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Exploring the human chorionic gonadotropin induced steroid secretion profile of mouse Leydig tumor cell line 1 by a 20 steroid LC-MS/MS panel

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ABSTRACT

The canonical androgen synthesis in Leydig cells involves Δ5 and Δ4 steroids. Besides, the backdoor pathway, eompassing 5α and 5α,3α steroids, is gaining interest in fetal and adult pathophysiology. Moreover, the role of androgen epimers and progesterone metabolites is still unknown. We developed a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for measuring 20 steroids and used it to investigate the steroid secretion induced by human chorionic gonadotropin (hCG) in the mouse Leydig tumor cell line 1 (mLTC1).

Steroids were extracted from 500 μL supernatants from unstimulated or 100 pM hCG-exposed mLTC1 cells, separated on a Luna C8 100 × 3 mm, 3 μm column, with 100 μM NH4F and methanol as mobile phases, and analyzed by positive electrospray ionization and multiple reaction monitoring.

Sensitivity ranged within 0.012–38.0 nmol/L. Intra-assay and inter-assay imprecision were < 9.1% and 10.0%, respectively. Trueness, recovery and matrix factor were within 93.4–122.0, 55.6–104.1 and 76.4–106.3%, respectively. Levels of 16OH-progesterone, 11-deoxycortisol, androstenedione, 11-deoxycorticosterone, testosterone, 17OH-progesterone, androstenedione, epitestosterone, dihydrotestosterone, progesterone, androsterone and 17OH-allopregnanolone were effectively measured. Traces of 17OH-dihydroprogesterone, androstanediol and dihydroprogesterone were found, whereas androstenediol, 17OH-pregnenolone, dehydroepiandrosterone, pregnenolone and allopregnanolone showed no peak. hCG induced an increase of 80.2–102.5 folds in 16OH-progesterone, androstenedione and testosterone, 16.6 in dihydrotestosterone, 12.2–27.5 in epitestosterone, progesterone and metabolites, 8.1 in 17OH-allopregnanolone and ≤ 3.3 in 5α and 5α,3α steroids.

Abbreviation: 11-DOC, 11-deoxycorticosterone / 21OH-progesterone – 4-pregnen-21-ol-3, 20-dione; 11-S, 11-deoxycortisol – 4-pregnen-17, 21-diol-3, 20-dione; 16OH-P4, 16-hydroxyprogesterone – 4-pregnen-16α-ol-3, 20-dione; 17OH-Allo, 17-hydroxyallopregnanolone – 5α-pregnan-3α, 17-diol-20-one; 17OH-DHP4, 17-hydroxydihydroprogesterone – 5α-pregnan-17-ol-3, 20-dione; 17OH-P4, 17-hydroxyprogesterone – 4-pregnen-17-ol-3, 20-dione; 17OH-P5, 17-hydroxypregnenolone – 5-pregnen-3β, 17-diol-20-one; 3α-diol, androstanediol – 5α-androstane-3α, 17β-diol; 5α-DHP4, dihydroprogesterone – 5α-pregnan-3, 20-dione; 5α-dione, androstanedione – 5α-androstan-3, 17-dione; A4, androstenedione – 4-androsten-3, 17-dione; A5, androstenediol – 5-androsten-3β, 17β-diol; Allo, allopregnanolone – 5α-pregnan-3α-ol-20-one; AN, androsterone – 5α-androstan-3α-ol-17-one; DHEA, dehydroepiandrosterone – 5-androsten-3β-ol-17-one; DHT, dihydrotestosterone – 5α-androstan-17β-ol-3-one; Epi-T, epitestosterone – 4-androsten-17α-ol-3-one; hCG, human chorionic gonadotropin; IS, internal standard; LC-MS/MS, liquid chromatography – tandem mass spectrometry; LH, luteinizing hormone; LLOQ, lower limit of quantification; LOD, limit of detection; mLTC1, mouse Leydig tumor cell line 1; MRM, multiple reaction monitoring; P4, progesterone – 4-pregnen-3, 20-dione; P5, pregnenolone – 5-pregnen-3β-ol-20-one; QC, quality control; RT, retention time; S/N, signal to noise; T, testosterone – 4-Androsten-17β-ol-3-one.

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In conclusion, our LC-MS/MS method allows exploring the Leydig steroidogenesis flow according to multiple pathways. Beside the expected stimulation of the canonical pathway, hCG increased progesterone metabolism and, to a low extent, the backdoor route.

1. Introduction

Leydig cells are the major male androgenic district, accounting for 95% of circulating testosterone (T) and 20% of dihydrotestosterone (DHT) [1]. According to the canonical route, DHT synthesis in humans occurs from T *via* Δ5 precursors, whereas the Δ4 pathway, involving androstenedione (A4), is preferred in rodents (Fig. 1) [2,3]. Recently, an alternative route for DHT production, encompassing progesterone (P4) metabolism through 5α and 5α,3α steroids (Fig. 1), is gaining renewed interest [4,5]. This so-called "backdoor pathway" was discovered in the Tammar Wallaby [6,7] and afterwards confirmed in humans, in which its key role for fetal sex development was hypothesized [8–10]. However, the relevance of this route in adult pathophysiology is still unclear. Leydig androgen profile is further complicated by the presence of epimers, such as 17α-epitestosterone (Epi-T), capable of antagonizing or mimicking T function in different contexts [11,12] (Fig. 1). Evidences also suggest the presence of a complex P4 metabolism, however this has only partly been elucidated. 16OH-progesterone (16OH-P4) has been shown to modulate the P4 receptor and to accumulate in immature testis [13]. Moreover, 21- and 11-hydroxylase activities were described in testis from rodents and in particular human diseases, however, available information are scarce [14–17] (Fig. 1).

The testicular androgen synthesis is naturally stimulated by the luteinizing hormone (LH) and, in clinics, by the human chorionic gonadotropin (hCG), both hormones acting through the same membrane G protein-coupled receptor (LHCGR) [18]. The differential impact of gonadotropins over the canonical and backdoor androgen routes and over P4 metabolism is far from being elucidated.

In such a frame, there is a lack of effective tools to investigate pathological contexts in which classical and/or backdoor steroidogenic pathways might be altered, such as in defects of male fetus masculinization [4], or to evaluate differences between LH- *vs* hCG-induced steroid patterns, which might be relevant in the pharmacological treatment of male reproductive defects [18].

Liquid chromatography – tandem mass spectrometry (LC-MS/MS) is the ideal technology for identifying and quantifying panels of steroids in biological fluids. While this technique has widely been applied to Δ4 androgens, such as T and A4, and C21 steroids, including progestogens, mineralocorticoids and glucocorticoids [19], only a paucity of LC-MS/MS applications to neutral steroids were reported in literature, most often including Δ5 precursors, such as pregnenolone (P5), and DHT [20,21], sometimes 5α,3α steroids, such as allopregnanolone (Allo) [22], and rarely 5α steroids such as 5α-dihydroprogesterone (5α-DHP4) [23,24]. Overall, the panel proposed by these methods do not offer a comprehensive view of the canonical and backdoor pathways, and are often burdened with a complex sample preparation [19].

Here, we developed a LC-MS/MS method to investigate a panel of twenty among the most relevant steroids from Δ5, Δ4, 5α and 5α,3α classes. The method was validated for the application to a model of mouse Leydig cells and used to evaluate the steroid secretion in basal and hCG-stimulated conditions.

