Article

Risk assessment of transgender people: development of rodent models mimicking gender-affirming hormone therapies and identification of sex-dimorphic liver genes as novel biomarkers of sex transition

Roberta Tassinari[1*], Alessia Tammaro[1], Gabriele Lori[1], Sabrina Tait[1], Andrea Martinelli[2], Luigia Cangemi[2], Paolo Frassanito[2], Francesca Maranghi[1]

[1] Istituto Superiore di Sanità, Center for Gender Specific Medicine, Rome, Italy;
[2] Istituto Superiore di Sanità, Experimental Animal Welfare Sector, Rome, Italy.
* Correspondence: roberta.tassinari@iss.it; Tel.:+390649902528, francesca.maranghi@iss.it Tel.:+390649902527

Abstract. Transgender (TG) describes individuals whose gender identity differs from the social norms. Some TG people undergo gender-affirming hormone therapy (HT) and may be considered as a sub-group of population susceptible to environmental contaminants for their targets and modes of action. Aim of the work is to set appropriate HT doses and identify specific biomarkers to implement TG animal models. Four adult rats/group/sex are subcutaneously exposed to 3 doses of HT (plus control) selected starting from available data. Demasculinizing-feminizing model (dMF): β-estradiol plus cyproterone acetate: 0.09+0.33, 0.09+0.93 and 0.18+0.33 mg, 5 times/week. Defeminizing-masculinizing model (dFM): testosterone 0.45, 0.95 and 2.05 mg, 2 times/week. Clitoral gain and sperm count, histophatological analysis of reproductive organs and liver, hormone serum levels and gene expression of sex-dimorphic CYP450 are evaluated. In dMF model, the selected doses – leading to T serum levels at the range of the corresponding cisgender - induced strong general toxicity and cannot be used in long-term studies. In dFM model, 0.45 mg of testosterone represents the correct dose. In addition, the endpoints selected are considered suitable and reliable to implement the animal model. The sex-specific CYP expression is a suitable biomarker to set proper (de)masculinizing/(de)feminizing HT and to implement TG animal models.

Keywords: testosterone, estrogen, cyprotenone acetate, masculinizing, feminizing, cytochrome P450, sex-specific genes

1. Introduction

Transgender people (TG) represent a sub-group of population with a gender identity that differs from the assigned sex at birth [1]. They may show binary gender identities (i.e., TG men or TG women) or nonbinary; this last is an umbrella term entailing gender identities falling outside the binary gender frame. Some non-binary people identify themselves as predominantly male or female, though with aspects of the "other" gender (e.g., genderqueer) [2]. Recent estimations recorded that 25 million people were TG, but the number is still increasing; this poses serious problems in term of how to include proper variables for an inclusive investigation of the health concerns [3,4]. TG people often undergo the gender-affirming/confirming hormone therapy (HT) but global medical regulatory agencies, e.g., European Medicines Agency or Food and Drug Administration, haven't approved yet any hormonal agents or clinical protocols for TG medicine. Therefore, 'off-label' HTs are based on guidance from The Endocrine Society or similar [5]. Usually, masculinizing HT uses testosterone (T) treatment and for TG women guidelines suggest estrogen (E2) + androgen lowering drugs [6]. Indeed, scarce data are still available on potential HT impact on the health of TG people and on its potential long-lasting effects. In

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this context, toxicological issues can be also included since one of the main goals of risk assessment is to characterize the chemical risks in potentially sensitive sub-population groups and to ensure the choice of proper safety factors [7]. In fact, TG people - as the general population groups - are daily exposed to food and environmental contaminants among which Endocrine Disrupters share targets and mode of actions with HT, making TG a sub-group of population specifically susceptible and vulnerable to their effects. In this frame, specific animal models should be set and used to perform a reliable hazard identification of TG people undergoing HT [6].

In this context, aim of the present study is the development of two innovative TG animal models – demasculinizing-feminizing model (dMF) and defeminizing-masculinizing model (dFM) - through the selection of suitable dose levels, time and way of administration of (de)masculinizing/(de)feminizing HTs on the basis of the current human therapies. Beyond the T serum level in the cisgender range, that is considered the best biomarker to evaluate the success of gender-affirming HT for both TG men and women, other specific functional and tissue biomarkers are studied and used to characterize the models, in particular: sperm count, clitoral dimensions, histopathological analysis of reproductive organs and liver, androgen receptor (AR) gene expression, E2 and luteinizing hormone (LH) serum levels. Specific attention is posed on liver: in fact, beyond its pivotal role in mammalian metabolism regulated by steroid hormones, a bidirectional link has been recently discovered connecting sex hormones and liver activity, due to the presence of both estrogen (ERs) and androgen (AR) receptors in the liver of both sexes [8]. In this study, a panel of sex-specific liver cytochrome P450 isoforms (CYP450s) are selected to evaluate the plasticity of liver metabolism in relation with HTs and their potential application as biomarkers. Indeed, sexually dimorphic genes could be used to verify the trend/success/safety of HTs, taking also into account that the potential long-lasting effects of gender affirming HTs on the metabolism of TG man and woman have not been established yet. For this purpose, the following CYPs are studied: CYP2C11, is the major male-specific androgen 16α- and 2α-hydroxylase in adult rat liver, induced at puberty following the neonatal androgenic programming in males but not females; CYP3A18 showing a similar profile, which mRNA levels are 25-fold higher in male rat liver compared to female liver [9]; the steroid sulfate 15p-hydroxylase CYP2C12 is expressed in a female-specific way in adult rats; interestingly, CYP2C12 is present in both male and female rats at 3 weeks of age, but at puberty, it is increased only in females and fully suppressed in males [10]; CYP2C6 is a female predominant isoform; male rat liver expresses about the 60% of the levels found in female liver [11].

2. Materials and Methods

2.1 Ethical approval

Animal studies were performed in accordance with the Directive 2010/63/EU, the Italian Legislative Decree n. 26 of 4 March 2014 and the OECD Principles of Good Laboratory Practice (GLP). The study protocol was approved by the Italian Ministry of Health (authorization n° 806/2021-PR).

2.2 Experimental design of animal study

Sixteen young sexually mature Sprague-Dawley rats of each sex (304 ± 13 g male rats and 190 ± 7 g female rats, 8/9 weeks old) were purchased from Envigo (Italy). They were housed two/cage, under standard laboratory conditions (22 ± 0.5 °C, 50–60% relative humidity, 12 h of dark-light alternation with 12–14 air changes per hour) with water and food (2018 Global Diet purchased from Mucedola, Milan, Italy) available ad libitum. In all cages, wood gnawing blocks were inserted for environmental enrichment and replaced weekly. After 1 week of acclimatization, same-sex rats were divided into two experimental groups (dFM and dMF, 4 rats/group), each composed by one control group and three dose levels of the selected HT.

The groups size was calculated using G*Power 3.1.9.7 software and starting from the serum levels of T, the key parameter to assess the success of HT, identified in previous in

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vivo studies after 2 weeks of therapy [12,13]. A power at 0.80 and a significance level (α) at 0.0167, equal to α=0.05 was used to calculate the sample size considering the differences among the control and treated groups by applying the Mann-Whiteny test with Bonferroni correction.

