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Central and peripheral effects of Dihydrotestosterone (DHT) in females: a preclinical perspective

Settore Scientifico Disciplinare MED/13

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Premise

Women are dramatically exposed to fluctuations in estrogen and androgen levels (e.g. during the menstrual cycle and at menopausal transition), which play a central role in modulating many aspects of behavior, in particular sexuality. Estrogens have long been known to be essential for women's sexual health, and natural or surgical menopause-related decline in estrogen levels has well-established detrimental consequences in this area. On the other hand, androgens have only recently come back in the spotlight as pivotal regulators of female sexuality. Their direct positive effect on desire and on female genitourinary tissue structure and function, independent of aromatization, has been consistently suggested by preclinical and clinical research.

Since androgens seem to influence sexual function and dysfunction also in women, targeting androgen signaling represents an interesting therapeutic strategy for common sexual disorders, including Hypoactive sexual desire disorder (HSDD) and Genitourinary syndrome of menopause (GSM). However, the underpinning mechanisms of the androgenic action on female sexual behavior and on the integrity and functionality of genital tissues have not been elucidated.

Part 1 of this work deals with the findings of a study aimed at clarifying the mechanisms of testosterone facilitation of female sexual desire by assessing the effect of *in vivo* administration of a non-aromatizable androgen (Dihydrotestosterone, DHT) on sexual behavior in a validated animal model of surgical menopause. In Part 2, the potential beneficial effects of DHT in a model of genitourinary disorder characterized by sex steroids deprivation and chronic inflammation, namely the GSM, was explored through DHT *in vitro* treatment of rat and human vagina smooth muscle cells.

Part 1

The non-aromatizable androgen dihydrotestosterone (DHT) facilitates sexual behavior in ovariectomized female rats primed with estradiol

1. Introduction and aim

Menopause is an ineluctable event in a woman's life, featuring a consistent drop in sex hormonal levels. However, while hormonal levels gradually decline in natural menopause, a sudden and even more pronounced shift of all ovarian hormones (estrogen, progesterone and androgens) is observed in surgical menopause (Davison et al., 2005). These menopause-related hormonal changes result in symptoms that are generally more severe and frequent in women with surgical relative to natural menopause (Bachmann et al., 2001). Among these is a persistent and distressing decline in sexual desire (Hypoactive Sexual Desire Disorder, HSDD), which affects approximately 9% of naturally menopausal women, but up to 16-22% of surgically postmenopausal women (Leiblum et al. 2006; West et al., 2008).

In both naturally or surgically menopausal women, administration of testosterone (T), either alone or in combination with estradiol (E2) as a hormonal replacement therapy (HRT), has been consistently effective in improving many domains of sexual response, especially sexual desire and satisfaction (Davis et al., 2006; Panay et al., 2010; Simon et al., 2005). However, the neuroendocrine mechanisms underlying the influence of T on female sexual behavior are still not completely understood. One complicating factor stems from the ability of T to be converted to both androgenic and estrogenic metabolites. For example, T serves as a precursor hormone that can be converted in both brain and

periphery by the enzyme aromatase into 17β-estradiol, or by the enzyme 5α-reductase into dihydrotestosterone (DHT). DHT is more potent than T itself, due to its higher affinity for the androgen receptor (AR) and its inability to be aromatized to E2 (Askew et al., 2007). Moreover, either AR and estrogen receptors (ERα and ERβ) are expressed abundantly in relevant brain regions associated with both appetitive and consummatory sexual behavior in females (Pfaff, 1980), making it unclear whether T positively modulates sexual desire and satisfaction through a stimulation of the AR, through its conversion to estrogen and the stimulation of ERs, or some combination of the two (Handa and McGivern, 2002).

