.77]
Testosterone (pg/mL)	6019.26 ± 2860.82	5410.95 [3330.24-8563.37]	6901.04 ± 3404.22	6392.94 [4318.49-8404.84]
Epitestosterone (pg/mL)	27.37 ± 14.65	24.01 [19.56-31.83]	16.44 ± 8.58	12.52 [11.13-22.18]
Estrone (pg/mL)	32.53 ± 22.4	24.62 [20.07-40.43]	16.43 ± 8.58	31.23 [21.79-44.98]
Allopregnanolone (pg/mL)	554.8 ± 493.61	221.14 [198.18-1085.45]	4243.92 ± 5068.67	3000.18 [636.62-5829.24]
Pregnanetriol (pg/mL)	2109.24 ± 1214.02	1850.18 [1247.41-2954.31]	5301.17 ± 4379.24	4268.56 [2419.44-5726.48]
Pregnanolone (pg/mL)	1305.21 ± 644.36	1156.71 [819.61-1737.39]	1364.31 ± 911.44	1210.76 [663.22-1525.65]
Pregnenolone (pg/mL)	2009.24 ± 1690.18	1721.25 [1035.53-2470.17]	7576.84 ± 6472.49	4872.77 [2741.26-12327.4]
Progesterone (pg/mL)	95.71 ± 54.42	74.7 [66.96-115.26]	86.98 ± 46.81	69.56 [53.26-104.22]

Table 6. Mean and median values of free steroids of the assigned female groups

Free Steroids	**AFAB with GI (Mean ± SD)**	**AFAB with GI (Median [25-75])**	**PCOS (Mean ± SD)**	**PCOS (Median [25-75])**
11β-Hydroxyandrostenedione (pg/mL)	824.71 ± 435.96	719.8 [458.0-1110.01]	875.26 ± 647.76	716.88 [442.8-1325.92]
11β-Hydroxytestosterone (pg/mL)	46.1 ± 28.37	42.34 [22.38-62.08]	71.03 ± 42.17	58.38 [34.12-113.69]

11-Ketoandrostenedione (pg/mL)	306.03 ± 233.99	213.84 [146.83-482.31]	397.84 ± 396.31	321.5 [139.03-579.31]
11-Ketotestosterone (pg/mL)	227.34 ± 153.6	203.73 [106.3-314.3]	330.71 ± 224.78	352.42 [146.14-431.56]
16α-Hydroxyandrostenedione (pg/mL)	82.7 ± 171.98	25.85 [20.15-47.43]	98.81 ± 97.27	62.34 [27.76-165.7]
16α-Hydroxyprogesterone (pg/mL)	94.74 ± 68.08	82.62 [47.25-111.58]	88.76 ± 76.06	70.25 [39.47-105.16]
16α-Hydroxytestosterone (pg/mL)	45.25 ± 18.63	41.32 [28.55-59.65]	53.67 ± 26.48	46.95 [30.77-80.24]
17α,20α-Dihydroxyprogesterone (pg/mL)	195.95 ± 136.55	162.8 [98.9-253.43]	198.97 ± 151.61	170.42 [95.65-214.04]
17-Hydroxypregnenolone (pg/mL)	3079.31 ± 3007.5	2417.43 [1080.78-3891.91]	1980.78 ± 1587.9	2180.55 [350.91-3022.1]
17-Hydroxyprogesterone (pg/mL)	551.63 ± 515.46	455.22 [215.28-643.79]	513.15 ± 498.52	318.71 [203.29-609.23]
20α-Dihydroprogesterone (pg/mL)	128.81 ± 257.96	57.12 [41.8-89.49]	190.43 ± 487.12	53.29 [40.0-99.0]
2β-Hydroxytestosterone (pg/mL)	10.01 ± 3.08	9.34 [7.19-13.07]	11.01 ± 5.94	8.88 [6.82-14.1]
5α-Androstanedione (pg/mL)	102.08 ± 80.30	75.53 [32.62-140.84]	102.70 ± 76.30	65.11 [45.47-148.7]
5-Androstenediol (pg/mL)	9746.6 ± 4971.94	8630.37 [6010.67-11377.43]	7928.44 ± 4399.15	7275.92 [4268.41-11366.74]
5α-Dihydroprogesterone (pg/mL)	132.88 ± 156.02	61.49 [39.0-160.13]	141.65 ± 166.57	75.77 [35.61-201.46]
5β-Dihydroprogesterone (pg/mL)	18718.27 ± 12894.28	13627.82 [9844.73-23926.95]	19736.43 ± 17644.65	12710.8 [9276.0-23440.12]
7α-Hydroxydehydroepiandrosterone (pg/mL)	208.24 ± 139.69	151.8 [90.14-309.95]	255.78 ± 197.14	233.28 [58.02-418.36]
DHEA (pg/mL)	4293.64 ± 2942.07	2985.6 [2404.23-5158.53]	4747.66 ± 3740.19	3047.5 [2101.84-6402.05]