The calculated number of rats/groups:

- for dFM model n=4 based on T serum levels reported in Kinnear H.M. et al. (control group: 0.2 ± 0.3 and 0.45 mg of T group: 16 ± 5 ng/ml - mean ± standard deviation (SD)) [12];

- for dMF model n=3 according to Gomez A. et at. that report T serum levels of 1.901 ± 0.413 and 0.043 ± 0.023 ng/ml (mean ± standard error (SE)) in control and E2 plus CPA group (0.2 + 0.8 mg/kw bw day), respectively [13].

A group size of 4 rats was selected to even out the two rat models.

Two weeks of treatment and the subcutaneous injection as way of administration have been chosen considering the similarities with human HTs, the age correlation between rats and humans and the available literature data [6].

For both models, the doses were selected taking into account the main clinical guidelines used for TG people [5,14] and relevant literature data concerning rodent studies [12,13]. Moreover, a virtual calculator for dosage conversion between human and rat or mice and rat was used (DoseCal, https://dosecal.cftri.res.in/index.php).

During the experimental procedures, all rats were monitored twice a day (at 8:30 a.m. and 4:00 p.m) for general health conditions and potentially aggressiveness due to HT administration. Body weight (bw) and food consumption were recorded two times a week. In dFM model, diameter of rat clitoral was measured the first day of treatment and at the day of sacrifice by precision digital caliper. Twenty-four hours after the last treatment, rats were anaesthetized with a gaseous solution of isoflurane and blood samples were collected by intracardiac puncture for the determination of serum hormones. Subsequently, animals were sacrificed by CO2 inhalation, necropsy and gross pathology were performed. Liver and reproductive organs (testes, ovaries and uteri) were excised and weighted. Right epididymis was used for sperm count analysis. For histopathological analysis, target tissues were immediately fixed in 10% buffered formalin, except testes and epididymis fixed in Bouin's solution. A portion of liver was flash frozen in liquid nitrogen and stored at -80 °C for gene expression analysis.

2.2.1 Demasculinizing-feminizing model (dMF)

16 male rats were divided into four treatment groups (4 rats/group) receiving 200-µL subcutaneous injections of β-Estradiol 17-valerate (Sigma-Aldrich, N. CAS: 979-32-8) plus cyproterone acetate (Sigma-Aldrich, N. CAS: 427-51-0) in sesame oil (ACROS, N. CAS: 8008-74-0) or sesame oil alone five times a week for two weeks as follows:

- Control group (CM): sesame oil (vehicle);
- Dose 1 (D1M): 0.09 + 0.33 mg per dose;
- Dose 2 (D2M): 0.09 + 0.93 mg per dose;
- Dose 3 (D3M): 0.18 + 0.33 mg per dose.

2.2.2 Defeminizing-masculinizing model (dFM)

16 female rats were divided into four treatment groups (4 rats/group) receiving 100-µL subcutaneous injections of testosterone enanthate (Sigma-Aldrich, N. CAS: 315-37-7) in sesame oil or sesame oil alone twice a week (Monday and Thursday am) for two weeks as follows:

- Control group (CF): sesame oil (vehicle);
- Dose 1 (D1F): 0.45 mg per dose;
- Dose 2 (D2F): 0.95 mg per dose;
- Dose 3 (D3F): 2.05 mg per dose.

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2.3 Sperm count

Right caudae epididymides were excised, rinsed with D-MEM medium (gibco), transferred on a Petri dish containing 1 mL D-MEM medium and minced with scissors. Epididymal pieces were fluxed through a Pasteur pipette to facilitate sperm extrusion. Sperm suspension was diluted up to 10 mL. Sperm were then counted by a Neubauer chamber under a light microscopy (Nikon Eclipse Ts2) [15].

2.4 Blood collection and biochemical evaluation of hormones

Blood was always sampled from 9 to 10 am, with stratification across treatment groups, in order to reduce the potential impact of circadian rhythm and pulsatility. Blood samples were left to coagulate at room temperature for 1h, centrifuged for 15 min at 2000 rpm twice in a cooled bench-top centrifuge (Microlite Microfuge, Thermo Electron Corporation) and stored at -80 °C until use. Serum levels of all hormones were measured in the same analytical section by the following commercial ELISA kits of the same lot(s):

-E2 Rat kit (RTC009R - BioVendor Brno, Czech Republic), LOD 2.5 pg/ml

-T Mouse/Rat kit (RTC001R - BioVendor Brno, Czech Republic), LOD 2.5 pg/ml;

-LH Rat Kit (ELK2367 - ELK Biotechnology, China), LOD 37.59 pg/ml

Each kit provided a standard solution of the hormone and serial dilutions were prepared to derive a standard curve and define the range of linearity of each test. For all the analyses, the manufacturer's instructions were followed. Each sample was assessed in duplicate, and absorbance was read at 450 nm on a VICTOR3 Multilabel reader (Perkin Elmer, USA). The unknown hormone concentrations in samples were derived using the standard curve of each hormone using the software GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA).

2.5 Histological and histomorphometrical analysis

Immediately after the sacrifice, to avoid any possible post-mortem artefacts, liver, ovary and uterus were fixed in 10% buffered formalin, testes in Bouin's solution and stored in 80% ethyl alcohol. They were dehydrated in a graded series of alcohol baths and embedded in paraffin by tissue processor (Shandon Excelsior ES, Thermo Scientific). The 5-µm-thick histological sections were prepared using the Microm HM 325 (Thermo Scientific) and stained with hematoxylin/eosin for the examination under a light microscopy (Nikon Microphot FX) at various magnifications to evaluate the histopathological alterations. The scoring of the lesions was semi-quantitative, using a 5-point grading scale (0 to 4), taking into consideration the severity of the changes based on the criteria explained by Shackelfold C et al. [16] and summarized as follows: Grade 0: No change; Grade 1 (+1): Minimal; Grade 2 (+2): Mild; Grade 3 (+3): Moderate; Grade 4 (+4): Marked [17].

The quantitative histomorphometrical analyses were performed on ovaries, uterus and testesicles by means of an image analysis system (Nis-Elements BR) applied to an optical microscope (Nikon Microphot FX). Briefly, testis tubular diameters and the relative area of the seminiferous tubules and lumen were measured in 20 randomly selected tubules (10x objective); in uterus, a cross-section was taken from the right uterine horn, 1 cm above the uterine bifurcation and ratio between the area of endometrium and myometrium as relative percentage of both uterine tissue components was calculated (2x objective) [18]. Moreover, the luminal epithelial cell height of the uterus was measured (64x objective). Ovarian classification of the different follicles was performed according to Fortune J.E. [19]. Using one of the largest sections in a central position of the ovary, primary and secondary follicles, corpora lutea, Graaf follicles and atretic follicles were counted in the whole ovarian section (2x objective) [18].

2.6 Gene expression analysis

10 mg of each liver samples were mechanically disgregated by the Miccra D-1 homogenizer (ART-moderne Labortechnik, Germany) and total RNA content was extracted

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with the Norgen kit (Norgen, Canada) according to the manufacturer's instructions. RNA quantity was assessed by Nabi Nano Spectrophotometer (MicroDigital Co. Ltd., Korea) whereas integrity was evaluated by 1% agarose gel electrophoresis. All the samples met quality criteria (integrity, A260/A280≥1.8) to proceed with real-time PCR analysis. One microgram of total RNA from each sample was reverse transcribed to cDNA using the Tetro cDNA Synthesis Kit (Quantace, UK) according to the manufacturer's instructions. Specific primers for CYP2C11, CYP3A18, CYP2C12, CYP2C6, AR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as reference gene, were designed using the Primer-BLAST web application (www.ncbi.nlm.nih.gov/tools/primer-blast) and purchased from Metabion (Metabion International AG, Germany) as follows: CYP2C11 forward (fw) ACAGAGCTTTGGGAGAGGGA, reverse (rev) CCATGCAACACCACAAAGGG; CYP3A18 fw CAGCTGGGAAGGAAACTTGG, rev AGGCCATGAGAATAGGTCCC; CYP2C12 fw CTTGCCCCAAATGGTTTGTTG, rev GGTCAGGAACAAAAACAGCTC; CYP2C6 fw CCACGTTTATCCTGGGCTGT, rev GTGTCCAGGGACTGCTCAAA and AR fw TAGGGCTGGGAAGGGTCTAC, rev CCTCTGATGTGGGCTTGAGG.