2. Materials and methods

2.1. Chemicals

16OH-P4, 11-deoxycortisol (11-S), A4, 11-deoxycorticosterone/21OH-progesterone (11-DOC), T, androstenediol (A5), 17OH-progesterone (17OH-P4), 17OH-pregnenolone (17OH-P5), dehydroepiandrosterone (DHEA), androstanedione (5α-dione), Epi-T, DHT, 17OH-dihydroprogesterone (17OH-DHP4), P4, androstanediol (3α-diol), androsterone (AN), P5, 5α-DHP4, 17OH-allopregnanolone (17OH-Allo), Allo, cortisol, corticosterone, 21-deoxycortisol, 11αOH-progesterone, 11βOH-progesterone, estrone and estradiol were from Steraloids (Newport, RI, USA). T-[2,2,4,6,6–2H5] (d5-T, 98.7% deuterium content) and A4-[2,2,4,6,6–2H5] (d5-A4, 98%) were from Cambridge Isotope Laboratories (Tewksbury, MA, USA); 17OH-P4-[2,2,4,6,6,21,21,21–2H8] (d8–17OH-P4, 98.7%), P4-[2,2,4,6,6,17α,21,21,21–2H9] (d9-P4, >98%) and 11-S-[4-Pregnen-17α,21-diol-3,20-dione-21,21–2H2] (d2–11-S, >98%) were from CDN

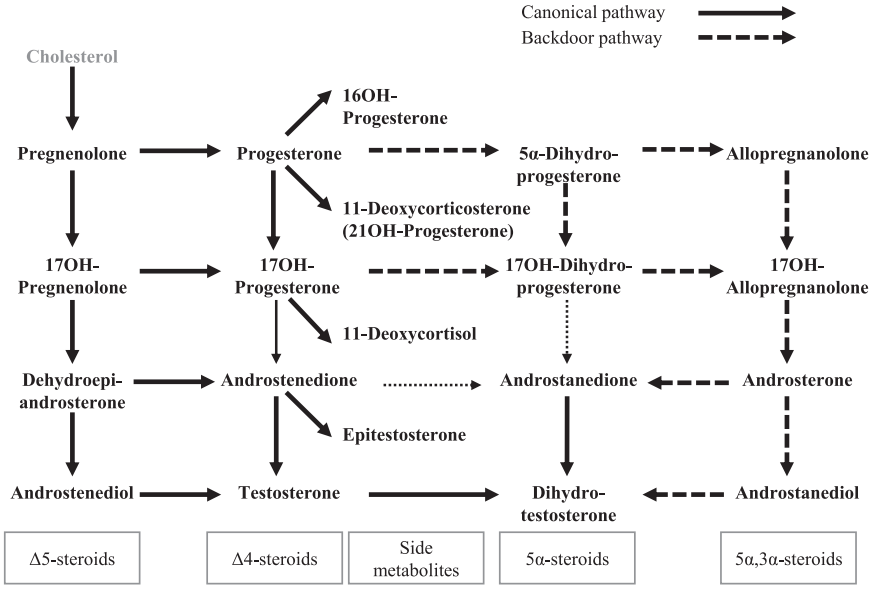

Fig. 1. Scheme of the investigated Leydig steroidogenesis. Continuous lines: canonical pathway; dashed lines: backdoor pathway; bold lines: main flux; thin lines: poor flux.

Isotopes (Pointe-Claire, Canada). Standards and isotopes were provided as lyophilic. LiChroSolv grade methanol, chloroform, N-hexane and ethyl-acetate were from Merck KGaA (Darmstadt, Germany). LC-MS grade ammonium fluoride was from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was produced by MilliQ Gradient A10 system (Burlington, MA, USA). Recombinant hCG (Ovitrelle, Merck KGaA) was provided in injectable saline buffer. The phosphodiesterase 5 inhibitors sildenafil, vardenafil and tadalafil (Sigma-Aldrich) were provided as methanol solution.

2.2. Standard solutions, calibrators and internal standards

Stock solutions were gravimetrically determined by the AX105 DeltaRange® analytical balance (Mettler-Toledo, Columbus, OH, USA) and dissolved in the mg/mL range in methanol, except 17OH-DHP4 and 5α-DHP4 which were diluted in methanol:chloroform (1:1). Working solutions were prepared in methanol from stock solutions. Microman® positive displacement pipettes (Gilson Inc, Middleton, WI, USA) and screw-top (2 mL) borosilicate V-Vials/PTFE-faced caps (Wheaton Industries Inc, NJ, USA) were used. The calibrating mixture was obtained by mixing working solutions at the following concentrations (μmol/L): 16OH-P4, 6.81; 11-S, 2.16; A4, 261.9; 11-DOC, 13.6; T, 26.0; A5, 516.5; 17OH-P4, 4.54; 17OH-P5, 676.7; DHEA, 312.0; 5α-dione, 624.1; Epi-T, 62.4; DHT, 154.9; 17OH-DHP4, 75.2; P4, 4.77; 3α-diol, 769.3; AN, 258.2; P5, 23.7; 5α-DHP4, 47.4; 17OH-Allo, 22.4 and Allo, 23.5. The internal standard (IS) mixture was prepared in 75% methanol with d2–11-S, d5-A, d5-T, d8–17OH-P4 and d9-P4 at 14, 17, 17, 15 and 15 nmol/L, respectively. All were stored at − 20 °C. The day of the assay, 10 μL of the calibrating mixture were diluted in 0.5 mL IS mixture. Eleven further calibrators were obtained by serial dilutions; zero consisted in the IS mixture. Low, medium and high-level quality controls (QCs) were prepared by diluting 50 μL of pure analyte mixtures at proper concentrations in 450 μL of culture medium.

2.3. Cell culture and treatments

The mouse Leydig tumor cell line 1 (mLTC1) was handled as previously described [25,26]. Briefly, mLTC1 cells were cultured in RPMI medium without phenol red, supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, 0.1 mg/mL streptomycin, 2 mM glutamine, and 1 mM HEPES (Invitrogen, Carlsbad, CA) and maintained at 37 °C and 5.0% CO_2. 3×10^4 cells/well were seeded in 24 multi-well plates 24 h before treatments. Over-night serum-starved cells were washed twice with 37 °C phosphate buffered saline and treated 24 h with 100 pM hCG diluted in RPMI medium without phenol red added with 0.2% bovine serum albumin (Sigma-Aldrich) [27–29]. Control cells were treated with the same solution lacking hCG.

2.4. Sample preparation

Samples were thawed, vortexed and centrifuged at room temperature for 5 min at 5500 g. Five-hundreds μL of each sample and QC were pipetted into 13 × 100 mm Pyrex® tubes (Sigma-Aldrich), spiked with 100 μL IS and vortexed 1 min. Afterwards, tubes were added 0.5 mL of water and vortexed 1 min. Two mL of N-hexane:ethyl-acetate (8:2) were added before tubes were vigorously vortexed for 5 min and centrifuged (5 min, 3000 g, room temperature). The lower aqueous layer was frozen in ice bath, while the upper organic layer was decanted in 12 × 75 mm glass tubes (Laboindustria, Arzergrande, Italy) and dried under nitrogen flow. Samples were reconstituted with 100 μL of 75% methanol and transferred into autosampler glass vials (Agilent Technology, Santa Clara, CA). Each batch included supernatants, calibrators and three QC replicates placed at the beginning, middle and end of the batch.

2.5. Liquid chromatography

The PerkinElmer Series 200 (Waltham, MA, USA) HPLC was used, equipped with the LUNA® C8(2) 100 Å 100 × 3.0 mm, 3 μm column and C8 4 × 2.0 mm guard column (Phenomenex, Torrance, CA, USA), maintained at 45 °C. Solvent A was 100 μM ammonium fluoride in water and solvent B was methanol. The gradient, operated at 0.4 mL/min, started with 45% B, increased to 62% B from 0.3 to 0.6 min and to 78.3% B until 10 min; 100% B was achieved at 10.2 and maintained until 11.2 min, before reconditioning to 45% B until 13 min. The autosampler was set at 8 °C. Injections were performed at 1 and 10 μL.

2.6. Mass spectrometry

Multiple reaction monitoring (MRM) was performed by the API-4000 QTrap triple-quadrupole (Sciex, Framingham, MA, USA) in electrospray positive ionization mode operated at 750 °C and 5500 V. Nebulizing, heating gas (air), curtain and collision activated dissociation gas (nitrogen) were set at 60, 65, 20 psi and "medium", respectively. Data were processed by Analyst v1.7 (Sciex).

2.7. LC-MS/MS method development and validation

The method was validated according to European Medicines Agency guidelines with some modifications [30].