Androstenedione (pg/mL)	1774.7 ± 1004.08	1608.73 [970.17-2304.8]	2182.58 ± 1359.69	1663.67 [1160.55-3135.21]
DHT (pg/mL)	147.28 ± 102.29	104.27 [74.16-222.89]	208.43 ± 163.53	157.82 [103.28-285.75]
11-Ketodihydrotestosterone (pg/mL)	1188.9 ± 690.37	995.15 [719.06-1382.79]	1381.37 ± 2182.15	832.32 [225.13-1481.93]
Testosterone (pg/mL)	406.67 ± 238.02	414.78 [188.04-517.59]	602.7 ± 246.96	585.54 [421.26-695.62]
Epitestosterone (pg/mL)	12.51 ± 0.67	12.51 [12.03-nan]	22.01 ± 10.5	
Estrone (pg/mL)	318.75 ± 1597.54	38.92 [17.49-55.88]	474.76 ± 374.02	43.89 [39.66-87.09]
Allopregnanolone (pg/mL)	1026.61 ± 931.96	644.26 [339.47-1632.71]	1841.68 ± 3424.22	298.52 [127.37-2210.5]
Pregnanetriol (pg/mL)	3402.9 ± 3019.0	2547.79 [1521.65-4317.92]	2650.26 ± 1933.42	2487.72 [1332.96-3143.31]
Pregnanolone (pg/mL)	1245.8 ± 1118.64	935.68 [544.58-1231.47]	1330.05 ± 704.45	1229.25 [860.27-2147.89]
Pregnenolone (pg/mL)	2533.23 ± 2769.1	1407.48 [659.4-3406.06]	1825.28 ± 2267.87	811.14 [657.63-2008.98]
Progesterone (pg/mL)	297.16 ± 831.8	96.43 [70.32-161.63]	559.56 ± 1693.13	87.52 [63.39-105.78]

Table 7. Mean and median values of phase II metabolites of the assigned male groups

Phase II Metabolites	AMAB with GI (Mean ± SD)	AMAB with GI (Median [25-75])	INFERTILITY (Mean ± SD)	INFERTILITY (Median [25-75])
17-Hydroxypregnanolone Gluc (ng/mL)	0.3727 ± 0.2181	0.3471 [0.2187-0.5134]	0.7929 ± 1.1573	0.3384 [0.1794-1.0212]
5aab-diol 17-Gluc (ng/mL)	2.6226 ± 1.3314	2.5463 [1.6470-2.9466]	2.7971 ± 1.4644	2.1339 [1.7027-3.7590]
5bab-diol 17-Gluc (ng/mL)	5.8125 ± 5.1184	4.3203 [2.3401-7.8221]	5.7644 ± 5.7406	3.3053 [2.3946-6.5453]
5xab-diol 3-Gluc (ng/mL)	2.1336 ± 0.8563	2.2026 [1.4396-2.4993]	1.9264 ± 1.0269	1.5334 [1.3531-2.1809]
Androsterone Gluc (ng/mL)	12.5046 ± 4.1852	12.9437 [8.9070-14.5197]	10.9774 ± 5.4909	8.3135 [6.4410-14.7562]
Etiocholanolone Gluc (ng/mL)	19.3965 ± 9.0618	17.2099 [14.7505-22.9548]	17.2902 ± 8.7545	16.8045 [10.7249-20.5939]
Pregnanediol Gluc (ng/mL)	7.6297 ± 3.2624	6.7522 [5.5011-9.8151]	8.8840 ± 11.3880	4.4122 [3.4443-8.5964]
Testosterone Gluc (pg/mL)	262.77 ± 150.09	240.55 [143.11-326.09]	263.46 ± 248.87	196.35 [124.08-286.28]
16a-HydroxyDHEA Sulf (ng/mL)	103.59 ± 96.22	70.29 [40.72-152.40]	62.38 ± 61.43	37.83 [26.00-99.22]
17a,20b-Dihydroxyprogesterone Sulf (ng/mL)	5.0648 ± 3.5607	4.1352 [2.4412-7.3983]	3.1336 ± 2.1802	2.1272 [1.5846-4.2690]
17-Hydroxypregnenolone 3-Sulf (ng/mL)	7.3912 ± 3.5170	7.1299 [5.4189-9.5675]	5.6931 ± 5.1164	4.3781 [3.3615-7.7060]
5-Androstenediol Sulf (ng/mL)	82.91 ± 53.57	58.86 [45.03-129.26]	51.71 ± 23.44	43.55 [36.59-67.58]
Allopregnanolone Sulf (ng/mL)	1.1985 ± 0.7732	0.9401 [0.6932-1.4981]	1.4267 ± 1.1411	0.9644 [0.4841-2.4411]
Androsterone Sulf (ng/mL)	887.14 ± 654.88	716.88 [442.15-1283.59]	386.78 ± 145.78	328.08 [273.00-482.94]

Dehydroandrosterone Sulf (ng/mL)	9.8906 ± 8.7780	7.1299 [5.8464-10.2128]	6.1709 ± 2.4996	5.5555 [4.8207-8.8880]
DHEAS (ng/mL)	2964.87 ± 1643.28	2701.67 [1772.25-3382.19]	1830.45 ± 869.98	1624.07 [1263.40-2759.65]
Epiallopregnanolone Sulf (ng/mL)	1.25 ± 1.32	0.83 [0.45-1.47]	1.09 ± 1.22	0.49 [0.37-1.47]
Epiandrosterone Sulf (ng/mL)	77.04 ± 41.56	64.34 [47.00-110.87]	46.02 ± 15.56	44.42 [31.96-55.66]
Epipregnanolone Sulf (pg/mL)	219.21 ± 158.40	174.62 [118.02-270.92]	444.05 ± 693.23	130.75 [90.49-572.33]
Epitestosterone Sulf (pg/mL)	558.85 ± 542.58	390.95 [262.84-703.14]	510.20 ± 217.68	482.44 [331.16-533.97]
Etiocholanolone Sulf (ng/mL)	32.47 ± 26.78	28.64 [6.05-56.39]	17.57 ± 22.20	5.33 [4.03-23.32]
Pregnanolone Sulf (ng/mL)	0.32 ± 0.18	0.28 [0.18-0.41]	0.39 ± 0.41	0.19 [0.13-0.51]
Pregnenolone Sulf (ng/mL)	35.75 ± 23.15	27.86 [16.52-57.49]	26.17 ± 17.89	19.50 [8.77-46.89]
Testosterone Sulf (pg/mL)	631.27 ± 750.76	332.44 [156.21-867.38]	853.66 ± 795.29	374.43 [276.32-1474.54]