Female rat models offer an effective tool for untangling this issue, not only because rat appetitive sexual behaviors, like hops and darts or solicitations, are homologous to sexual interest/initiation in women (Gelez et al., 2013; Pfaus et al., 2003; Pfaus et al., 2015), but also because of the ease in manipulating their hormones (Giuliano et al., 2010). Bilateral ovariectomy (OVX) in female rats does indeed approximate sex steroid alterations in natural and surgical menopause (Pfaus et al., 2015). Although acute administration of E2 or estradiol benzoate (EB) alone to OVX rats facilitates the display of lordosis when females are mounted by males, the combination of EB and progesterone classically restores not only full lordosis behavior, but also appetitive sexual behaviors in OVX rats (Boling and Blandau, 1939; Pfaff, 1980; Pfaus et al., 1999; Pfaus et al., 2015). Indeed, it has recently been shown that T propionate also facilitates lordosis and appetitive sexual behaviors in OVX rats treated with EB (Jones et al., 2017), suggesting that OVX female rats may constitute a predictive model of women with surgical menopause.

The aim of the present study was to examine whether the administration of the non-aromatizable androgen receptor agonist DHT would facilitate appetitive and consummatory aspects of sexual behavior in OVX rats treated with EB, to levels equivalent to rats primed with EB and progesterone (P), used as positive controls. DHT alone was also compared with a neutral vehicle in non EB-primed rats. 3-month-old OVX

rats were used as a model of surgical menopause, which often takes place in women younger than those naturally experiencing menopause. Given that there are multiple aspects of sexual behavior that cannot be captured by the direct measure of a discrete action itself, this study also examined sexual behavior dynamics. The effects of different hormonal treatments on the various stages of sexual behavior as it unfolds over time were therefore assessed using sequential analyses related to the elaboration of transition matrices and T-pattern analysis (Vanderschuren et al., 1995; Magnusson et al., 2016).

2. Material and Methods

2.1 Animals

The Long-Evans strain was selected given its proposed superiority in the study of androgen-induced facilitation of desire, due to a high level of baseline sexual inhibition (Jones et al., 2017). Animals were purchased from Charles River Canada (St-Constant, QC) and housed in colony rooms maintained at 21°C on a 12-h reverse day-night cycle (lights off at 8AM). Upon arrival, females (weight 150-250 g) were housed in pairs in Plexiglas® shoebox cages lined with Beta Chip®, whereas males (200-250 g) were group-housed (4/cage) in large Plexiglas® cages. Standard laboratory chow and tap water were freely available. Environmental enrichment was provided.

The experiments were conducted in accordance with the ethical standards established by the Canadian Council on Animal Care (CCAC) and approved by Concordia University's Animal Research Ethics Committee.

2.2 Ovariectomy

One week after arrival, females were anesthetized with a 2:1 mixture of ketamine hydrochloride (50 mg/ml; Ketaset©; Wyeth Canada, Guelph, Ontario, Canada) and xylazine hydrochloride (4 mg/ml; Rompum©; Bayer Health-Care, Toronto, Ontario,

Canada) injected intraperitoneally (IP; mL/kg body weight), and OVX bilaterally through a single or double lumbar incision, and numbered by tail marking. Post-operative care was given with subcutaneous (SC) injections of Penicillin G (0.1 ml), ketoprophen (0.03ml of a 100 mg/ml solution per rat per day for 5 days), 1 ml of a 0.9% saline solution, and polysporin ointment applied to cover the incision sites.

2.3 Hormone administration

Estradiol benzoate (EB), progesterone (P) and DHT were dissolved in 0.1 mL reagent grade sesame oil vehicle (Sigma-Aldrich) and administered by SC injection. EB was administered 48 h, while P and DHT administered 4 h, prior to sexual behavior training and test sessions. An equal volume of sesame oil was administered in the control conditions. For tests involving only DHT treatment, the sesame oil vehicle was administered 48 h before testing. All hormone regimens were administered and scored by two trained observers blind to the hormone treatment groups.