The Excel TaqTM Fast Q-PCR Master Mix SYBR (SMOBIO Technology Inc., Hsinchu City, Taiwan) was used to perform real-time PCR assays running reactions on a Bioer LineGene 9600 (Bioer, China) with the following thermal program: one cycle at 95°C for 20 s; 40 cycles at 95°C for 3 s, 58°C for 15 s and 72°C for 15 s; one melting cycle from 55 to 95°C to verify amplification products. Experiments were performed in duplicate on 96-well PCR plates. Threshold cycles were calculated by the LineGen9620 software (Bioer). Data are expressed as ΔΔCt ± SD values for each target gene with control samples as calibrator and GAPDH as reference gene.

2.7 Data Analysis

Data management, data enter and statistical analyses were performed by a single operator using Microsoft Excel 2013. Data were analyzed using the software JMP 10 (SAS Institute Inc., Cary, NC, USA). A non-parametric Kruskal-Wallis analysis was performed to analyze data followed by post-hoc pairwise comparisons (Mann-Whitney test). Quantal data were analyzed by 2-way Fisher Exact Test to assess significant differences with respect to control groups. The Cochran-Armitage Trend Test was used to evaluate a dose-response trend. Differences between groups were considered significant if the P-value was < 0.05. The GraphPad Prism 6.0 software was used to design all graphics.

3. Results

3.1. General toxicity, sperm count and clitoral gain

3.1.1. Demasculinizing-feminizing model

No death, clinical effects or aggressive behaviour have been recorded. The bw gain, bw at treatment day 5, 8, 12 and 13, feed consumption, testis absolute weight and sperm count were significantly decreased in comparison to CM in all treatment groups (D1M, D2M and D3M) (Figure 1; Figure 2; Table 1). Testis relative weight was significantly decreased in D2M and D3M in comparison to CM group (Table 1). Liver absolute weight was significantly reduced in D3M and relative weight was significantly increased in D2M compared to CM group (Table 1).

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Figure 1. Body weight (bw) of male rats subcutaneously treated, with different doses of estradiol valerate plus cyproterone acetate five times a week, for 2 weeks: CM: 0 – sesame oil, D1M 0.09+0.33, D2M 0.09 + 0.93 and D3M 0.18+0.33 mg. Panel (A) bw gain and (B) bw at treatment days 5, 8, 12 and 13. Data are presented as mean ± SD. Statistical significance: * $p < 0.05$ Mann-Whitney test.

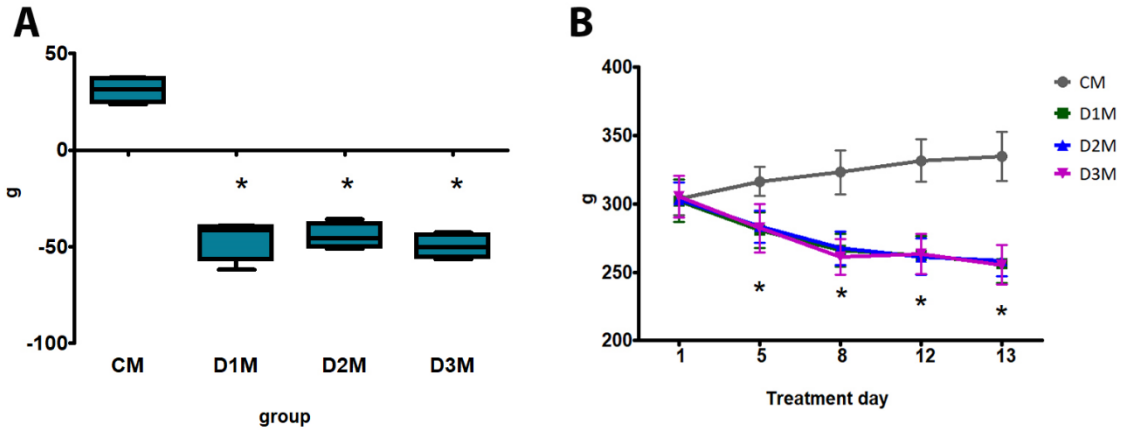

Figure 2. Sperm Count of male rats subcutaneously treated with different doses of estradiol valerate plus cyproterone acetate five times a week, for 2 weeks: CM 0 – sesame oil, D1M 0.09+0.33, D2M 0.09 + 0.93 and D3M 0.18+0.33 mg. Panel (A) sperm count and (B) picture of caudae epididymides. Data are presented as mean ± SD. Statistical significance: * $p < 0.05$ Mann-Whitney test. N: sperm number.

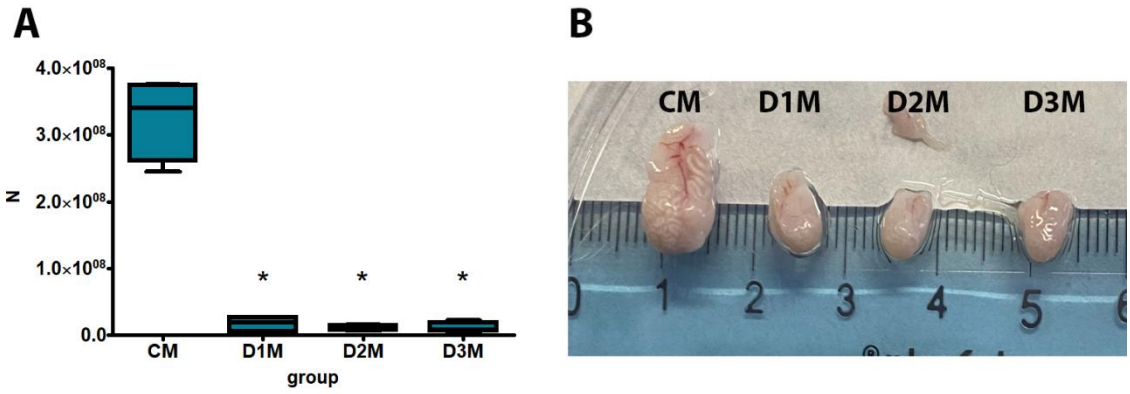

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Table 1. General toxicity data of male rats subcutaneously treated with different doses of estradiol valerate plus cyproterone acetate five times a week, for 2 weeks: CM 0 – sesame oil, D1M 0.09+0.33, D2M 0.09 + 0.93 and D3M 0.18+0.33 mg. Statistical significance: * $p < 0.05$ Mann-Whitney test.