Table 8. Mean and median values of phase II metabolites of the assigned female groups

Phase II Metabolites	**AFAB with GI (Mean ± SD)**	**AFAB with GI (Median [25-75])**	**PCOS (Mean ± SD)**	**PCOS (Median [25-75])**
17-Hydroxypregnanolone Gluc (ng/mL)	0.75 ± 0.97	0.42 [0.28-0.75]	0.73 ± 0.63	0.49 [0.38-0.85]
5aab-diol 17-Gluc (ng/mL)	1.19 ± 0.85	1.05 [0.62-1.61]	1.37 ± 1.00	0.90 [0.53-2.45]
5bab-diol 17-Gluc (ng/mL)	1.10 ± 0.98	0.68 [0.41-1.69]	3.09 ± 3.15	1.86 [0.67-5.39]
5xab-diol 3-Gluc (ng/mL)	1.26 ± 1.12	0.85 [0.58-1.65]	1.53 ± 0.79	1.08 [0.88-2.21]
Androsterone Gluc (ng/mL)	10.22 ± 6.70	8.15 [5.10-14.35]	9.58 ± 6.08	7.23 [5.10-14.89]
Etiocholanolone Gluc (ng/mL)	17.58 ± 9.47	13.81 [9.50-27.30]	21.50 ± 8.72	22.25 [14.52-27.33]

Pregnanediol Gluc (ng/mL)	11.29 ± 12.01	6.65 [4.96-12.12]	11.30 ± 10.18	8.36 [6.35-12.63]
Testosterone Gluc (pg/mL)	63.58 ± 6.29	63.58 [59.13-63.58]	86.62 ± 29.36	84.03 [58.65-84.03]
16a-HydroxyDHEA Sulf (ng/mL)	97.99 ± 87.91	65.29 [49.15-118.15]	97.29 ± 51.13	78.39 [58.66-148.11]
17a,20b-Dihydroxyprogesterone Sulf (ng/mL)	3.50 ± 2.89	2.53 [1.80-3.89]	2.62 ± 2.59	2.57 [0.86-3.23]
17-Hydroxypregnenolone 3-Sulf (ng/mL)	3.80 ± 2.96	2.45 [1.74-5.11]	4.57 ± 3.00	4.48 [2.90-5.50]
5-Androstenediol Sulf (ng/mL)	44.49 ± 29.16	32.60 [22.63-59.98]	61.31 ± 28.08	60.26 [47.75-64.37]
Allopregnanolone Sulf (ng/mL)	2.74 ± 5.90	1.01 [0.54-2.30]	2.00 ± 2.84	0.95 [0.69-1.83]
Androsterone Sulf (ng/mL)	675.38 ± 590.65	519.37 [347.54-754.35]	722.88 ± 421.83	609.80 [467.04-817.00]
Dehydroandrosterone Sulf (ng/mL)	8.89 ± 6.80	7.48 [4.20-11.60]	10.90 ± 9.51	7.26 [6.26-12.06]
DHEAS (ng/mL)	1964.32 ± 1025.53	1690.67 [1156.52-2660.71]	2318.98 ± 855.03	2236.08 [1632.75-2886.83]
Epiallopregnanolone Sulf (ng/mL)	2.62 ± 6.39	1.02 [0.42-2.35]	1.69 ± 2.44	0.38 [0.29-2.56]
Epiandrosterone Sulf (ng/mL)	55.37 ± 40.34	46.57 [33.72-61.14]	65.02 ± 27.76	54.51 [44.58-83.13]
Epipregnanolone Sulf (pg/mL)	246.37 ± 195.24	172.20 [100.73-317.88]	191.24 ± 80.61	170.87 [121.82-241.95]
Epitestosterone Sulf (pg/mL)	215.41 ± 168.96	167.27 [120.77-227.54]	133.07 ± 93.50	107.47 [57.94-196.77]
Etiocholanolone Sulf (ng/mL)	36.43 ± 33.56	25.79 [15.08-52.50]	48.12 ± 52.70	26.74 [13.85-65.20]
Pregnanolone Sulf (ng/mL)	0.71 ± 1.02	0.42 [0.23-0.67]	0.58 ± 0.44	0.47 [0.33-0.67]
Pregnenolone Sulf (ng/mL)	25.04 ± 16.21	18.75 [13.42-29.78]	18.86 ± 11.93	18.76 [9.01-28.03]
Testosterone Sulf (pg/mL)	118.11 ± 86.79	92.81 [60.28-163.05]	107.76 ± 44.51	115.15 [62.58-145.56]

Table 9. Comparisons of phase II metabolites between assigned male groups and reference male groups