2.4 Sexual training

Starting at least one week after surgery, females were made fully sexually receptive by administration of 10 μg EB 48 h, and 500 μg P 4 h prior to each of the 5 sexual training sessions in unilevel 4-hole pacing chambers. Training sessions occurred at 4-day intervals to approximate the normal estrous cycle duration, which has been shown previously to induce stable baseline rates of sexual behavior and to reduce variability in sexual responding (Pfaus et al., 1999). Females were then given a 2-week hormone washout period before testing, to reduce residual effects of prior hormonal treatments (Jones et al., 2017; Kow and Pfaff, 1975).

Behavioral training and testing were performed in unilevel pacing chambers (38 × 60 × 38 cm) bisected by a clear Plexiglas divider with 4 square holes (4 x 4 cm) in the

bottom that rest on bedding. The holes were adjusted to the size of the female in order to allow her to cross to the male's compartment but to prevent the male from doing the same. This allowed the female to initiate and pace the rate of copulatory contact with the male. Prior to sexual contact, randomly selected sexually vigorous males were placed into one compartment for a 5-minute acclimation period and only ever used once during a session. Females were then placed into the opposite side for a 30-minute copulatory session.

All training and testing occurred during the middle-third of the dark circadian cycle. The 30-minute test sessions were recorded digitally on a GoPro HERO® camera for subsequent scoring with the Behavioral Observation Program customized for rat sexual behavior (Cabilio, 1996).

2.5 Testing procedures

Following sexual training and washout, OVX Long-Evans rats (n = 60) were assigned randomly to one of following experimental groups: oil (O) + O (n = 12), 10 μg estradiol benzoate (EB) + O (n = 12), 10 μg EB + 500 μg progesterone (P) (n = 12); O + 500 μg DHT (n = 12); or 10 μg EB + 500 μg DHT (n = 12). According to previously established protocols (Jones et al., 2017), O + O and EB + O groups were used as negative controls, whereas the group of OVX rats receiving EB + P was used as a positive control. The doses of EB and P were chosen according to previously established protocols (Jones et al., 2013). The dose of DHT was chosen according to previous studies (Beyer et al., 1972). Subsequently, all rats from the different experimental groups were tested at 8-day intervals for 4 tests, to minimize sensitization to estradiol (Jones et al., 2013).

2.6 Behavioral outcome measures

The frequency of partial (hops/darts) and full solicitations were considered as the main outcome measure and analyzed after being combined into a general measure of

appetitive sexual behaviors (as in Jones et al., 2015). In unilevel chambers, hops and darts are defined as quick short runs in front of the male (Afonso and Pfaus, 2006), whereas full solicitations are defined as a headwise orientation of the female toward the male followed by an abrupt runaway, regardless of whether the female stays on the male's side or exits through the divider (Pfaus et al., 1999). Female-male mounting, previously reported as a "super-solicitational" estrogen-dependent behavior displayed when the males do not mount following a normal bout of solicitation, was also coded (Afonso and Pfaus, 2006).

As a secondary outcome measure, indices of sexual receptivity were examined, specifically lordosis reflex magnitudes and the lordosis quotient. Lordosis reflex magnitudes were scored on a 3-point scale (Hardy and Debold, 1971) and presented as a mean lordosis rating (LR = sum of points/[mounts + intromissions + ejaculations]). Lordosis quotients (LQ) were calculated as total number of lordosis reflexes/[mounts + intromissions + ejaculations]. If a female did not display lordosis when mounted, she received a score of 0, which was not entered into the LR (as it would thus be redundant with the LQ), but was included in the analyses of sexual behavior dynamics (see below). In the event of a male not mounting a female, a division by zero occurred, and the subject was removed from the analysis on lordosis, but was included in all other analyses. Mounts (front paws on female's flanks), intromissions (pelvic thrust with a dismount) and ejaculations received from the male were also coded, as they are known to provide further insight into the females' sexual receptivity (Pfaus et al., 1999). Finally, the frequency of visits to the male's side, time spent in the male's side and rejection responses [(kicks, side-ways takedowns, boxing postures, prone positions, as in Barnett (1967)] were measured.