2.7.1. MS/MS detection

MRM transitions were manually optimized by syringe pump infusion at 10 μL/min of pure analyte and isotopes ranging 1–100 μg/mL. Three or more MRM transitions were optimized for each of the 20 validated steroids and for cortisol, 21-deoxycortisol, corticosterone, 11αOH-progesterone, 11βOH-progesterone, estradiol and estrone.

2.7.2. Selectivity and specificity

The MS cross-interference among steroids included in the panel was verified. In addition, the potential interference from cortisol, 21-deoxycortisol, corticosterone, 11αOH-progesterone, 11βOH-progesterone, estradiol and estrone, sildenafil, vardenafil and tadalafil was tested. Each compound was individually injected. Then, the peak area produced in MRM transitions of monitored analytes and IS was checked. LC gradient was optimized in order to separate analytes showing cross-interference. Quantitative and qualitative MRM transitions were selected as the most sensitive transitions whose ion ratio in tested sample was within ± 20% of ion ratio in analyte standards.

2.7.3. Ammonium fluoride optimization

Ammonium fluoride was added to solvent A at 0, 20, 50, 100 and 200 μM. Peak areas at each level were compared.

2.7.4. Retention time (RT) repeatability, carry-over and IS purity

RT repeatability, accepted within 1% deviation, was evaluated within-run and among-runs across consecutive weeks. Carry-over was determined as the analyte and IS peak area in the blank following the highest calibration point, and was accepted when < 20% of the analyte area at the lower limit of quantification (LLOQ) and when < 5% of the IS area. The presence of unlabeled analytes in IS injections was checked.

2.7.5. Calibration, quantitation range and sensitivity

Three independent calibration curves, each consisting of three replicates of each calibrator, were run in consecutive weeks. The quantitation range was defined by continuous calibration points showing trueness within 85–115% and CV< 15%, with the LLOQ defined as the lowest calibration point showing trueness within 85–115%, CV< 20% and signal-to-noise (S/N) ≥ 5. The limit of detection (LOD) was defined as the lowest analyte amount yielding a S/N ≥ 3.

2.7.6. Recovery and matrix factor

Recovery and matrix factor were evaluated in cell medium containing 100 pM hCG. Fifty μL of methanolic analyte mixture at low, medium and high level were spiked in 450 μL medium before extraction. In addition, 50 μL of the same mixtures were added to 50 μL of 50% methanol and used to reconstitute dried extracts of unspiked medium, or were injected as a matrix-free reference. All were prepared in triplicates. Recovery was calculated as the percentage ratio between peak areas in pre- *vs* post-extraction spiked test samples. Matrix factor was calculated as the percentage ratio between peak areas in post-extraction *vs* matrix-free reference. Deviations from 100% indicated the presence of ion suppression or enhancement. Matrix effect was also tested by post-column infusion. A syringe pump was connected to the LC eluate by a T-piece upstream the ionization source. The analyte mixture in 75% methanol was infused during LC injections of methanol and of extracts of culture media as such or containing 100 pM hCG, or 1 μM sildenafil, vardenafil or tadalafil.

2.7.7. Imprecision and trueness

Five replicates of QCs at low, medium and high levels were injected within the same day and in three independent runs in consecutive weeks. Imprecision was determined as the CV% calculated within run (intra-assay) and among runs (inter-assay). Trueness was calculated as the percentage ratio between the observed and the expected concentration.

2.7.8. Stability

Amounts of steroids in the middle range of the calibration curve were spiked in culture medium and incubated at 37 °C for 0, 1, 4, 8 and 24 h before freezing at − 80 °C. Freshly reconstituted extracts were injected immediately and after 24 h in autosampler at 8 °C. All were tested in triplicate.

2.8. Statistics

Means and standard deviations were computed. Variables were not normally distributed, therefore, values from unstimulated *vs* hCG-stimulated cells were compared by the Wilcoxon test for paired samples (MedCalc, v.18.2.1; Mariakerke, Belgium). $P < 0.050$ was considered statistically significant.

3. Results

3.1. LC-MS/MS method development and validation

3.1.1. LC-MS/MS detection, specificity and selectivity

Compound-dependent parameters are reported in Table 1. A sub-optimal collision energy was selected for A4 and T to avoid signal oversaturation in study samples. Analyte peaks are reported in Fig. 2. Baseline separation was obtained within groups of isobars or cross-interfering compounds including 16OH-P4, 17OH-P4, 11-DOC, 11αOH-progesterone and 11βOH-progesterone; 11-S, corticosterone and 21-deoxycortisol; T, DHEA, 5α-dione and Epi-T; 17OHP5, 17OH-DHP4 and P4; A5 and AN; P5, 5α-DHP4 and 17OH-Allo. Selective detection of 5α-DHP4 and 17OH-Allo was achieved by choosing specific Q3 ions. No interference could be observed from cortisol, estrone, estradiol, sildenafil, vardenafil or tadalafil. Ion ratio consistency was verified in all tested samples.

3.1.2. Ammonium fluoride optimization

One-hundred μM was chosen as the best compromise to optimize sensitivity within the whole panel, increasing the signal to 288–859% in respect to signal at 0 μM. Exception was found for A5 and 3α-diol, whose signal was reduced to 82% and 28%, respectively (Supplemental Fig. 1).

3.1.3. Retention time repeatability, carry-over and IS purity

RT variability was < 0.4% for all analytes. The carry-over was absent or < 0.1% of the area of the highest calibrator. For 11-DOC, Epi-T, DHT and P4, carry-over was occasionally observed up to 20%, 170%, 200% and 16% of the LLOQ, respectively. Therefore, a blank was always injected after the highest calibrator.

3.1.4. Calibration, quantitation range and sensitivity

Curve and quantitation parameters are reported in Table 2. Isotopic

Table 1
Compound-dependent LC-MS/MS detection parameters.

Analyte	Molecular weight	Retention Time	Quantifier MRM				Qualifier MRM			
	(g/mol)	(min)	Q1/Q3	DP	CE	CXP	Q1/Q3	DP	CE	CXP
16OH-Progesterone (16OH-P4)	330.47	4.42	331.4/97.1	110	40	2	331.4/109.1	110	40	2
11-Deoxycortisol (11-S)	346.46	4.44	347.3/109.3	100	35	5	347.3/97.2	100	38	7
Androstenedione (A4)	286.41	5.18	287.3/97.2	90	13 *	5	287.3/109.2	90	14 *	5
11-Deoxycorticosterone (11-DOC)	330.46	5.33	331.4/109.1	130	40	2	331.4/97.2	130	40	3
Testosterone (T)	288.42	5.75	289.2/97.2	80	16 *	4	289.2/109.1	80	17 *	3
Androstenediol (A5)	290.44	5.85	273.4/159.2	60	30	6	273.4/145.2	60	26	6
17OH-Progesterone (17OH-P4)	330.46	5.90	331.4/97.2	80	40	4	331.4/109.2	80	40	2
17OH-Pregnenolone (17OH-P5)	332.48	6.01	315.2/159.2	55	35	9	315.2/91.2	50	75	4
Dehydroepiandrosterone (DHEA)	288.42	6.08	271.3/197.2	65	27	9	271.3/213.2	65	23	10
Androstanedione (5α-dione)	288.42	6.45	289.3/213.3	70	25	7	289.3/161.2	70	25	7
Epitestosterone (Epi-T)	288.42	6.78	289.3/97.2	130	35	6	289.3/109.1	130	35	7
Dihydrotestosterone (DHT)	290.44	6.97	291.3/159.3	110	30	6	291.3/255.3	110	25	3
17OH-Dihydroprogesterone (17OH-DHP4)	332.48	7.02	315.3/111.1	90	25	8	333.3/255.3	90	25	5
Progesterone (P4)	314.46	7.62	315.2/97.1	115	30	2	315.2/109.2	115	35	2
Androstanediol (3α-diol)	292.46	8.08	257.2/161.2	70	20	8	257.2/147.2	70	30	5
Androsterone (AN)	290.44	8.36	273.3/147.2	90	25	10	291.4/199.2	60	30	10
Pregnenolone (P5)	316.48	8.63	317.4/159.3	30	30	11	317.4/255.3	30	15	12
Dihydroprogesterone (5α-DHP4)	316.48	9.31	317.2/85.1	100	20	5	317.2/159.2	100	35	10
17OH-Allopregnanolone (17OH-Allo)	334.49	9.40	317.3/111.2	45	25	5	299.3/135.2	90	30	5
Allopregnanolone (Allo)	318.49	10.68	319.3/257.3	60	20	8	319.3/135.2	60	30	5
d2–11-Deoxycortisol (d2–11-S)	348.46	4.43	349.3/109.2	120	40	5	349.3/97.1	120	40	5
d5-Androstenedione (d5-A4)	291.44	5.14	292.3/100.2	110	35	2	292.3/113.2	110	30	3
d5-Testosterone (d5-T)	293.46	5.68	294.3/100.2	110	40	2	294.3/113.2	110	30	3
d8–17OH-Progesterone (d8–17OH-P4)	338.46	5.84	339.6/100.2	100	40	2	339.6/113.2	100	40	3
d9-Progesterone (d9-P4)	323.52	7.51	324.4/100.2	110	35	2	324.4/113.2	110	40	3