Metabolites	AMAB with GI (Median [25-75])	INFERTILITY (Median [25-75])	CONTROL MEN Median [25-75])	Z_Score AMAB with GI vs CONTROLS	p_value AMAB with GI vs CONTROLS	Z score INFERTILITY vs CONTROLS	p_value INFERTILITY vs CONTROLS
5aab-diol 17-Gluc (ng/mL)	2.55 [1.65-2.95]	2.13 [1.70-3.76]	1.36 [0.85 - 2.12]	**4.69**	**<0.001**	**2.4**	**0.016**
5bab-diol 17-Gluc (ng/mL)	4.32 [2.34-7.82]	3.30 [2.39-6.54]	3.75 [2.43 - 5.00]	1.11	0.27	-0.69	0.49
Androsterone Gluc (ng/mL)	12.94 [8.90-14.52]	8.31 [6.44-14.76]	29.0 [22.5 - 37.7]	**-5.29**	**<0.001**	**-5.4**	**<0.001**
Etiocholanolone Gluc (ng/mL)	17.21 [14.75-22.95]	16.8045 [10.72-20.59]	19.2 [13.6 - 27.1]	-0.74	0.46	-0.7	0.48
Testosterone Gluc (ng/mL)	0.24 [0.14-1.1]	0.20 [0.12-2.9]	0.27 [0.19 - 0.35]	-0.94	0.35	-1.73	0.08
Androsterone Sulf (ng/mL) *	887.14 ± 654.88	386.78 ± 145.78	376.4 ± 212	**3.48**	**0.002**	0.19	0.85
DHEAS (ng/mL)	2701.67 [1772.25-3382.19]	1624.07 [1263.40-2759.65]	1450 [940 - 2140]	**5.23**	**<0.001**	0.58	0.29
Epiandrosterone Sulf (ng/mL)	64.34 [47.00-110.87]	44.42 [31.96-55.66]	126.7 [82.8 - 167.1]	**-3.71**	**<0.001**	**-3.87**	**<0.001**
Epitestosterone Sulf (ng/mL)	0.39 [0.26-0.7]	0.48 [0.33-0.53]	0.20 [0.12 - 0.27]	**6.35**	**<0.001**	**7.45**	**<0.001**
Etiocholanolone Sulf (ng/mL)	28.64 [6.05-56.39]	5.33 [4.03-23.32]	15.9 [5.80 - 51.5]	1.4	0.16	-0.92	0.35
Testosterone Sulf (ng/mL)	0.33 [0.16-0.87]	0.37 [0.27-1.47]	0.42 [0.28 - 0.67]	-1.16	0.24	-0.51	0.61

*expressed as mean ± SD, Welch test

Table 10. Comparisons of phase II metabolites between assigned female groups and reference female groups

Metabolite	AFAB with GI (Median [25-75])	PCOS (Median [25-75])	CONTROL WOMEN N=40	Z score AFAB with GI vs CONTROLS	p_value AFAB with GI vs CONTROLS	Z_Score PCOS vs CONTROLS	p_value PCOS vs CONTROLS
5aab-diol 17-Gluc (ng/mL)	1.05 [0.62-1.61]	0.90 [0.53-2.45]	0.29 [0.14 - 0.51]	**5.23**	**<0.001**	1.65	0.10
5bab-diol 17-Gluc (ng/mL)	0.68 [0.41-1.69]	1.86 [0.67-5.39]	1.25 [0.65 - 2.85]	-1.82	0.07	0.65	0.52
Androsterone Gluc (ng/mL)	8.15 [5.10-14.35]	7.23 [5.10-14.89]	17.7 [9.4 - 23.8]	**-4.49**	**<0.001**	**-4.15**	**0.03**
Etiocholanolone Gluc (ng/mL)	13.81 [9.50-27.30]	22.25 [14.52-27.33]	13.1 [9.2 - 22.0]	0.24	0.80	**3.19**	**0. 001**
Testosterone Gluc (pg/mL)	63.58 [59.13-63.58]	84.03 [58.65-84.03]	<50	N/A	N/A	N/A	N/A
Androsterone Sulf (ng/mL)	519.37 [347.54-754.35]	609.80 [467.04-817.00]	285.9 [174.4 - 478.1]	**3.48**	**<0.001**	**4.27**	**< 0.001**
DHEAS (ng/mL)	1690.67 [1156.52-2660.71]	2236.08 [1632.75-2886.83]	1300 [770-1660]	1.66	0.10	**3.58**	**< 0.001**
Epiandrosterone Sulf (ng/mL)	46.57 [33.72-61.14]	54.51 [44.58-83.13]	68.9 [40.4 - 123.1]	**-2.14**	**0.03**	-1.18	0.24
Epitestosterone Sulf (pg/mL)	167.27 [120.77-227.54]	107.47 [57.94-196.77]	<50	N/A	N/A	N/A	N/A
Etiocholanolone Sulf (ng/mL)	25.79 [15.08-52.50]	26.74 [13.85-65.20]	31.8 [16.4 - 43.7]	-0.98	0.33	-0.49	0.62

Testosterone Sulf (pg/mL)	92.81 [60.28-163.05]	115.15 [62.58-145.56]	<100	N/A	N/A	N/A	N/A

Discussion

In this study we aimed to collect samples from clinical conditions potentially related to androgen metabolism and outline an extended steroid target panel in the enrolled individuals. Talking about the method, this LC MS/MS tandem analysis was able to target 49 free steroids, including classic androgens and phase I metabolites, 29 of them directly related to androgen metabolites. Furthermore, we were able to simultaneously assess 28 phase II steroids, of which 24 androgen related.