	CM	D1M	D2M	D3M
N	4	4	4	4
Feed consumption (g, mean ± SD)	21.04 ± 0.08	13.48 ± 0.40*	15.69 ± 0.77*	14.97 ± 1.22*
Testis absolute weight (g, mean ± SD)	3.49 ± 0.15	2.20 ± 0.50*	2.16 ± 0.16*	2.21 ± 0.21*
Testis relative weight (mean ± SD)	1.05 ± 0.06	0.86 ± 0.22	0.83 ± 0.07*	0.86 ± 0.07*
Liver absolute weight (g, mean ± SD)	12.23 ± 1.17	10.03 ± 1.50	11.20 ± 0.96	10.14 ± 0.66*
Liver relative weight (mean ± SD)	3.65 ± 0.26	3.91 ± 0.37	4.33 ± 0.24*	3.97 ± 0.24

3.1.2 Defeminizing-masculinizing model

No death, clinical effects or aggressive behavior have been recorded. The bw gain was significantly increased in D2F and D3F; bw at treatment days 12 and 13 was significantly increased compared to controls in D3F (Figure 3). Feed consumption was significantly increased in D3F compared to CF group (Table 2). At necropsy, a dose-dependent increase of haemorrhagic ovaries and uteri, significant in D3F in comparison to CF group (Table 2), was recorded. Clitoral gain was increased in all treatment groups although not significantly (Table 2). Ovary absolute and relative weight showed a dose-dependent decrease, significantly in D2M and D3M in comparison to CF group (Table 2). Uterus absolute weight was significantly decreased in D1F and D2F, whereas the relative weight was unaffected (Table 2). No treatment-related alterations were observed in absolute and relative liver weight (Table 2).

Figure 3. Body weight (bw) of female rats subcutaneously treated with different doses of testosterone enanthate two times a week, for 2 weeks: CF: 0 – sesame oil; D1F: 0.45; D2F: 0.95; and LD3F: 2.05 mg. Panel (A) bw gain and (B) bw at treatment days 5, 8, 12 and 13. Data are presented as mean ± SD. Statistical significance: * $p < 0.05$ Mann-Whitney test.

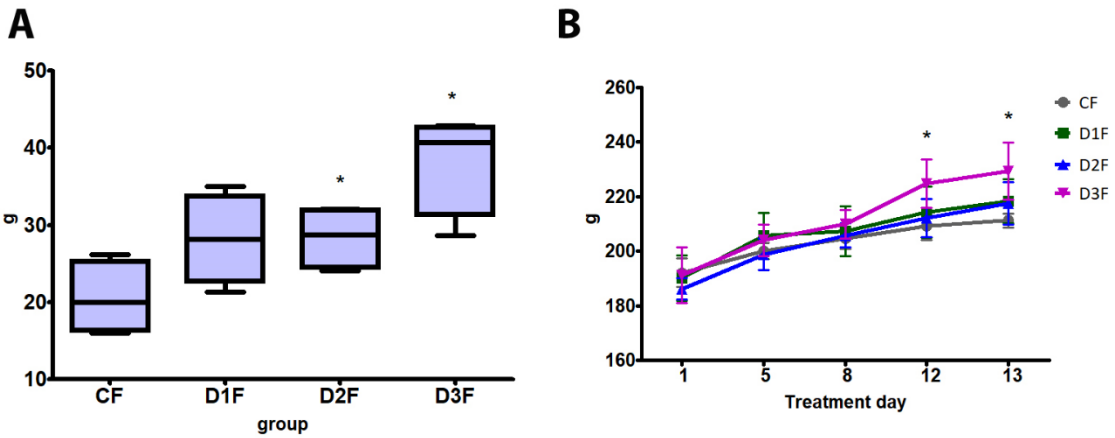

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Table 2. General toxicity data of female rats subcutaneously treated with different doses of testosterone enanthate two times a week, for 2 weeks: CF 0 – sesame oil; D1F 0.45, D2F 0.95 and D3F 2.05 mg. Statistical significance: § $p < 0.05$ Fisher exact test; # $p < 0.05$ linear trend; * $p < 0.05$ Mann-Whitney test.

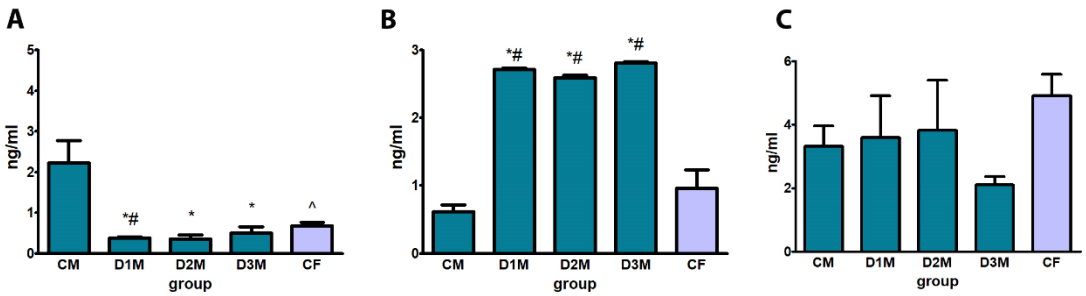
	CF	D1F	D2F	D3F
N	4	4	4	4
Feed consumption (g, mean ± SD)	15.8 ± 0.2	16.8 ± 1.0	15.4 ± 0.5	17.9 ± 1.4*
Haemorrhagic ovaries and uteri	0/4 (0%) #	0/4 (0%)	1/4 (20%)	4/4 (100%) §
Clitoral gain (mm, mean ± SD)	-0.12 ± 0.47	0.48 ± 0.43	0.24 ± 0.20	0.39 ± 0.36
Ovary absolute weight (g, mean ± SD)	0.17 ± 0.02	0.14± 0.12	0.12 ± 0.02*	0.10 ± 0.01*
Ovary relative weight (mean ± SD)	0.08 ± 0.01	0.07± 0.01	0.06 ± 0.01*	0.05 ± 0.01*
Uterus absolute weight (g, mean ± SD)	0.52 ± 0.07	0.36 ± 0.02*	0.37 ± 0.03*	0.41 ± 0.10
Uterus relative weight (mean ± SD)	0.21 ± 0.04	0.17± 0.01	0.17 ± 0.02	0.18 ± 0.05
Liver absolute weight (g, mean ± SD)	6.94 ± 0.14	7.36 ± 0.59	7.47 ±0.61	7.25 ± 1.45
Liver relative weight (mean ± SD)	3.25 ± 0.07	3.37 ± 0.25	3.48 ± 0.20	3.16 ± 0.63

3.2 Biochemical evaluation of hormones

3.2.1 demasculinizing-feminizing model

T serum levels were significantly decreased in all treatment groups (D1M, D2M and D3M) in comparison to CM. E2 serum levels was statistically significant increased in all treatment groups (D1M, D2M and D3M) in comparison to CM and in comparison to CF. No treatment-related alterations were observed in LH serum levels (Figure 4).

Figure 4. Biochemical evaluation of hormones by ELISA of male rats subcutaneously treated with different doses of estradiol valerate plus cyproterone acetate five times a week, for 2 weeks: CM 0 – sesame oil, D1M 0.09+0.33, D2M 0.09 + 0.93, D3M 0.18+0.33 and CF (control female) 0 mg. Panel (A) testosterone, (B) estradiol and (C) luteinizing hormone. Data are presented as mean ± SD. Statistical significance: * $p < 0.05$ "CM" vs "DM" groups; #$p < 0.05$ "CF" vs "DM" groups; ^ $p < 0.05$ "CM" vs "CF" groups Mann-Whitney test.