Positive electrospray ionization and 10 eV entrance potential were used for all analytes. MRM: multiple reaction monitoring; DP: declustering potential; CE: collision Energy; CXP: cell exit potential. *For avoiding oversaturation, a sub-optimal CE was selected, inducing about the 10% of the highest achievable signal.

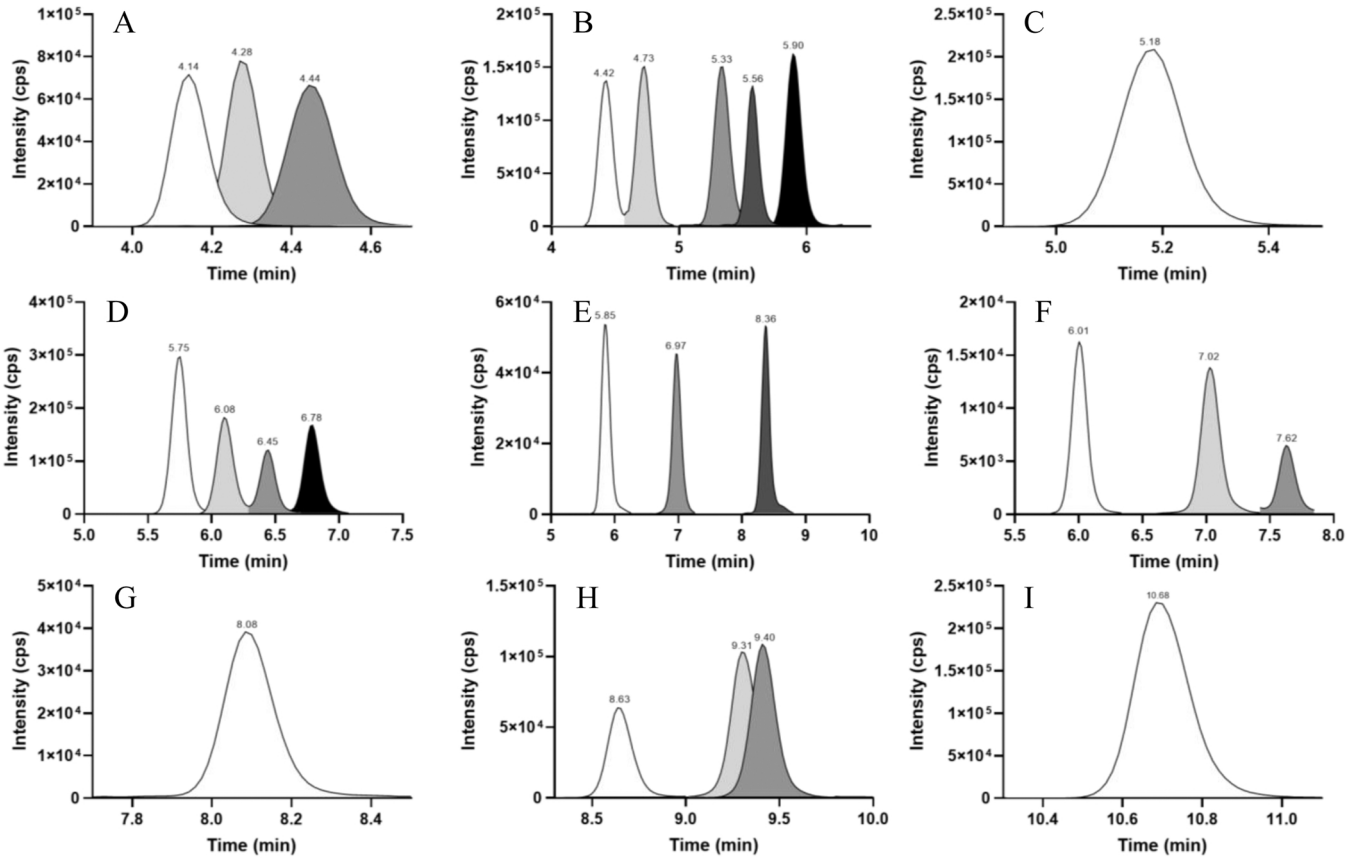

Fig. 2. LC peak separation with focus of cross interfering compounds. A: 21-deoxycortisol (4.14 min), corticosterone (4.28 min) and 11-deoxycortisol (4.44 min); B: 16OH-progesterone (4.42 min), 11αOH-progesterone (4.73 min), 11-deoxycorticosterone (5.33 min), 11βOH-progesterone (5.56 min) and 17OH-progesterone (5.90 min); C: androstenedione (5.18 min); D: testosterone (5.75 min), dehydroepiandrosterone (6.08 min), androstanedione (6.45 min) and epitestosterone (6.78 min); E: androstenediol (5.85 min), dihydrotestosterone (6.97 min) and androsterone (8.36 min); F: 17OH-pregnenolone (6.01 min), 17OH-dihydroprogesterone (7.02 min) and progesterone (7.62 min); G: androstanediol (8.08 min); H: pregnenolone (8.63 min), 5α-dihydroprogesterone (9.31 min) and 17OH-allopregnanolone (9.40 min); I: allopregnanolone.

dilution quantitation was obtained by 1/x weighted linear regression using d2–11-S as IS for 16OH-P4 and 11-S, d5-A for A4 and 11-DOC, d8–17OH-P4 for 17OH-P4, d5-T for A5, T, 17OH-P5, DHEA, 5α-dione and Epi-T, d9-P4 for DHT, 17OH-DHP4, P4, 3α-diol, AN, P5, 5α-DHP4, 17OH-Allo and Allo. Quantitation was performed in 10 μL injections, with some exceptions. Indeed, injections at 1 μL were used for upper calibrators showing loss of linearity due to signal oversaturation for A5, 17OH-P5, DHEA, 5α-dione, 17OH-DHP4, 3α-diol and AN. Moreover, 1 μL injections were used to measure A4 and T, because of their very large concentration in the study samples, and 11-DOC, because of ion suppression from the coeluting A4. Five to seven calibration points were defined for all analytes, except for 17OH-Allo, achieving acceptable performance in four points. The sensitivity in supernatants ranged from 0.012 nmol/L of 11-S to 38.0 nmol/L of 3α-diol.

3.1.5. Recovery and matrix factor

Recovery ranged within 55.6–63.1% for the early eluting analytes 16OH-P4, 11-S and d2–11-S, and within 88.6–101.1% for other compounds. Matrix factor ranged within 94.9–104.7% for all compounds (Supplemental Table 1). The post-column infusion experiment showed a region of signal suppression around 6.5 min which did not impact on RT of any analyte. Among tested drugs, sildenafil caused a slight signal suppression around min 5 (Supplemental Fig. 2).

3.1.6. Imprecision and trueness

Intra- and inter-assay CVs were $< 9.1\%$ and 10.0%, respectively, and trueness within 93.4–122.0% for all analytes at the three tested concentrations (Supplemental Table 2).