This represents a novel approach to further investigate the physiology and pathophysiology underlying clinical conditions potentially linked to variations in sex steroid metabolism. In current clinical practice, hormonal assessments typically focus on a predefined set of androgens, such as testosterone, androstenedione, DHEAS, and 17OHP. While this is often sufficient to characterize the patient, adopting an amplified perspective could provide a more comprehensive understanding of the clinical case. Specifically, expanding the analysis to include additional metabolites within the androgen panel could offer valuable support in patient management.

This extended steroid assessment is already performed in the context of doping controls, where the use of various detection methods, both in urine and plasma, has proven effective in identifying even minimal levels of steroid misuse (Abushareeda et al., 2014). Therefore, a tailored panel is more representative of the investigated person, even for smaller concentrations of metabolites that otherwise would not be routinely assessed (Pozo et al., 2008). Furthermore, such methodology takes also into account the “intracrinology” concept. We are used to thinking of steroid production and metabolism as a mechanism only related to adrenal and gonadal glands. Nevertheless, researchers have shown how peripheral organs, such as adipose tissue, brain, bone, even cancers own their enzymatic machinery that further locally metabolize steroids, particularly androgens (Labrie et al., 2003). This further enriches our understanding of the individual’s metabolism, although it may also present a downside: incorporating additional information can make the overall picture more complex and challenging to interpret. Using this LC-MS/MS method, we were able to unveil the concentration of first pass metabolites and gluco- and sulfated analytes in certain specific conditions. The inclusion of both unconjugated and conjugated metabolites in a single analytical workflow offers a holistic view of steroid metabolism, providing insights into both active hormones and their excretory forms, thus broadening the scope of clinical and research applications.

Regarding some of the investigated metabolites, it has to be considered that there is no previous robust knowledge in literature; this is due to challenging steroids to assess, due to their similar steric and conformation characteristics, or due to really low concentrations that are hard to detect; in fact for some metabolites, such as 2b-Hydroxytestosterone, 5a-Androstanedione, 5a-Dihydroprogesterone, Allopregnanolone, the method was not able to detect them in the whole bunch of samples, in particular in born female subjects (supplementary table 3 and 4).

Considering phase II metabolites, the most complex analytes to investigate were the testosterone derivatives testosterone sulfate and glucuronide, due to really low concentrations in female samples (Olesti et al., 2021).
The continuous improvement of the method, refined over three years through internal and external quality controls, successfully addressed potential challenges. In particular, it was able to separate isobaric isomers that present similar molecular mass and charge, such as the 3-alpha-glucuronide, that was able to split it into its 3- and 17- derivatives; achieving efficient chromatographic separation enhances the accuracy and specificity of the analysis, ensuring reliable identification and quantification of the target compounds. Second, the sensitivity and the linearity were remarkable, and they were able to assess metabolites at vastly different concentrations, from a few picograms per milliliter (e.g., DHT in women) to micrograms per milliliter (e.g., dehydroepiandrosterone sulfate DHEAS); lastly, it was able to detect a wide range of free steroids and phase II metabolites in the same run, granting robustness to the method itself. Furthermore, the method was consistent with the hormonal sex dimorphism that one should expect. Considering the overall group of assigned male and female people, the former showed higher levels of testosterone, DHT, and their testosterone related analytes testosterone Glucuronide, Epitestosterone Sulfate, Testosterone Sulfate and DHT derivatives 5aab-diol 17-Glucuronide, 5bab-diol 17- Glucuronide, 5xab-diol 3-Glucuronide. The latter showed higher levels of DHEA and androstenedione. Therefore, the method can be considered reliable even considering the other metabolites investigated.
These achievements come with the experience gained during the validation of a previous method which used a similar approach and analyzed a smaller steroid profile, including glucocorticoids, androgens and some of their phase II metabolites, in healthy women and men (Ponzetto et al., 2023).
Gender identity is considered as a multifactorial aspect of an individual. This consists in making an individual define themselves, with an inner sense of self, as a female or a male or, occasionally, some categories different from male and female (*Diagnostic and Statistical Manual of Mental Disorders*, 2013). Gender identity is constituted by a complex interplay of genetic, familiar, social and somewhat hormonal aspects. Regarding hormonal assessment, no definitive evidence regarding a disruption of modifications of sexual steroids have been highlighted in this population. It has been postulated an influence of certain sex hormones in brain development but also prenatal causes and puberty androgen exposure have been called into question. Studies that involved DSD 46, XY individuals, and DSD 46, XX with CAH, showed in the first case, a tendency to identify as a female in the presence of a lack of AR activity, while in the latter, XX CAH showed a tendency to behave in a more “masculine” way regarding sport activities and lifestyle, and studies have reported a higher incidence of gender incongruence (Fisher et al., 2018).
We were able to assess an extended steroid profile of transgender AMAB and AFAB individuals before starting any hormonal therapies. Before enrollment, these individuals underwent a comprehensive psychological assessment to support the further use of gender affirming hormone therapy. We decided to include these people to avoid analysing samples of people who were not transgender and before any disruption that may be generated by the use of

external hormones. From the graphical and statistical analysis of the patterns, we were unable to identify any distinct profile of free steroids that could be considered characteristic. This study represents the first attempt to provide an extensive hormonal report on adult individuals with gender incongruence. It further confirms that, at present, it is not possible to identify a detectable hormonal pattern specific to the transgender population, regardless of whether they were assigned male or female at birth.