Outcome measures were analyzed for distribution and found not-normally distributed; therefore, the appropriate non-parametric tests were used for statistical analyses.

2.7 Analysis of sexual-behavior dynamics: Transition Matrices and T-pattern analyses

Two different approaches to test the effect of hormonal treatments on sexual-behavior dynamics were carried out. Elaboration and analysis of Transition Matrices (TM) provided a full picture describing the probability of transition among the behaviors included in the study (Casarrubea et al., 2009; Spruijt and Gispen, 1984). On the other hand, T-pattern analysis was employed to detect and describe different real-time sequences characterizing the sexual behavior of the study groups (Magnusson et al., 2016).

2.7.1 Transition Matrices

TMs are numerical tables expressing the number of sequential transitions that occurred among behaviors during the observation. TMs were collected per rat and summed in a total TM per group. Results from TMs were provided transforming original TMs in Probability Matrices (PMs) and Adjusted Residuals Matrices (ARMs).

PMs were obtained by transforming transitions into probabilities of transition. To this aim any transition from a given behavior "A" to another behavior "B" was expressed as a ratio of the total number of transitions included in the original TMs. Probabilities of transition were displayed per group by the mean of Path Diagrams. When the number of transitions was > the total sample size, a non-parametric Kruskal-Wallis test followed by Dunn's post-hoc was employed to test differences among groups. Bonferroni correction was applied to reduce the risk of type-I error.

To refine the results of TMs from a statistical point of view, ARMs were obtained for the EB + P and EB + DHT groups. ARMs compared for each behavioral transition the observed values versus the expected values of a given equal distribution of transitions per each row of the matrix (Vanderschuren et al., 1996; van den Berg et al., 1999; van Lier et al., 2003). Positive or negative residual values indicated transitions occurring respectively more often or less often than expected. ARMs can be expressed according to a Z-distribution and therefore p values can be found in a Z-table. Considering an $\alpha = 0.05$, a

residual value ≥ + 1.96 indicates transitions occurring significantly more often and residual values, whereas a residual value ≤ - 1.96 indicates transitions occurring significantly less often than expected. A square-root normalization of residual values was performed to facilitate graphical representation so that the new statistical cut-off was ± "square-root (1.96)" = ± 1.40. Adjusted Residuals of EB + P group and EB + DHT group are illustrated by means of histograms (Casarrubea et al., 2012).

2.7.2 T-pattern analysis

TPA is largely employed to study animal and human behavior (Magnusson et al., 2016; Casarrubea et al., 2018). This method was conceived to detect recurring sequences of events characterized by statistically significant constraints among the interval length separating them (Magnusson, 2000). To this purpose, we used Theme, a specifically developed software (PatternVision, Ltd, Iceland). The Theme algorithm performs comparisons of the distribution of each possible pair of behaviors along the observation window. When a recurring sequence of two given "A" and "B" behaviors is found, such a sequence, indicated with a textual string as "(A B)", basically represents a first level T-pattern encompassing two-events. Hence, the algorithm considers the "(A B)" pattern as an "A" or "B" term of other possible hierarchically higher T-patterns such as, for instance, "(A B) C)". This bottom-up procedure continues up to any level and finishes when no more statistically significant relationships are found.

Theme software requires specific criteria to be selected according to the experimental set-up to search and detect T-patterns in the dataset. The following parameters were selected: significance level $p < 0.0001$; minimum sample performing a t-pattern in each group = 50 %; lumping factor = 0.80; Free algorithm procedure. For further information and details on T-pattern analysis see Casarrubea et al. (2018) and Magnusson et al. (2016).