3.1.7. Stability

Analyte stability in experimental conditions is shown in Supplemental Table 3. The maximum deviation observed in culture medium after 8 h at 37 °C was 86.7%, whereas in extracts kept at 8 °C for 24 h it was 93.9%.

3.2. Steroid levels in supernatants from unstimulated and hCG-stimulated mLTC1 cell

Steroid levels observed in study samples are reported in Fig. 3 and Supplemental Table 4. Levels within the measurement range were found for 16OH-P4, 11-S, A4, 11-DOC, T, 17OH-P4, 5α-dione, Epi-T, DHT, P4 and AN in both conditions, and for 17OH-Allo in hCG-treated samples. The highest concentrations were achieved by AN (290.5 ± 33.7 nmol/L), 5α-dione (56.4 ± 4.8 nmol/L) and A4 (8.54 ± 0.87 nmol/L) in unstimulated, and by A4 (726.8 ± 88.6 nmol/L), 5α-dione (773.8 ± 163.5 nmol/L) and AN (379.4 ± 80.0 nmol/L) in hCG-treated samples. A trace signal slightly below or above the LOD could be observed for 3α-diol, 5α-DHP4, 17OH-DHP4 and 17OH-Allo. Although a reliable quantitation below the LLOQ is not possible, we reported the concentrations referred to those traces in an attempt to roughly estimate the effect of hCG. Hence, we found that hCG induced a modest increase of AN, 3α-diol, 5α-DHP4, 17OH-DHP4 and 17OH-Allo (1.3–8.1 folds), moderate increase of Epi-T, P4, 5α-dione, 17OH-P4, DHT, 11-S and 11-DOC (12.2–27.5 folds), and a large increase of 16OH-P4, A4 and T

Table 2
Parameters of the calibration curve and assay sensitivity.

Analyte	Internal standard	Calibration points	Range	Slope	Intercept	R^2	LOD		LLOQ				Sensitivity in supernatants
			nmol/L				fmol o.c.	S/N	nmol/L	CV (%)	Bias (%)	S/N	nmol/L
16OH-Progesterone (16OH-P4)	d2–11-S	7	0.187 – 136.2	0.2250 ± 0.0044	0.0119 ± 0.0017	0.9994	0.62	3.7	0.187	10.6	102.1	10.7	0.037
11-Deoxycortisol (11-S)	d2–11-S	6	0.059 – 14.43	0.3887 ± 0.0116	0.0000 ± 0.0000	0.9998	0.30	3.1	0.059	3.8	110.2	8.5	0.012
Androstenedione (A4)*	d5-A4	6	21.6 – 5237	0.0115 ± 0.0006	0.0000 ± 0.0000	0.9999	7.18	4.0	21.5	16.4	103.9	14.7	4.31
11-Deoxycorticosterone (11-DOC)*	d5-A4	6	0.374 – 90.8	0.2597 ± 0.0055	0.0003 ± 0.0005	0.9995	0.13	3.5	0.374	6.6	106.1	12.8	0.075
Testosterone (T)*	d5-T	7	0.713 – 520.1	0.0332 ± 0.0022	0.0000 ± 0.0000	0.9999	5.31	3.6	0.713	12.0	100.4	5.6	0.143
Androstenediol (A5)	d5-T	6	42.5 – 10,329	0.0005 ± 0.0002	0.0000 ± 0.0000	0.9989	318	3.2	42.5	5.2	96.4	5.1	8.50
17OH-Progesterone (17OH-P4)	d8–17OH-P4	7	0.125 – 90.8	0.2610 ± 0.0145	0.0000 ± 0.0000	0.9999	0.95	3.1	0.125	16.7	98.9	5.3	0.025
17OH-Pregnenolone (17OH-P5)	d5-T	6	18.6 – 4512	0.0008 ± 0.0001	0.0000 ± 0.0000	0.9992	141	3.5	18.6	17.7	98.0	5.4	3.71
Dehydroepiandrosterone (DHEA)	d5-T	6	25.7 – 6241	0.0017 ± 0.0006	0.0000 ± 0.0000	0.9994	192	3.3	25.7	16.5	93.0	6.4	5.14
Androstanedione (5α-dione)	d5-T	7	17.1 – 12,482	0.0026 ± 0.000	0.0000 ± 0.0000	0.9997	57.1	4.0	17.1	7.7	94.2	12.4	3.42
Epitestosterone (Epi-T)	d5-T	7	0.190 – 138.7	0.07360 ± 0.0010	0.0000 ± 0.0000	0.9999	0.63	3.6	0.190	4.1	101.2	14.5	0.038
Dihydrotestosterone (DHT)	d9-P4	6	1.42 – 344.3	0.0099 ± 0.0007	0.0000 ± 0.0000	0.9995	8.52	3.4	1.42	14.5	94.1	9.4	0.283
17OH-Dihydroprogesterone (17OH-DHP4)	d9-P4	6	6.19 – 1504	0.0045 ± 0.0007	0.0000 ± 0.0000	0.9997	20.6	3.7	6.19	3.2	83.6	8.8	1.24
Progesterone (P4)	d9-P4	7	0.131 – 95.4	0.1777 ± 0.0106	0.0000 ± 0.0000	0.9999	0.44	4.0	0.131	2.1	102.4	11.3	0.026
Androstanediol (3α-diol)	d9-P4	5	190.0 – 15387	0.0002 ± 0.0000	0.0000 ± 0.0000	0.9980	633	3.5	190	0.1	81.5	7.1	38.0
Androsterone (AN)	d9-P4	7	7.08 – 5165	0.0077 ± 0.0003	0.0000 ± 0.0000	0.9996	23.6	3.5	7.08	11.4	93.8	10.3	1.42
Pregnenolone (P5)	d9-P4	5	5.85 – 474.0	0.0028 ± 0.0003	0.0000 ± 0.0000	0.9993	29.3	3.2	5.85	11.1	94.7	5.9	1.17
Dihydroprogesterone (5α-DHP4)	d9-P4	5	11.7 – 947.9	0.0020 ± 0.0006	0.0000 ± 0.0000	0.9997	39.0	3.6	11.7	3.5	90.4	8.1	2.34
17OH-Allopregnanolone (17OH-Allo)	d9-P4	4	16.6 – 448.4	0.0028 ± 0.0001	0.0000 ± 0.0000	0.9997	55.3	4.6	16.6	2.9	94.1	9.2	3.32
Allopregnanolone (Allo)	d9-P4	5	5.81 – 471.0	0.0038 ± 0.0010	0.0000 ± 0.0000	0.9996	34.7	3.0	5.81	3.3	99.5	5.9	1.16

LOD: limit of detection; LLOQ: lower limit of quantification; o.c.: on column; S/N: signal-to-noise ratio; d2-11-S: d2-11-deoxycortisol; d5A4: d5-androstenedione; d5-T: d5-testosterone; d9-P4: d9-progesterone. *data referred to 1 μL injections.

(80.2–102.5 folds).

No peak of 11αOH-progesterone and 11βOH-progesterone, corticosterone, 21-deoxycortisol, cortisol, estrone and estradiol could be found in any tested culture condition (Supplemental Table 5), therefore, these analytes were not included in the panel to ease the practicability of the method.

4. Discussion and conclusions

We developed a LC-MS/MS method to quantify a panel of 20 steroids belonging to the canonical and backdoor androgen pathways, plus P4 metabolites. We validated the method for its application to the study of the mLTC1 model [31]. mLTC1 cells are permanently expressing the murine Lh receptor, which is structurally similar to the human receptor and capable of binding human LH and hCG [32,33]. Most importantly, human ligands trigger steroidogenic signals mainly activating the synthesis of Δ4 hormones [34], although Δ5 steroid production was described as well [35]. However, comparative analyses between mLTC1 and human Leydig steroidogenesis must be considered carefully, as the two models differ for their enzymatic *milieu* [36].