It has to be highlighted that, comparing these profiles to controls from a previous study, a derivative of DHT and androsterone sulfate resulted to be higher in both AFAB and AMAB individuals, while epitestosterone sulfate just in the latter individuals; this represents a hint that could be further analyzed. It has to be considered, however, that these comparisons could not be corrected for age and BMI of the sample due to statistical limitations.

A work by the Ghent gender team aimed to evaluate the hormonal changes undergone during GAHT in a group of transgender women. They highlighted that the classic hormonal panel that included testosterone, calculated free testosterone, androstenedione, DHEA, and DHEAS dropped extensively after one year and then remained stable, even after gonadectomy (Collet et al., 2022). This underlines the importance to avoid an extensive panel before starting any hormonal therapies; however, this can represent an interesting topic to deepen in further studies, to analyze how androgen metabolites changes during different gender affirming hormones both in assigned female and male people.

Men with idiopathic infertility showed higher levels of progesterone related precursors (pregnenolone) and higher levels of its related metabolites, in particular the ones considered neurosteroids, such as allopregnenolone, 5-dyidroprogesterone and pregnanetriol. Moreover, they showed lower levels of 7a,hydroxydehydroepiandrosterone compared to the other male and female groups, which presented serum levels coherent with what was already reported in literature (Hill et al., 2001)(Delbert A. Fisher, 2007)

Infertility is a condition related to the health of the couple, due to potential male or female factors involved. Looking at the male side, 30–50% of male infertility cases are considered idiopathic, with no discernible cause or contributory female infertility (Agarwal et al., 2021). In these cases, the potential causes are related to oxydative stress (Agarwal et al., 2019). No specific therapies have been identified as helpful, suggesting that men should focus on ameliorating their lifestyle, use medical empirical drugs and, as a definitive solution, resort to assisted reproductive technologies. Among the potential drugs some hormonal ones, such as clomiphene citrate and aromatase inhibitors, could be used, with a few RCT supporting their use without strong evidence (Minhas et al., 2021). Surely these therapies modify steroid metabolism and in particular androgen milieu. The question that remains to be addressed is whether the hormonal background in these men is already altered. It is renowned that men with idiopathic infertility seem not to show any disruption in hormonal, physical and genetic assessment. There is no direct evaluation of steroid panels in infertile men, especially in idiopathic cases. Low levels of neurosteroids are associated with depression and anxiety, but no clue was found linking any specific condition to higher levels of these hormones. The synthesis of neurosteroids occurs in peripheral tissues such as gonads, liver, and skin; then, because of their high lipophilia, they cross the blood–brain barrier and are stored or can be

locally metabolized. They exert a broad range of physiological regulation functions, such as neuroendocrine control of reproduction and sex behavior, synaptic plasticity, myelination, the morphology of neurons and astrocytes, adult neurogenesis, and cognition-related functions (Melcangi et al., 2017). In animal models, allopregenolone has shown to boost the GABA activity on brain areas related to sexual interest and sexual behavior, while its sulfate metabolite, being less active, retains the opposite action (King, 2008). Moreover 7a,hydroxydehydroepiandrosterone is thought to modulate inflammation. A previous study which analyzed metabolites via immunoassay both in serum and in sperm in fertile men, was able to detect this analyte in both samples. This analyte has also been studied as a neurosteroid, with potential neuroprotective effect related to balancing local cortisol action (Stárka, 2017).

Among the phase II metabolites, epitestosterone sulfate showed to be higher in infertile men. This analyte is the sulphated molecule of epitestosterone, or isotestosterone, an endogenous steroid and an epimer of testosterone; this means that they differ for the configuration of carbon C17. It is a weak competitive antagonist of the AR and a potent 5α-reductase inhibitor. Furthermore, the assessment of this analyte is performed in anti-doping setting, where a significant alteration in the epitestosterone/testosterone ratio can indicate doping use (García-Arnés & García-Casares, 2022). Epitestosterone showed to be higher in this cohort compared to control men, and this finding should be further investigated considering also the conceivable age-dependent serum variation of this molecule, still not well addressed in the literature.

PCOS has been the subject of increasing attention over the years, both from a diagnostic and therapeutic point of view (Teede et al., 2023). The evaluation of 11-hydroxysteroids, in particular, has shown to be useful to resolve cases where the classic steroid pattern did not seem to show any abnormalities. In particular, a study from O'Reilly et al., 2017, has investigated the magnitude of serum and urinary 11-hydroxy and ketosteroids both in serum and urine (O'Reilly et al., 2017). Studies regarding the potential androgenic activity of these metabolites have determined that 11OHandrostenedione presents no androgenic activity while many studies have shown that 11hydroxy- and keto-testosterone act as AR agonists; 11KetoDHT and 11hydroxyDHT also present an agonist tropism, however, their assessments in serum are still difficult (Naamneh Elzenaty et al., 2022).