3.2.2 Defeminizing-masculinizing model

T serum levels were dose-dependently and significantly increased in all treatment groups (D1F, D2F and D3F) in comparison to CF. No treatment-related alterations were

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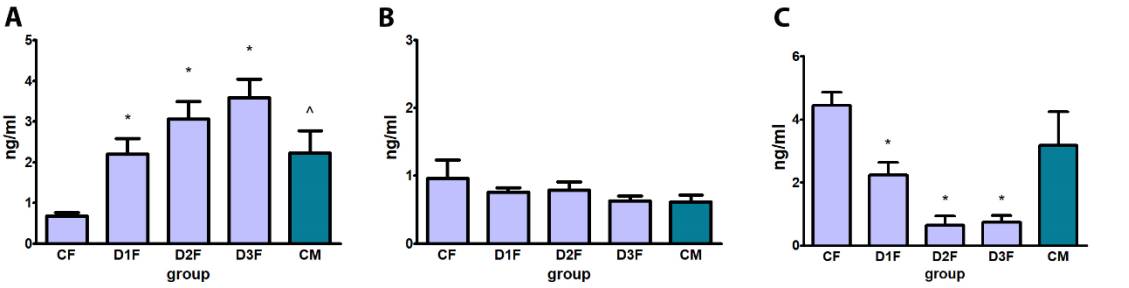
observed in E2 serum levels. Serum levels of LH were significantly decreased in all treatment groups (D1F, D2F and D3F) in comparison to CF (Figure 5).

Figure 5. Biochemical evaluation of hormones by ELISA of female rats subcutaneously treated with different doses of testosterone enanthate two times a week, for 2 weeks: CF: 0 – sesame oil, D1F: 0.45, D2F: 0.95, D3F: 2.05 and CM (control male) 0 mg. Panel (A) testosterone, (B) estradiol, (C) luteinizing hormone. Data are presented as mean ± SD. Statistical significance: * $p < 0.05$ "CF" vs "DF" groups; #$p < 0$. "CM" vs "DF" groups; ^ $p < 0.05$ "CF" vs "CM" groups Mann-Whitney test.



3.3 Histological and histomorphometrical analysis

3.3.1 Demasculinizing-feminizing model

Testes showed a significant increase of tubule degeneration with germinal epithelium degeneration in all treatment groups (D1M, D2M and D3M) compared to CM group (Figure 6; Table 3). Histomorphometrical analysis of testes showed a significant reduction of tubule area in D3M group (Table 3). Liver showed a significant increase of sinusoidal dilatation (enlargement of the hepatic capillaries) and hepatocyte vacuolation (steatosis) in all treatment groups (D1M, D2M and D3M) compared to CM group (Figure 7; Table 3).

Figure 6. Testes of male rats subcutaneously treated five times a week, for 2 weeks with 0 – sesame oil (CM panel A) and 0.18+0.33 mg of estradiol valerate plus cyproterone acetate (D3M panel B). Tubule degeneration with germinal epithelium degeneration. Bar 10 ⊚m (original magnification 10X; haematoxylin and eosin stain).

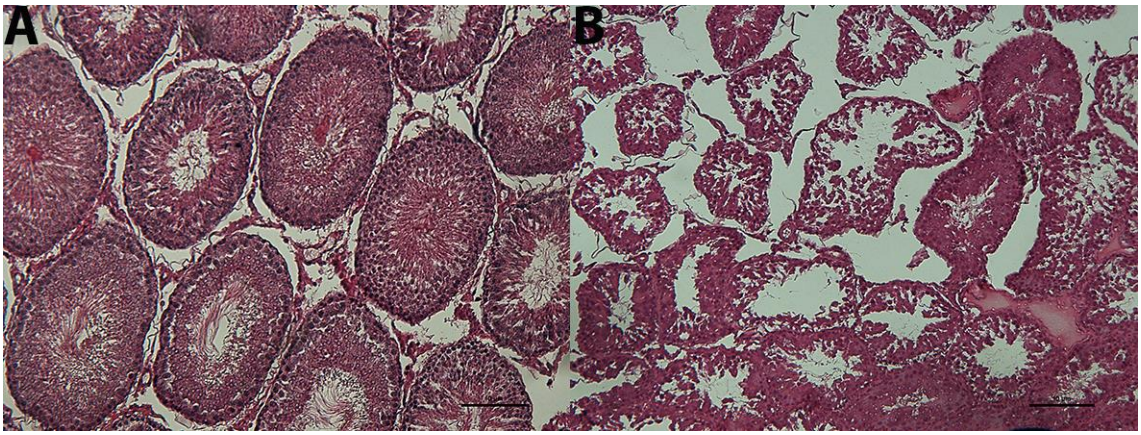


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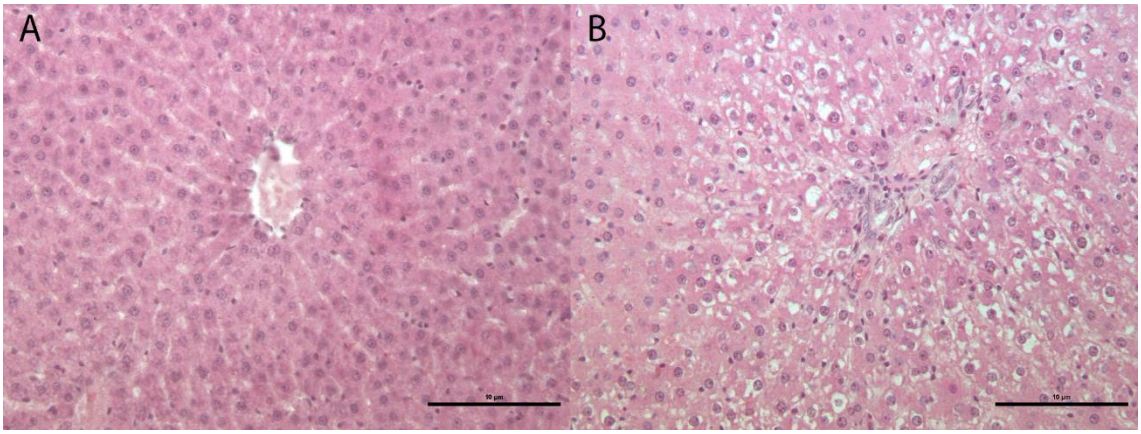
Table 3. Histopathological endpoints in target organs of male rats subcutaneously treated with different doses of estradiol valerate plus cyproterone acetate, five times a week, for 2 weeks: CM 0 – sesame oil, D1M 0.09+0.33, D2M 0.09 + 0.93; and D3M 0.18+0.33 mg. Statistical significance: § $p < 0.05$ Fisher exact test; * $p < 0.05$ Mann-Whitney test.

ORGAN/Observation		CM	D1M	D2M	D3M
	N	4	4	4	4
TESTIS: tubule degeneration with germinal epithelium degeneration					
	0	4			
	2		4		
	3			4	
	4				4
	Total Finding Incidence	0	4§	4§	4§
TESTIS: tuble area [µm²; mean ± SD]		422.3 ± 74.3	271.9 ± 147,1	264.8 ± 85,8	223.0 ± 30,7*
LIVER: sinusoidal dilatation					
	0	4			
	1		2		
	2		2	4	
	Total Finding Incidence	0	4§	4§	4§
LIVER: hepatocyte vacuolation					
	0	4			
	1		4		
	2			2	
	3			2	4
	Total Finding Incidence	0	4§	4§	4§

Figure 7. Liver of male rats subcutaneously treated five times a week, for 2 weeks with 0 – sesame oil (CM panel A) and 0.09 + 0.93 mg of estradiol valerate plus cyproterone acetate (D2M panel B). Hepatocyte vacuolation (steatosis). Bar 10 ◎m (original magnification 20X; haematoxylin and eosin stain).