The method overall showed good recovery, precision, trueness and stability, with no relevant matrix effect. Specificity among isobars was achieved by careful LC separation and fragment ion selection. Moreover, to guarantee the reliability of steroid results in the present study and in future *in vitro* as well as *in vivo* studies, we verified the absence of interferences from steroids not included in the panel and from phosphodiesterase 5 inhibitors. As expected, Δ4 steroids exhibited much higher sensitivity than neutral Δ5, 5α and 5α,3α steroids. Notably, the sensitivity of A4 and T was purposely detuned for avoiding signal oversaturation. Previous LC-MS/MS applications to neutral steroids often used derivatization to enhance sensitivity [20–23]. However, given the chemical diversity within our panel, derivatization is hardly practicable and would also complicate the preanalytical stage. As similarly reported [37,38], ammonium fluoride enhanced the signal of 3–9 folds for the overall panel. Unfortunately, it diminished the signal of A5 and 3α-diol. In future, the sensitivity of our method could be ameliorated by moving to a high-end MS instrument. Indeed, a recent study used atmospheric pressure photoionization and a last generation triple quadrupole to measure a similar panel in serum [24].

AN, 5α-dione and A4 were the most abundant steroids secreted by mLTC1 both in unstimulated and hCG-stimulated conditions, although in different relative abundance, *i.e.* 35:6:1 and 1:2:2, respectively. The accumulation of these intermediates could be explained by the fact that they all are substrates of the 17β-HSD3, which was found to be minimally expressed in murine Leydig cells [36]. On the opposite hand, levels of Δ5 steroids were undetectable.

Interestingly, unstimulated cells secreted DHT and T at similar levels. Moreover, T is 20–100 folds less abundant than other DHT precursors 3α-diol and 5α-dione. Notably, hCG induced about 100 fold increase in A4 and T, but only 16.6-fold increase in DHT. A modest increase was also observed in intermediates from the backdoor pathway. However, results about 17OH-DHP4, 3α-diol and 5α-DHP4 are to be taken with caution as only trace levels were found in tested samples.

Taken together, these data suggest that, in absence of gonadotropin, minimal DHT production is, at least in part, maintained through the backdoor pathway. hCG strongly activated the canonical pathway, with an important effect on the generation of T rather than DHT. This may be due to a weak upregulation of 5α-reductase by hCG in mouse Leydig cells, leading to relatively low T-to-DHT conversion rate [39]. Additionally, albeit weak, we observed an effect of hCG on the backdoor

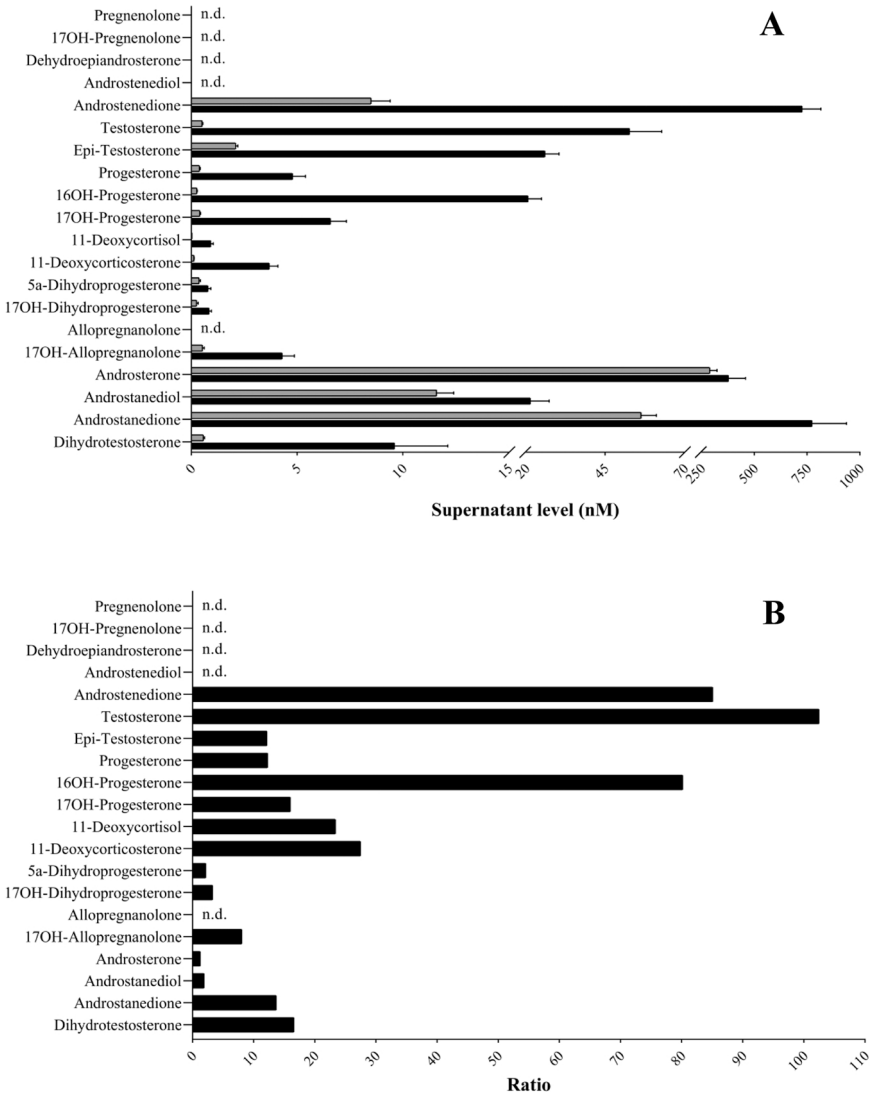

Fig. 3. A: Steroid levels in the culture medium of mouse Leydig tumor cell line 1 in unstimulated condition (grey bars) or upon stimulation with 100 pM human chorionic gonadotropin (black bars). B: Fold increase of steroid levels in culture medium of 100 pM hCG-stimulated compared to unstimulated mouse Leydig tumor cell line 1.

pathway, supporting previous hypothesis about the relevance of the maternal hormone in upregulating the backdoor route during the fetal development [4,5].

Unstimulated mLTC1 cells basally secrete Epi-T levels four times higher than T. Interestingly, hCG enhanced T secretion more than Epi-T, inducing a final T/Epi-T ratio of 2:1. This is consistent with *in vivo* T/Epi-T circulating ratio, increasing during puberty upon the raise in LH secretion [11].

hCG induced about 15-fold elevation of P4 and 17OH-P4 levels. While not corroborating the overall modest increase of backdoor pathway steroids, this finding is consistent with the hCG-induced 25-fold increase of the 21-hydroxylated metabolites 11-DOC and 11-S. The expression of adrenal-specific enzymes in mice testis was previously described [40]. For this reason, in the early stage of method development, we tested the presence of other steroids derived by the 11-hydroxylation of P4, such as 11OHα-P4 and 11OHβ-P4, of 11-DOC, such as corticosterone, of 17OH-P4, such as 21-deoxycortisol, and of 11-S, such as cortisol, and found they were undetectable.

In contrast with previous studies suggesting 16OH-P4 is specific for the primate testis [41], we reported relevant levels of this P4 metabolite in our murine cells. Moreover, in agreement with Storbeck et al. [13], we found 16OH-P4 and 17OH-P4 are secreted in a 1:2 ratio from untreated mLTC1 cells. Surprisingly, the ratio changed to 3:1 upon hCG stimulation. Of note, the extent of the increase in 16OH-P4, about 80-fold, is similar to the increase observed for Δ4 androgens, somehow suggesting a relevant physiologic role of the former. Unfortunately, very little is known about 16OH-P4 [13,41]. We hypothesize that this metabolite may counteract the effect of increasing P4 levels at its receptor, possibly favoring the utilization of the latter as backdoor precursor.

In a previous study in hCG-treated mLTC1 cells, T, A4 and P4 secretion was reported in 1:20:60 relative concentration, respectively [42]. In contrast, in our hands, these steroids are in 11:152:1 proportion, respectively. Such differences may depend on the lower hCG exposure time of 1 h [42] instead of 24 h as here, possibly not allowing the full downstream metabolization of P4.