3. Results

Preliminary analyses using a Mann-Whitney U test for non-parametric variables were conducted to test whether there were differences between the two negative controls (OVX rats treated with O + O and OVX rats treated with EB + O) in the main outcome measure (hops/darts and solicitations). Confirming previous findings (Jones et al., 2013), no differences were detected between the two negative control groups ($U= 101.00$, $p= 0.10$, $r= 0.18$). Conversely, OVX rats treated with EB + DHT showed a significant increase in appetite behaviors as compared to EB + O ($U = 132.00$, $p < 0.0001$, $r = 0.85$). In order to simplify the presentation of the results, we decided to exclude the EB + O group from further analyses, and to consider only O + O as the negative control group.

A Kruskal-Wallis test was conducted in order to analyze overall differences among all the measures between the remaining groups, followed by post-hoc analyses using the Mann-Whitney U test (comparing the EB + DHT group to the 3 control groups), applying a Bonferroni correction. The corrected level of significance was set at $0.05/4= 0.01$, whereas statistical trends were considered at $p < 0.05$. Effect sizes were calculated on the Mann-Whitney tests with the formula $r = Z/(\sqrt{(n)})$. Finally, the percent distribution of total T-patterns was compared using the Chi-square test; measures of effect size were calculated as odds ratios.

3.1 Appetitive behaviors

A Kruskal-Wallis H test showed that there was a statistically significant difference in appetitive sexual behaviors (hops/darts and solicitations) among the different subgroups (χ^2 (3) = 38.32, $p<0.0001$). As expected, Long-Evans OVX rats treated with EB + P showed a significant increase in appetite behaviors (hops/darts and solicitations) as compared to both oil groups ($U = 144.00$, $p < 0.0001$, $r = 0.85$; $U = 144.00$, $p < 0.0001$, $r = 0.85$ vs. O + O and O + DHT, respectively). Interestingly, a higher number of appetitive

behaviors was observed in the O + DHT group compared to the O + O group ($U = 137.5$, $p < 0.0001$, $r = 0.78$; Fig. 1A). In addition, OVX rats treated with EB followed by DHT displayed significantly more appetitive behaviors compared to both oil groups (O + O: $U = 120.00$, $p < 0.0001$, $r = 0.85$; O + DHT: $U = 120.00$, $p < 0.0001$, $r = 0.84$), reaching a level that was similar to EB+ P rats. In fact, no difference was observed between OVX females treated with EB + DHT and OVX rats treated with EB + P ($U = 37.00$, $p = 0.14$, $r = 0.32$) (Fig. 1A).

3.2 Female-male mounting

The number of female-male mounts was not affected by different hormonal treatments (χ^2 (3) = 6.50, p=0.09; not shown).

3.3 Lordosis

As shown in Fig. 1B and 1C, statistically significant differences were detected in LR (χ^2 (3) = 36.38, p<0.0001) or LQ (χ^2 (3) = 37.65, p<0.0001) values among groups. OVX treated with EB + DHT displayed a significantly higher LR when compared to OVX treated with O + O ($U = 144.00$, $p < 0.0001$, $r = 0.88$) and O + DHT ($U = 144.00$, $p < 0.0001$, $r = 0.85$), whereas they did not differ from OVX rats treated with EB + P ($U = 57.00$, $p = 0.41$, $r = -0.18$; Fig. 1B).

Rats treated with EB + DHT also displayed a significant increase in LQ when compared to O + O (U 144.00, p<0.0001, r = 0.88) and O + DHT group (U = 142.00, p<0.0001, r = 0.83) (Fig. 1C). No difference was found in LQ when comparing the EB + DHT and the EB + P groups ($U = 47.00$, $p = 0.16$, $r = -0.30$) (Fig. 1C).