In this cohort we were able, among the others, to identify nine different hydroxy androgens, including five 11-oxy (11b-Hydroxyandrostenedione, 11b-Hydroxytestosterone
11-Ketoandrostenedione, 11-Ketotestosterone, 11-Ketodihydrotestosterone). However, in our analysis, we did not report the same androgen pattern that was highlighted in other works. This may be related, apart from the limited study sample, to different methodological approaches. PCOS can be characterized by oligomenorrhea, therefore making it challenging to standardize blood samples collection; usually this methodological part is not clearly stated in the various articles. We performed the assessment in a standardized way, namely in the strict early follicular phase, before the 5th day from the start of the menstrual period. It is renowned that during the ovarian cycle, sexual hormones begin to rise during the menstrual cycle up to the maximum reached around the ovulatory phase (Farage et al., 2009). In the previous work of O'Reilly and colleagues, the authors did not report the timing of blood collection; this may have

made them include women in their ovulation phase, implying that the results may be less reproducible as a consequence.
Testosterone showed to be higher in the PCOS group, even after correction for age. To further confirm this finding, even derivatives of DHT, such as 5bab-diol-3 and 5xabdiol-17-glucuronide showed to be higher in the PCOS group when compared to the group of transgender AFAB individuals, also after excluding the ones with any signs of hyperandrogenism and even after correction for age and BMI. Even if DHT levels were comparable between the groups, this finding could represent a reflection of major peripheral metabolic androgen activities in women with PCOS.
Furthermore, regarding other phase II metabolites, PCOS women showed higher levels of testosterone metabolites such as etiocholanolone glucuronide and androsterone sulfate and higher levels of DHEAS when compared to control women. It has to be considered that adrenal steroids, in particular DHEAS, lower with age and subsequently their phase II derivatives (Guazzo et al., 1996). Furthermore, in the PCOS group epiandrosterone sulfate was not lower when compared to control groups, differently from what was shown in all the other studied groups. This result may suggest an increased conversion of DHEA to this metabolite in this population.

Limitations and Strengths:
This study presents some limitations. The simultaneous analysis of multiple steroids, on the one hand amplifies the picture of the studied condition, but on the other hand can make it difficult to properly grasp the nuance of the findings, considering that a considerable number of analytes lack proper literature on their background or physiological function. Second, there was no control group that was previously defined, and the population could be broadened to further confirm the findings. Lastly, the analysis does not take into account the potential interactions on the cytochrome induced by antidepressants or antipsychotic medications taken within the sample group.
The strengths of this study are represented by the novelty of the method perfectioned and used, that was able to analyze a total of 53 androgen related metabolites in the same session using the LC MS/MS. We were able to obtain, for the first time, a wide steroid panel that describe transgender AMAB and AFAB people before taking any potential disrupting hormone therapy. It gave a hint on the potential role of neurosteroids in the group of men with idiopathic infertility that will be further investigated. The same approach could be applied also to women with PCOS to more deeply address the role of diol derivative of DHT, etiocholanolone glucuronide and androsterone sulfate.

Conclusions:

This study aimed to extensively assess the hormonal patterns associated with various androgen-related conditions. The method reliably identified a significant number of androgen analytes, expanding the hormonal profile of conditions such as gender incongruence, PCOS, and male idiopathic infertility. Future approaches to strengthen these findings will include enrolling a larger cohort of participants, focusing on the differences already outlined, and enhancing the method to incorporate additional metabolites while improving the identification of the most challenging ones.

Looking ahead, we aim to include individuals with DSD, both XX and XY, to investigate how enzymatic machinery and the hormonal milieu adapt, with the goal of enabling more tailored management of these conditions.

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Supplementary material:

Supplementary Table 1.
Full list of free steroids analyzed within the method

1. 11b-Hydroxyandrostenedione (pg/mL)
2. 11b-Hydroxytestosterone (pg/mL)
3. 11-Dehydrocorticosterone (pg/mL)
4. 11-Dehydrotetrahydrocorticosterone (pg/mL)
5. 11-Deoxycorticosterone (pg/mL)
6. 11-Deoxycortisol (pg/mL)
7. 11-Ketoandrostenedione (pg/mL)
8. 11-Ketotestosterone (pg/mL)
9. 16a-Hydroxyandrostenedione (pg/mL)
10. 16a-Hydroxyprogesterone (pg/mL)
11. 16a-Hydroxytestosterone (pg/mL)
12. 17a,20a-Dihydroxyprogesterone (pg/mL)
13. 17-Hydroxypregnenolone (pg/mL)
14. 17-Hydroxyprogesterone (pg/mL)
15. 18-Hydroxycortisol (pg/mL)
16. 20a-Cortolone (pg/mL)
17. 20a-Dihydrocorticosterone (pg/mL)
18. 20a-Dihydrocortisone (pg/mL)
19. 20a-Dihydroprogesterone (pg/mL)
20. 20b-Cortol (pg/mL)
21. 20b_Cortolone (pg/mL)
22. 20b-Dihydrocorticosterone (pg/mL)
23. 21-Deoxycortisol (pg/mL)
24. 2b-Hydroxytestosterone (pg/mL)
25. 3a,5b-Tetrahydrocortisone (pg/mL)
26. 5a-Androstanedione (pg/mL)
27. 5a-Dihydrocortisol (pg/mL)
28. 5a-Dihydroprogesterone (pg/mL)
29. 5-Androstenediol (pg/mL)
30. 5b-Dihydroprogesterone (pg/mL)
31. 7a-Hydroxydehydroepiandrosterone (pg/mL)
32. Allopregnanolone (pg/mL)
33. Allotetrahydro11-dehydrocorticosterone (pg/mL)
34. Tetrahydrocortisol (pg/mL)
35. Androstenedione (pg/mL)
36. Corticosterone (pg/mL)
37. Cortisol (ng/mL)
38. Cortisone (ng/mL)
39. DHEA (pg/mL)
40. DHT (pg/mL)
41. Epitestosterone (pg/mL)
42. Estrone (pg/mL)
43. Pregnanetriol (pg/mL)