Finally, in the early development stage we also tested the presence of estrogens in mLTC1 supernatants, and found they were undetectable. It cannot be excluded, however, that an upgrade of instrumental sensitivity would result in measurable levels of both Δ5 and estrogen classes.

In conclusion, our LC-MS/MS method allows exploring the steroidogenesis flow according to multiple pathways. To our knowledge, no previous study has provided such a broad characterization of steroid secretion of Leydig cells *in vitro*. Future studies are needed to explore the

steroid secretion pattern of other Leydig cell lines or primary cultures from animal models and humans, and to characterize how the different routes are modulated by hormones or drugs. In addition, our steroid profiling tool could be applied to the *in vitro* and *in vivo* characterization of diseases featured by a deranged androgen synthesis, such as abnormalities of fetal masculinization [4], or of conditions requiring hCG administration, such as hypogonadic hypogonadism and maldescended testes [18].

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Author contributions

FF and LC conceived, designed and coordinated the study. mL contributed to the study design. MMa, MMe and AT performed the LC-MS/MS experiments. SL performed the cell culture experiments. FF, MMa and LC wrote the manuscript. CP, DS, ML, MS, UP and LC contributed in interpreting results and in writing the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jsbmb.2023.106270.

References

[1] D.J. Handelsman, Androgen Physiology, Pharmacology, Use and Misuse [Updated 2020], in: K.R. Feingold, B. Anawalt, A. Boyce A, et al., Endotext, South Dartmouth (MA): MDText.com, Inc., 2000-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK279000/.

[2] W.L. Miller, R.J. Auchus, The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders, Endocr. Rev. 32 (2011) 81–151, https://doi.org/10.1210/er.2010-0013.

[3] S. Connan-Perrot, T. Léger, P. Lelandais, C. Desdoits-Lethimonier, A. David, P. A. Fowler, S. Mazaud-Guittot, Six decades of research on human fetal gonadal steroids, Int. J. Mol. Sci. 22 (2021) 6681, https://doi.org/10.3390/ijms22136681.

[4] P.J. O'Shaughnessy, J.P. Antignac, B. Le Bizec, M.L. Morvan, K. Svechnikov, O. Söder, I. Savchuk, A. Monteiro, U. Soffientini, Z.C. Johnston, M. Bellingham, D. Hough, N. Walker, P. Filis, P.A. Fowler, Alternative (backdoor) androgen production and masculinization in the human fetus, PloS Biol. 17 (2019), e3000002, https://doi.org/10.1371/journal.pbio.3000002.

[5] W.L. Miller, R.J. Auchus, The "backdoor pathway" of androgen synthesis in human male sexual development, PLoS Biol. 17 (2019), e3000198, https://doi.org/10.1371/journal.pbio.3000198.

[6] J.D. Wilson, R.J. Auchus, M.W. Leihy, O.L. Guryev, R.W. Estabrook, S.M. Osborn, G. Shaw, M.B. Renfree, 5α-androstane-3α,17β-diol is formed in tammar wallaby pouch young testes by a pathway involving 5α-pregnane-3α,17α-diol-20-one as a key intermediate, Endocrinology 144 (2003) 575–580, https://doi.org/10.1210/en.2002-220721.

[7] R.J. Auchus, The backdoor pathway to dihydrotestosterone, Trends Endocrinol. Metab. 15 (2004) 432–438.

[8] C.E. Flück, A.V. Pandey, Steroidogenesis of the testis – new genes and pathways, Ann. Endocrinol. (Paris) 75 (2014) 40–47, https://doi.org/10.1016/j.ando.2014.03.002.

[9] M. Fukami, K. Homma, T. Hasegawa, T. Ogata, Backdoor pathway for dihydrotestosterone biosynthesis: implications for normal and abnormal human sex development, Dev. Dyn. 242 (2013) 320–329, https://doi.org/10.1002/dvdy.23892.

[10] R.J. Auchus, W.L. Miller, Defects in androgen biosynthesis causing 46,XY disorders of sexual development, Semin. Reprod. Med. 30 (2012) 417–426, https://doi.org/10.1055/s-0032-1324726.

[11] F.C. Cavalari, L.A. da Rosa, G.M. Escott, T. Dourado, A.L. de Castro, M.B.D. F. Kohek, M.F.M. Ribeiro, W.A. Partata, L.S. de Fraga, E.D.S. Loss, Epitestosterone- and testosterone-replacement in immature castrated rats changes main testicular developmental characteristics, Mol. Cell. Endocrinol. 461 (2018) 112–121, https://doi.org/10.1016/j.mce.2017.08.023.

[12] L.A. da Rosa, G.M. Escott, R.B. Simonetti, J.C.D. da Silva, I.C.R. Werlang, M. Z. Goldani, L.S. de Fraga, E.D.S. Loss, Role of non-classical effects of testosterone and epitestosterone on AMH balance and testicular development parameters, Mol. Cell. Endocrinol. 511 (2020), 110850, https://doi.org/10.1016/j.mce.2020.110850.

[13] K.H. Storbeck, P. Swart, D. Africander, R. Conradie, R. Louw, A.C. Swart, 16α-hydroxyprogesterone: origin, biosynthesis and receptor interaction, Mol. Cell. Endocrinol. 336 (2011) 92–101, https://doi.org/10.1016/j.mce.2010.11.016.

[14] M. Namiki, E. Koh, N. Meguro, N. Kondoh, H. Kiyohara, A. Okuyama, S. Sakoda, K. Matsumoto, T. Sonoda, Extraadrenal expression of steroid 21-hydroxylase and 11 beta-hydroxylase by a benign testicular Leydig cell tumor, J. Steroid Biochem. Mol. Biol. 39 (1991) 897–901, https://doi.org/10.1016/0960-0760(91)90347-8.

[15] G.M. Wang, R.S. Ge, S.A. Latif, D.J. Morris, M.P. Hardy, Expression of 11beta-hydroxylase in rat Leydig cells, Endocrinology 143 (2002) 621–626, https://doi.org/10.1210/endo.143.2.8638.

[16] T. Puar, M. Engels, A.E. van Herwaarden, F.C. Sweep, C. Hulsbergen-van de Kaa, K. Kamphuis-van Ulzen, V. Chortis, W. Arlt, N. Stikkelbroeck, H.L. Claahsen-van der Grinten, A.R. Hermus. Bilateral Testicular Tumors Resulting in Recurrent Cushing Disease After Bilateral Adrenalectomy. J. Clin. Endocrinol. Metab. 102 (2017) 339–344. https://doi.org/10.1210/jc.2016–2702.

[17] D. van Rooyen, R. Gent, L. Barnard, A.C. Swart, The in vitro metabolism of 11β-hydroxyprogesterone and 11-ketoprogesterone to 11-ketodihydrotestosterone in the backdoor pathway, J. Steroid Biochem. Mol. Biol. 178 (2018) 203–212, https://doi.org/10.1016/j.jsbmb.2017.12.014.

[18] L. Casarini, D. Santi, G. Brigante, M. Simoni, Two hormones for one receptor: evolution, biochemistry, actions, and pathophysiology of LH and hCG, Endocr. Rev. 39 (2018) 549–592, https://doi.org/10.1210/er.2018-00065.

[19] S.A. Wudy, G. Schuler, A. Sánchez-Guijo, M.F. Hartmann, The art of measuring steroids: principles and practice of current hormonal steroid analysis, J. Steroid Biochem. Mol. Biol. 179 (2018) 88–103, https://doi.org/10.1016/j.jsbmb.2017.09.003.

[20] U. Bussy, M. Huertas, Y.W. Chung-Davidson, K. Li, W. Li, Chemical derivatization of neurosteroids for their trace determination in sea lamprey by UPLC-MS/MS, Talanta 149 (2016) 326–334, https://doi.org/10.1016/j.talanta.2015.11.061.