3.3.2 Defeminizing-masculinizing model

Uterus showed a dose-dependent increase of endometrial and/or myometrial hemorrhage, significant in D3F in comparison to CF group. Histomorphometrical analysis showed a significant reduction of horn areas and myometrium areas in D1F and D2F groups and lumen areas in D3F only. Endometrium areas were significantly reduced in all treatment groups (D1F, D2F and D3F). The ratio of endometrium and myometrium

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areas was significantly reduced in D3F, whereas it was significantly increased in D2F and D3F in comparison to CF group. Luminal epithelium height was significantly decreased in D2F and D3F groups (Table 4). Ovary showed a dose-dependent increase of hemorrhage, significantly in D3M group in comparison to CF group. The number of primary and secondary follicles was significantly increased and Graaf follicles were significantly reduced in D3F group in comparison to CF group (Table 4). Liver showed a significant increase of sinusoidal dilatation (enlargement of the hepatic capillaries) in all treatment groups (D1F, D2F and D3F) compared to CF group (Figure 8; Table 4).

Figure 8. Liver of female rats subcutaneously treated two times a week, for 2 weeks with 0 - sesame oil (CF panel A) and 2.05 mg of testosterone enanthate (D3F panel B). Sinusoidal dilatation (enlargement of the hepatic capillaries). Bar 10 ◎m (original magnification. 20X; haematoxylin and eosin stain).

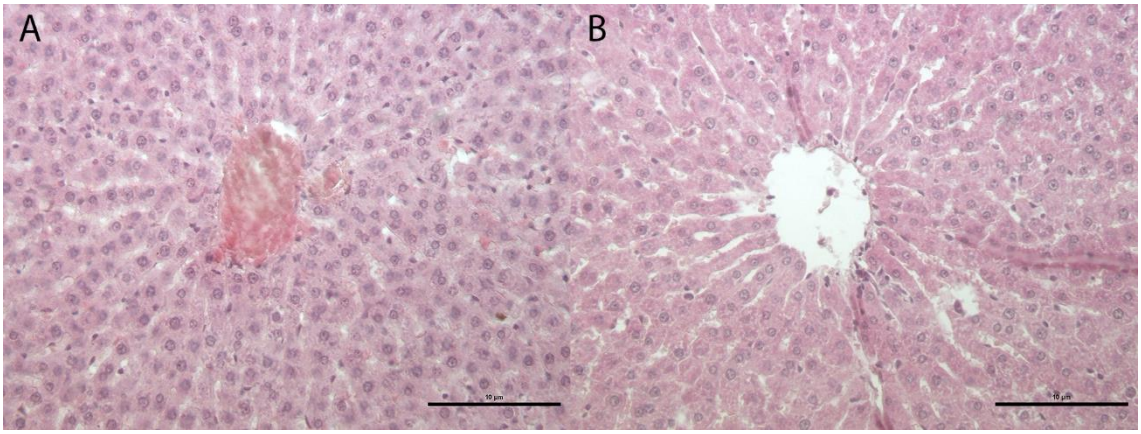

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Table 4. Histopathological endpoints in target organs of female rats subcutaneously treated with different doses of testosterone enanthate two times a week, for 2 weeks: CF 0 – sesame oil, D1F 0.45, D2F 0.95 and D3F 2.05 mg. Statistical significance: § $p < 0.05$ Fisher exact test; ## $p < 0.01$ linear trend; * $p < 0.05$ Mann-Whitney test.

ORGAN/Observation	**CF**	**D1F**	**D2F**	**D3F**
N	4	4	4	4
UTERUS: endometrial and/or myometrial hemorrhage				
0	4	4	3	
3			1	4
Total Finding Incidence	0##	0	1	4§
UTERUS: total horn areas [µm²; mean ± SD]	60651 ± 12149	33878 ± 3137*	36023 ± 8422*	37795 ± 11903
UTERUS: lumen areas [µm²; mean ± SD]	3864 ± 3878	1355 ± 514	1683 ± 1364	1032 ± 248*
UTERUS: myometrium areas [µm²; mean ± SD]	12614 ± 1158	7660 ± 1396*	7269 ± 2350*	9464 ± 2730
UTERUS: endometrium areas [µm²; mean ± SD]	26246 ± 3253	17261 ± 1939*	17495 ± 3596*	13519 ± 5230*
UTERUS: Endometrium/Myometrium areas	2.09 ± 0,28	2.31 ± 0,52*	2.52 ± 0,60*	1.44 ± 0,33*
UTERUS: luminal epithelium height [µm²; mean ± SD]	23.03 ± 1,56	18.10 ± 3,80	17.14 ± 2,23*	17.60 ± 1,98*
OVARY: hemorrhage				
0	4	4	2	
2			2	4
3				
Total Finding Incidence	0##	0	2	4§
OVARY: follicular density	0.08 ± 0.0	0.11 ± 0.1	0.14 ± 0.0	0.15 ± 0.0
OVARY: primary + secondary follicles [N; mean ± SD]	28.5 ± 11.7	21.25 ±10.5	47.3 ±14.2	65 ± 6.8*
OVARY: Graaf follicles [N; mean ± SD]	3.5 ±1.3	2.5 ± 1.0	2.5 ± 1.0	1.25 ± 0.5*
LIVER: sinusoidal dilatation				
0	4			
1		4		
2			3	
3			1	4
Total Finding Incidence	0	4§	4§	4§

3.4 Gene expression

3.4.1 Demasculinizing-feminizing model

Male-specific CYPs were down-regulated in all treatment groups; in particular, CYP2C11 expression was significantly down-regulated in all treatment groups (D1M, D2M and D3M); CYP3A18 was significantly down-regulated in D1M group in comparison to CM (Figure 9). No treatment related alterations were observed in AR gene expression (Figure 9). Considering the female-specific CYPs, an up-regulation of CYP2C12 gene expression was observed in all treatment groups, statistically significant in D2M and D3M. The same dose levels induced up-regulation of CYP2C6 with borderline significance in D2M ($p=0.0518$) (Figure 10).

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Figure 9. Comparison between gene expression analysis of sex (male)-specific genes CYP2C11 (Panels A, B), CYP3A18 (panels C, D) and AR (Panels E, F) by real-time PCR in rat liver. Male rats, panels (A), (C) and (E), subcutaneously treated with different doses of estradiol valerate plus cyproterone acetate five times a week, for 2 weeks: CM 0 – sesame oil, D1M 0.09+0.33, D2M 0.09+0.93, and D3M 0.18+0.33 mg. Female rats, panels (B), (D) and (F), subcutaneously treated with different doses of testosterone enanthate two times a week, for 2 weeks: CF 0 – sesame oil, D1F 0.45, D2F 0.95, and D3F 2.05 mg. Data are presented as mean ΔΔCt values ± SD, with control samples as calibrators and GAPDH as the reference gene. Statistical significance: * $p < 0.05$ Mann-Whitney test.