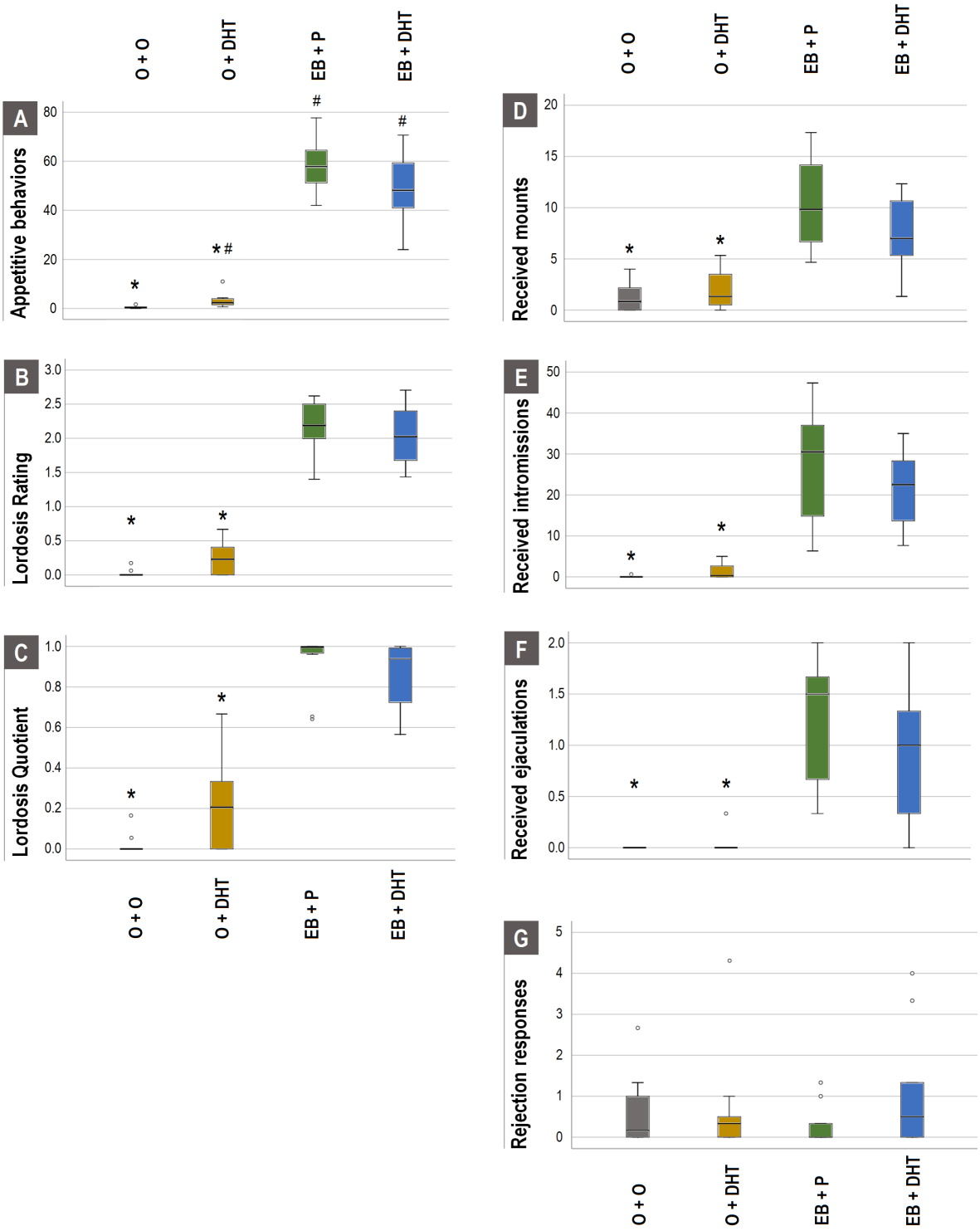


Figure 1. Median frequency of appetitive behaviors (hops/darts and solicitations; panel A), lordosis measures (panels B and C), received mounts (panel D), intromissions (panel E), or ejaculations (panel F) and defensive behaviors (panel G) of ovariectomized Long-Evans rats following various hormone treatment combinations. Data were analyzed using Kruskall-Wallis to detect differences between groups, and significant effects were followed up using Mann-Whitney U, with a corrected p-value set at $p < 0.01$. Boxes represent interquartile range, and whiskers each represent the top and bottom 25% of scores. O: sesame oil, EB: estradiol benzoate, 10 mcg; P: progesterone, 500 mcg; DHT: dihydrotestosterone, 500 mcg). ◦ = Outlier. *= Different from EB + DHT. # = Different from O + O. N = 12/group.