[21] M.R. Häkkinen, T. Murtola, R. Voutilainen, M. Poutanen, T. Linnanen, J. Koskivuori, T. Lakka, J. Jääskeläinen, S. Auriola, Simultaneous analysis by LC-MS/MS of 22 ketosteroids with hydroxylamine derivatization and underivatized estradiol from human plasma, serum and prostate tissue, J. Pharm. Biomed. Anal. 164 (2019) 642–652, https://doi.org/10.1016/j.jpba.2018.11.035.

[22] K. Saito, T. Matsuzaki, T. Iwasa, M. Miyado, H. Saito, T. Hasegawa, K. Homma, E. Inoue, Y. Miyashiro, T. Kubota, M. Irahara, T. Ogata, M. Fukami, Steroidogenic pathways involved in androgen biosynthesis in eumenorrheic women and patients with polycystic ovary syndrome, J. Steroid Biochem. Mol. Biol. 158 (2016) 31–37, https://doi.org/10.1016/j.jsbmb.2016.02.010.

[23] T. Higashi, N. Takido, K. Shimada. Studies on neurosteroids XVII. Analysis of stress-induced changes in neurosteroid levels in rat brains using liquid chromatography-electron capture atmospheric pressure chemical ionization-mass spectrometry. Steroids. 70(2005) 1–11. https://doi.org/10.1016/j.steroids.2004.08.001.

[24] R. Desai, D.T. Harwood, D.J. Handelsman, Simultaneous measurement of 18 steroids in human and mouse serum by liquid chromatography-mass spectrometry without derivatization to profile the classical and alternate pathways of androgen synthesis and metabolism, Clin. Mass. Spectrom. 11 (2019) 42–51, https://doi.org/10.1016/j.clinms.2018.12.003.

[25] L. Casarini, L. Riccetti, S. Limoncella, C. Lazzaretti, F. Barbagallo, S. Pacifico, R. Guerrini, S. Tagliavini, T. Trenti, M. Simoni, M. Sola, G. Di Rocco, Probing the effect of sildenafil on progesterone and testosterone production by an intracellular FRET/BRET combined approach, Biochemistry 58 (2019) 799–808, https://doi.org/10.1021/acs.biochem.8b01073.

[26] S. Limoncella, C. Lazzaretti, E. Paradiso, S. D'Alessandro, F. Barbagallo, S. Pacifico, R. Guerrini, S. Tagliavini, T. Trenti, D. Santi, M. Simoni, M. Sola, G. Di Rocco, L. Casarini, Phosphodiesterase (PDE) 5 inhibitors sildenafil, tadalafil and vardenafil impact cAMP-specific PDE8 isoforms-linked second messengers and

steroid production in a mouse Leydig tumor cell line, Mol. Cell. Endocrinol. 542 (2022), 111527, https://doi.org/10.1016/j.mce.2021.111527.
[27] L. Casarini, M. Lispi, S. Longobardi, F. Milosa, A. La Marca, D. Tagliasacchi, E. Pignatti, M. Simoni, LH and hCG action on the same receptor results in quantitatively and qualitatively different intracellular signalling, PLoS One 7 (2012), e46682, https://doi.org/10.1371/journal.pone.0046682.
[28] L. Riccetti, F. De Pascali, L. Gilioli, F. Potì, L.B. Giva, M. Marino, S. Tagliavini, T. Trenti, F. Fanelli, M. Mezzullo, U. Pagotto, M. Simoni, L. Casarini, Human LH and hCG stimulate differently the early signalling pathways but result in equal testosterone synthesis in mouse Leydig cells in vitro, Reprod. Biol. Endocrinol. 15 (2017) 2, https://doi.org/10.1186/s12958-016-0224-3.
[29] L. Casarini, L. Riccetti, E. Paradiso, R. Benevelli, C. Lazzaretti, S. Sperduti, B. Melli, S. Tagliavini, M. Varani, T. Trenti, D. Morini, A. Falbo, M.T. Villani, K.C. Jonas, M. Simoni, Two human menopausal gonadotrophin (hMG) preparations display different early signaling in vitro, Mol. Hum. Reprod. 26 (2020) 894–905, https://doi.org/10.1093/molehr/gaaa070.
[30] Guideline Bioanalytical method validation – European Medicines Agency, (2019), https://www.ema.europa.eu/en/bioanalytical-method-validation.
[31] R.V. Rebois, Establishment of gonadotropin-responsive murine leydig tumor cell line, J. Cell. Biol. 94 (1982) 70–76, https://doi.org/10.1083/jcb.94.1.70.
[32] M. Ascoli, F. Fanelli, D.L. Segaloff, The lutropin/choriogonadotropin receptor, a 2002 perspective, Endocr. Rev. 23 (2002) 141–174, https://doi.org/10.1210/edrv.23.2.0462.
[33] P.R. Manna, J. Kero, M. Tena-Sempere, P. Pakarinen, D.M. Stocco, I.T. Huhtaniemi, Assessment of mechanisms of thyroid hormone action in mouse Leydig cells: regulation of the steroidogenic acute regulatory protein, steroidogenesis, and luteinizing hormone receptor function, Endocrinology 142 (2001) 319–331, https://doi.org/10.1210/endo.142.1.7900.
[34] R.V. Rebois, S.K. Beckner, R.O. Brady, P.H. Fishman, Mechanism of action of glycopeptide hormones and cholera toxin: what is the role of ADP-ribosylation? Proc. Natl. Acad. Sci. U. S. A. 80 (1983) 1275–1279, https://doi.org/10.1073/pnas.80.5.1275.
[35] W.E. Rainey, R.E. Kramer, J.I. Mason, J.W. Shay, The effects of taxol, a microtubule-stabilizing drug, on steroidogenic cells, J. Cell. Physiol. 123 (1985) 17–24, https://doi.org/10.1002/jcp.1041230104.
[36] R.T. Engeli, C. Fürstenberger, D.V. Kratschmar, A. Odermatt, Currently available murine Leydig cell lines can be applied to study early steps of steroidogenesis but not testosterone synthesis, Heliyon 4 (2018), e00527, https://doi.org/10.1016/j.heliyon.2018.e00527.
[37] T. Fiers, B. Casetta, B. Bernaert, E. Vandersypt, M. Debock, J.M. Kaufman, Development of a highly sensitive method for the quantification of estrone and estradiol in serum by liquid chromatography tandem mass spectrometry without derivatization, J. Chromatogr. B. Anal. Technol. Biomed. Life Sci. 893–894 (2012) 57–62, https://doi.org/10.1016/j.jchromb.2012.02.034.
[38] M. Mezzullo, C. Pelusi, A. Fazzini, A. Repaci, G. Di Dalmazi, A. Gambineri, U. Pagotto, F. Fanelli, Female and male serum reference intervals for challenging sex and precursor steroids by liquid chromatography - tandem mass spectrometry, J. Steroid Biochem. Mol. Biol. 197 (2020), 105538, https://doi.org/10.1016/j.jsbmb.2019.105538.
[39] S. Connan-Perrot, T. Léger, P. Lelandais, C. Desdoits-Lethimonier, A. David, P. A. Fowler, S. Mazaud-Guittot, Six decades of research on human fetal gonadal steroids, Int. J. Mol. Sci. 22 (2021) 6681, https://doi.org/10.3390/ijms22136681.
[40] L. Hu, A. Monteiro, H. Johnston, P. King, P.J. O'Shaughnessy, Expression of Cyp21a1 and Cyp11b1 in the fetal mouse testis, Reproduction 134 (2007) 585–591, https://doi.org/10.1530/REP-07-0133.
[41] H. Inano, A. Kikuchi, B. Tamaoki, In vitro 16 alpha-hydroxylation of progesterone by testes of crab-eating monkey (Macaca irus): a characteristic of primates? Comp. Biochem. Physiol. B 81 (1985) 55–57, https://doi.org/10.1016/0305-0491(85)90161-0.
[42] N.S. Panesar, K.W. Chan, C.S. Ho, Mouse Leydig tumor cells produce C-19 steroids, including testosterone, Steroids 68 (2003) 245–251, https://doi.org/10.1016/s0039-128x(02)00183-6.