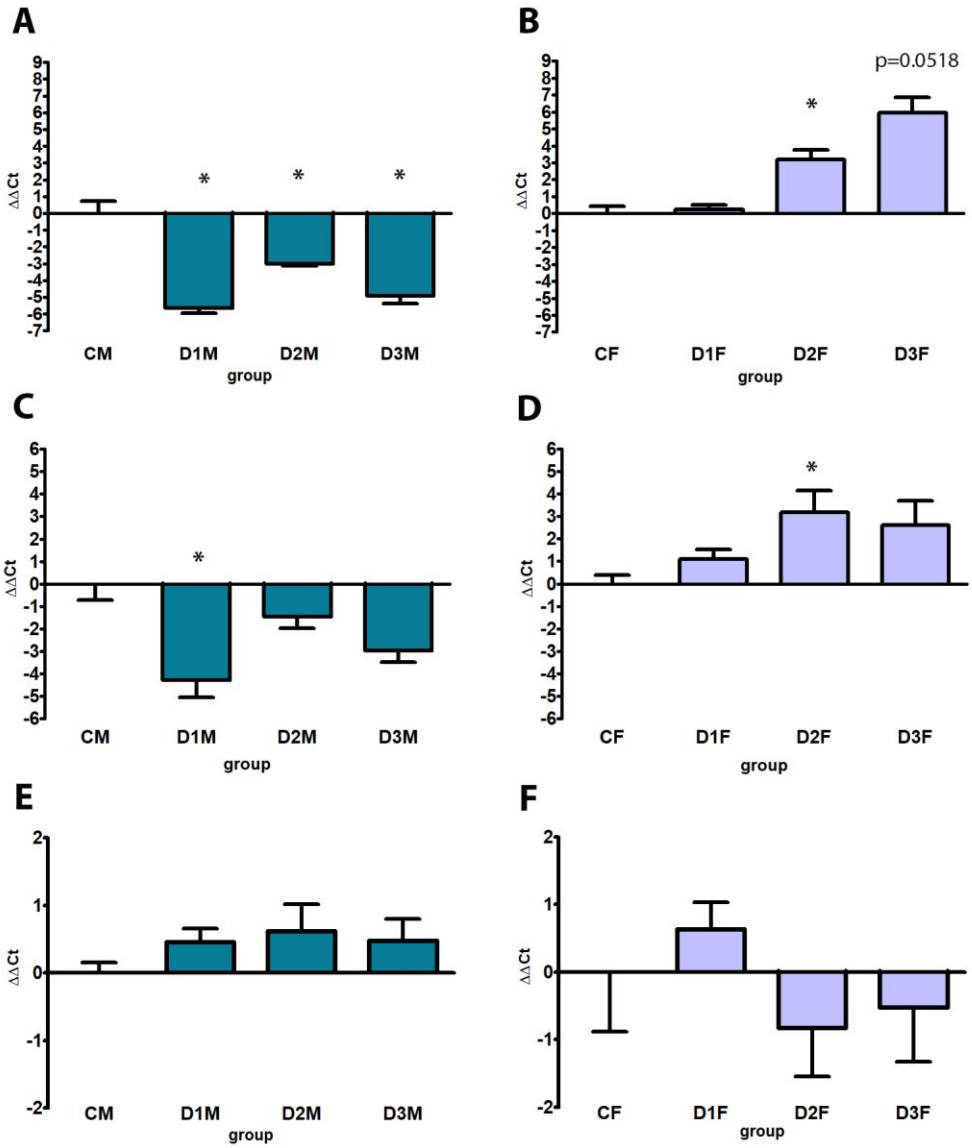

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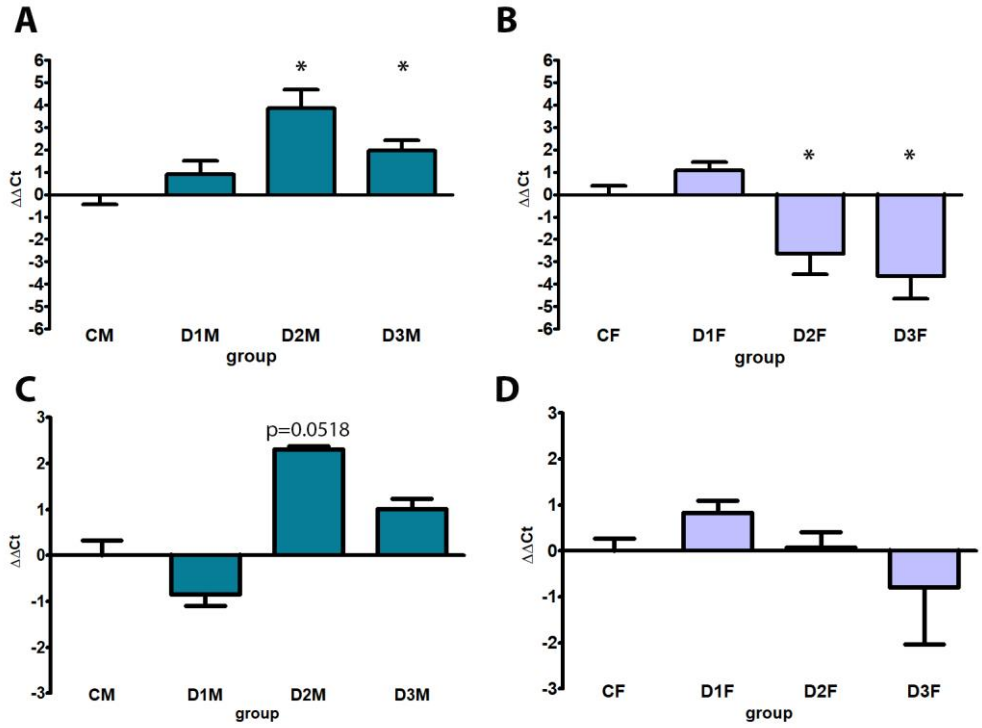
Figure 10. Comparison between gene expression analysis of sex (female)-specific genes CYP2C12 (Panels A, B) and CYP2C6 (Panels C, D) by real-time PCR in rat liver. Male rats, panels (A) and (C), subcutaneously treated with different doses of estradiol valerate plus cyproterone acetate five times a week, for 2 weeks: C 0 – sesame oil, D1M 0.09+0.33, D2M 0.09 + 0.93, and D3M 0.18+0.33 mg. Female rats, panels (B) and (D), subcutaneously treated with different dose of testosterone enanthate two times a week, for 2 weeks: CF: 0 – sesame oil, D1F 0.45, D2F 0.95, and D3F 2.05 mg. Data are presented as mean ΔΔCt values ± SD, with control samples as calibrators and GAPDH as the reference gene. Statistical significance: * $p < 0.05$ Mann-Whitney test.



3.4.2 Defeminizing-masculinizing model

Female-specific CYP2C12 gene expression was down-regulated, significantly in D2F and D3F groups. No treatment-related alteration was present in CYP2C6 gene expression (Figure 10). CYPs specifically expressed in male rats were up-regulated in all treatment groups, in particular CYP2C11 expression showed a dose-dependent up-regulation statistically significant in D2F and with borderline significance in D3F groups (p=0.0518); CYP3A18 was significantly up-regulated in D2F group in comparison to CF. No treatment-related alterations were observed in AR gene expression (Figure 9).