3.4 Male behaviors

The number of male stimulations received (mounts, intromissions, and ejaculations) was also significantly different among groups (mounts, χ^2 (3) = 31.10, $p < 0.0001$, Fig.1D; intromissions, χ^2 (3) = 37.08, $p < 0.0001$, Fig.1E; ejaculations, χ^2 (3) = 35.87, $p < 0.0001$, Fig.1F). Specifically, females treated with EB+DHT received from the male a higher number of mounts, intromissions, and ejaculations than those treated with O + O (U = 137.50, $p < 0.0001$, r = 0.78; U = 144.00, $p < 0.0001$, r = 0.89; U = 132.00, $p < 0.0001$, r = 0.79 for mounts, intromissions, and ejaculations, respectively) or with O + DHT (U = 134.50, $p < 0.0001$, r = 0.74; U = 144.00, $p < 0.0001$, r = 0.85; U = 129.00, $p < 0.0001$, r = 0.78 for mounts, intromissions, and ejaculation, respectively; Fig. 1D-F). The effects of EB+DHT on male behavior were not significantly different from those observed in rats treated with E + P (U = 50.00, p = 0.22, r = 0.26; U = 49.50, p=0.19, r = 0.26; U = 44.50, p = 0.11, r = 0.07 for mounts, intromissions, and ejaculation, respectively; Fig. 1D-F).

3.5 Visits to the male's side and time spent in the male's side

The number of visits to the male's side was significantly different among groups (χ^2 (3) = 11.68, p = 0.009). EB + DHT treatment was equivalent to EB + P in inducing visits to the male's side (U = 63.50, p = 0.63, r = -0.10; not shown). A tendency for EB + DHT to increase the number of visits when compared to O + O (U = 113.00, p = 0.02, r = 0.48; not shown) was observed. On the other hand, no difference emerged between EB + DHT and O + DHT (U = 100.00, p = 0.11, r = 0.33; not shown).

No difference in the total time spent in the male's compartment was found among the 4 groups (χ2 (3) = 3.56, p = 0.314).

3.6 Female rejection responses

A Kruskall-Wallis test failed to detect significant differences among groups on number of female rejection responses ($\chi^2 = 2.06$, $p = 0.56$; Fig. 1G).

3.7 Dynamics of Sexual Behavior

The impact of different hormonal treatments on sexual behavior dynamics was assessed by using analyses based on transition matrices (Fig. 2 and Fig. 3) and an approach based on the evaluation of the temporal structure of behavior, known as T-pattern analysis (Fig. 4 and Fig. 5).

3.7.1 Analysis based on transition matrices

Concerning the analyses based on the elaboration of transition matrices, first we evaluated the probability of transition among sexual behaviors by analyzing the Transition Matrices (TM) in the experimental groups. As shown in the Table 1, the Kruskal-Wallis test detected differences among groups in the vast majority (13 of 15) of TMs. Post-hoc analysis did not find any significant difference among transitions observed in the two oil groups (O + O vs. O + DHT; Table 1, column A). In contrast, significant variations were observed in transitions of EB + DHT rats when compared to O + O or to O + DHT groups. In particular, we found a significantly higher probability of transition from female appetitive behaviors (hops and darts, H/D) to the receipt of male mounts/intromissions/ejaculations (M/I/E) in OVX treated with EB+DHT when compared either with O + O or O + DHT (Table 1, columns B and D respectively). An increased probability of transition from female lordosis (receptive behavior), mainly lordosis of magnitude 1 or 2 (LM1, LM2), to H/D and to M/I/E was also observed in OVX treated with EB+DHT when compared to both oil-groups (O + O and O + DHT, Table 1, columns B and D, respectively). Opposite transitions from the receipt of male M/I/E to lordosis of different magnitudes (LM1, LM2, LM3) were also more frequent in the EB + DHT group as compared to the two oil-groups (O + O and O + DHT, Table 1, columns B and D,