44. Pregnanolone (pg/mL)
45. Pregnenolone (pg/mL)
46. Progesterone (pg/mL)
47. Testosterone (pg/mL)
48. 11-Ketodihydrotestosterone (pg/mL)
49. 18-hydroxycorticosterone (pg/mL)

Supplementary Table 2
Full list of analyzed phase II metabolites

1. 17-Hydroxypregnanolone Gluc (ng/mL)
2. 5aab-diol 17-Gluc (ng/mL)
3. 5bab-diol 17-Gluc (ng/mL)
4. 5xab-diol 3-Gluc (ng/mL)
5. Androsterone Gluc (ng/mL)
6. Cortisol 21_Gluc (pg/mL)
7. Etiocholanolone Gluc (ng/mL)
8. Pregnanediol Gluc (ng/mL)
9. Testosterone Gluc (pg/mL)
10. Tetrahydro-11-dehydrocorticosterone 21-Gluc (ng/mL)
11. 16a-HydroxyDHEA Sulf (ng/mL)
12. 17a,20b-Dihydroxyprogesterone Sulf (ng/mL)
13. 17-Hydroxypregnenolone 3-Sulf (ng/mL)
14. 5-Androstenediol Sulf (ng/mL)
15. Allopregnanolone Sulf (ng/mL)
16. Androsterone Sulf (ng/mL)
17. Dehydroandrosterone Sulf (ng/mL)
18. DHEAS (ng/mL)
19. Epiallopregnanolone Sulf (ng/mL)
20. Epiandrosterone Sulf (ng/mL)
21. Epipregnanolone Sulf (pg/mL)
22. Epitestosterone Sulf (pg/mL)
23. Etiocholanolone Sulf (ng/mL)
24. Pregnanolone Sulf (ng/mL)
25. Pregnenolone Sulf (ng/mL)
26. Testosterone Sulf (pg/mL)

Table 3 Supplementary material. Lower Limit of Quantification of free steroids

Steroids	**Below LLOQ for born male**	**Below LLOQ for born female**
11b-Hydroxyandrostenedione (pg/mL) comparison	0	1
11b-Hydroxytestosterone (pg/mL) comparison	6	3
11-Ketoandrostenedione (pg/mL) comparison O reilly	0	0
11-Ketotestosterone (pg/mL) comparison	2	1
16a-Hydroxyandrostenedione (pg/mL)	9	6
16a-Hydroxyprogesterone (pg/mL)	0	1
16a-Hydroxytestosterone (pg/mL)	0	1
17a,20a-Dihydroxyprogesterone (pg/mL)	0	0
17-Hydroxypregnenolone (pg/mL)	2	7
17-Hydroxyprogesterone (pg/mL)	0	0
20a-Dihydroprogesterone (pg/mL)	1	0
2b-Hydroxytestosterone (pg/mL)	8	15
5a-Androstanedione (pg/mL)	7	11
5a-Dihydroprogesterone (pg/mL)	9	5
5-Androstenediol (pg/mL)	0	0
5b-Dihydroprogesterone (pg/mL)	0	0
7a-Hydroxydehydroepiandrosterone (pg/mL)	7	2
Allopregnanolone (pg/mL)	12	16
Androstenedione (pg/mL)	0	0
DHEA (pg/mL)	0	0
DHT (pg/mL)	0	6
Epitestosterone (pg/mL)	18	46
Estrone (pg/mL)	2	0
Pregnanetriol (pg/mL)	0	0
Pregnanolone (pg/mL)	0	0
Pregnenolone (pg/mL)	4	1
Progesterone (pg/mL)	4	5

Testosterone (pg/mL)	0	1
11-Ketodihydrotestosterone (pg/mL)	0	0

Table 4 Supplementary material. Lower Limit of Quantification of phase II metabolites

Steroids	**Below LLOQ for born male**	**Below LLOQ for born female**
17-Hydroxypregnanolone Gluc (ng/mL)	2	2
5aab-diol 17-Gluc (ng/mL)	0	0
5bab-diol 17-Gluc (ng/mL)	0	0
5xab-diol 3-Gluc (ng/mL)	0	0
Androsterone Gluc (ng/mL)	0	0
Etiocholanolone Gluc (ng/mL)	0	0
Pregnanediol Gluc (ng/mL)	0	0
Testosterone Gluc (pg/mL)	4	43
16a-HydroxyDHEA Sulf (ng/mL)	0	0
17a,20b-Dihydroxyprogesterone Sulf (ng/mL)	0	0
17-Hydroxypregnenolone 3-Sulf (ng/mL)	0	0
5-Androstenediol Sulf (ng/mL)	0	0
Allopregnanolone Sulf (ng/mL)	1	0
Androsterone Sulf (ng/mL)	0	0
Dehydroandrosterone Sulf (ng/mL)	0	0
DHEAS (ng/mL)	0	0
Epiallopregnanolone Sulf (ng/mL)	2	4
Epiandrosterone Sulf (ng/mL)	0	0
Epipregnanolone Sulf (pg/mL)	7	1
Epitestosterone Sulf (pg/mL)	0	2
Etiocholanolone Sulf (ng/mL)	0	0
Pregnanolone Sulf (ng/mL)	0	2
Pregnenolone Sulf (ng/mL)	0	0
Testosterone Sulf (pg/mL)	0	39

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