3. Discussion and conclusions

In toxicological studies, the use of targeted animal models is of pivotal importance to obtain sound data for hazard identification of chemicals [20]. As an example, it is known that children are different from adults concerning chemical hazards due to different exposure scenarios, age-related metabolic capacity and biological susceptibility; the Juvenile toxicity test –intended for hazard identification in a children-specific perspective and already used in paediatric drug development – is based on animal model at the peripubertal phase of life corresponding to childhood [21]. TG people undergoing HT showed unique features in term of specific susceptibility and vulnerability to chemical contaminants; therefore, they need suitable models based on appropriate, novel biomarkers [15], and the development of these last represents the main aim of the present study.

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Considering the dMF rat transition model, although no death was recorded during the treatment period, the selected doses of E2 plus CPA are shown to be too high and, consequently, the model cannot be implemented and used for long-term studies. In fact, several marked toxicological effects are recorded in all the selected doses even if the T serum levels are decreased, reaching the corresponding cisgender value in D2M and D3M. As expected, the feminizing HT increased the levels of E2 and caused marked testis alterations (weight, dimensions, sperm count and histopathology). Moreover, the strong reduction of weight gain and food consumption observed in all treatment groups could be also due to the well-known estrogenic inhibition of eating, maybe enhanced by the increase in E2 levels resulting from the of the available circulating T [22]. Several modulators of appetite including e.g., ghrelin, cholecystokinin, glucagon, insulin and leptin are estradiol-sensitive under specific conditions, and they may mediate the estrogenic inhibition of eating; further analyses should than be performed in such direction to evaluate the metabolic impact of HT. Noteworthy, in the present case, the weight gain reduction is beyond the percentage of variation (<5%) considered of toxicological relevance [22].

At tissue level, liver of male rats showed sinusoidal dilatation and steatosis in all the doses. Several studies correlate E2 with hepatotoxicity. Ethynyl estradiol (a synthetic estrogen used as contraceptive drug) caused liver effects (congestion, focal areas of hemorrhage, vacuolation of cytoplasm, distended sinusoids with dilated central veins) in female rats and the extent and severity of the damage depends on the dose and the time of administration [23]. More recently, CPA demonstrated also to be an aryl hydrocarbon receptor (AhR) agonist in mouse and an AhR antagonist in human cells [24]. Indeed, in both male and female C57BL/10J mice, long-term exposure to 800 ppm of CPA in the diet induced hepatocyte hypertrophy and increased fat and glycogen [25]. Since the effects recorded in the present study are coherent with these data, they may serve as a support to develop more targeted HTs, e.g., with reduced concentrations of CPA [26].

Two experimental studies using feminizing HT in rodents are available up to now: Gomes et al study focused on brain morphology after feminization therapy [13] and Gusmão-Silva J.V. et al. study explored the effects of injectable steroid combination - frequently used by transwomen - on blood pressure and metabolic outcomes [27]. Although they are relevant for the health impact of HTs, none of them is specifically focused on the implementation of the rodent model for further applications.

Considering the masculinizing HT for female rat – dFM model – the selected doses of T didn't induce death or overt sign of toxicity; as expected, the body weight gain was increased together with food intake due to T administration [28]. T serum levels were in the range of cisgender at all the doses; nevertheless, at the highest doses, uteri and ovaries with strong hemorrhage were noted, leading to identify the D1F - 0.45 mg - as the suitable T concentration for log-term studies. Clitoral areas were increased although not significantly; in a previous study, three doses (0.225, 0.45 and 0.90 mg) of T enanthate were subcutaneously injected twice a week to post pubertal C57BL/6N female mice for 6 weeks, mimicking the HT for TG men [12]. Clitoral area was significantly increased at the highest doses, and the difference with the present data may be due to the shorter treatment period. Histopathological analysis of uteri showed atrophy in all treatment groups, compatible with T administration [29]. The alterations recorded in the ovary referred to increased hemorrhagic areas coherently with the observations at necropsy, and the delayed/reduced maturation of follicles in D3F. This last is again a typical effect of T administration in adult female mice [30]; indeed, the maturation of follicles in the ovary was not completely inhibited, as shown also by Yang M. et al suggesting that T therapy didn't deplete the ovarian reserve [12,30].

The LH suppression was already observed in mice treated with T, and E2 serum levels were decreased in the range of cisgender [12].

Liver alterations were similar but milder in comparison to those observed in the dMF model, above all concerning sinusoidal dilatation; interestingly, hepatocyte vacuolization was significantly reduced in the D2F and D3F groups in comparison to controls, indicating

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an apparent protective effect of T. Indeed, an interesting study of Uchida K. et al. showed that hepatic triglyceride accumulation diminished with sex maturation in male but not in female protein-restricted rats since endogenous T reduces hepatic lipid accumulation. It might be hypothesized that masculinizing T treatment protected female rats from hepatic lipid accumulation [31], providing also an indirect hint on the liver masculinization.

Other than the above cited studies, there are some others that administered T to female rodents with different aims; as an example, Barteles C.B. et al. provided evidence that female mice produce normal, fertilizable eggs after 6-weeks treatment with 400 μg T cypionate, whether T levels are low after a washout period or high during 2 active exposure [32]. In another study, female 12-week old mice masculinized by subcutaneous injections of 0.9 mg T twice a week for 3 weeks, showed long-lasting epigenetic modification in liver [33]. None of them provided specific data to implement a targeted animal model for TG people.

In the present study, CYP gene expression analysis provided interesting results: after just two weeks of HT in dMF model, an apparent demasculinization in the expression of sex-specific CYPs occurred either for CYP2C11 or CYP3A18 (preferentially expressed in male rats and up-regulated by the treatment), and CYP2C12 (preferentially expressed in female rats and down-regulated) both in D2M e D3M groups. The same for the dFM, where a reversion, in this case a defeminization, of sex-specific CYP expression with up-regulation of male-specific CYP3A18 and CYP2C11 and a down -regulation of female-specific CYP2C12 occurred, in particular in D2F and D3F. It should be also considered that the male and female hormonal profiles can be altered by several factors, including drug therapies, exposure to environmental chemicals and diseases such as diabetes and liver cirrhosis; these factors could consequently influence the expression of specific liver CYPs highly under hormone regulatory controls [10]. Nevertheless, in the present study, the marked shift of expression after a short (two weeks) exposure time might be considered as a biomarker of demasculization/defeminization induced by the treatment [34]. In both the models AR expression was unaffected by the treatment although it is known that its expression is sexually dimorphic and temporally patterned in rodent liver [35].

In conclusion, concerning the dMF model, the selected doses of drugs – although leading to T serum levels at the range of the corresponding cisgender - have shown to be induce strong general toxicity; for such reason, they cannot be used in long-term studies.

For the dFM model, the subcutaneous injection of 0.45 mg of T represents the correct dose to be administered for masculinizing female rodents also in long-term studies without appreciable adverse effects. In addition, the endpoints selected are considered suitable and reliable to implement the animal model.

CYP450 is a superfamily of membrane-bound enzymes expressed in almost all biological systems that play a crucial role in the homeostasis and metabolism. Sex steroids regulate CYP enzymes in vitro and in vivo models; given that sex steroid concentrations are markedly increased or decreased among TG people undergoing HT, and that differential CYP expression may lead to different, sex-specific susceptibility, the potential role of sex steroids on drug-metabolizing enzyme expression and activity should be carefully studied [34]. In the present paper, for the first time, the sex-specific CYP gene expression has been used as a biomarker supporting the set of proper (de)masculinizing or (de)feminizing HT in order to obtain a reliable animal model for TG people. This novel endpoint may have potential further applications in the frame of risk assessment for TG people.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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