respectively). Significant differences were observed also in EB + P as compared with the two oil groups (O + O and O + DHT, Table 1, columns C and E, respectively). Interestingly, OVX rats treated with EB + P also showed a higher, even though not statistically significant, frequency of all these patterns of behavioral transitions when compared to OVX rats treated with EB + DHT (Table 1, column F). The four path diagrams illustrated in Fig. 2 graphically summarizes these results, representing the probability of transitions among behaviors in the experimental groups. It is evident that the highest probabilities of transition do occur among behavioral components belonging to EB + P and EB + DHT groups.

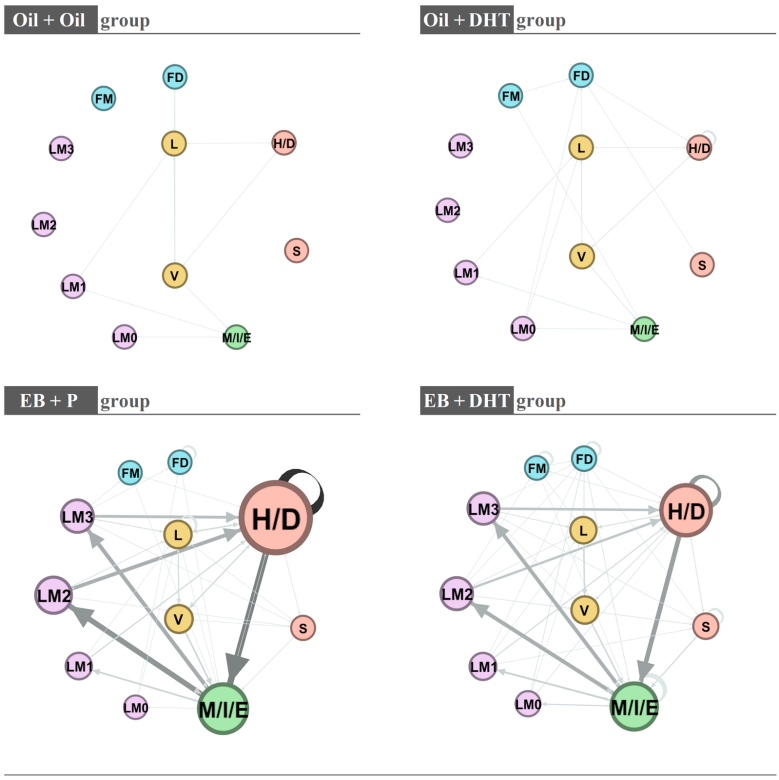


Figure 2. Path diagrams representing transition probabilities among the behavioral components for each group of the study. The arrows of different thickness illustrate the likelihood of transition. Circles encompass the components of the behavioral repertoire: "V" = Visiting and "L" = Leaving the male's compartment of the chamber; "H/D" = Hops/Darts and "S" = Solicitations are appetitive components of female sexual behavior; "M/I/E" = Mounts/Intromissions/Ejaculations stand for male copulatory behaviors; "LM 0 – 3" = Lordosis Magnitude, from 0 to 3; "FM" = Female mounting Male; "FD" = Female Defences. Circle size is related to the overall occurrences of each behavioral node. Top - Left: Oil + Oil group, n = 11. Top - Right: Oil + DHT group, n = 12. Bottom - Left: EB + P group, n = 12. Bottom - Right: EB + DHT group, n = 12.

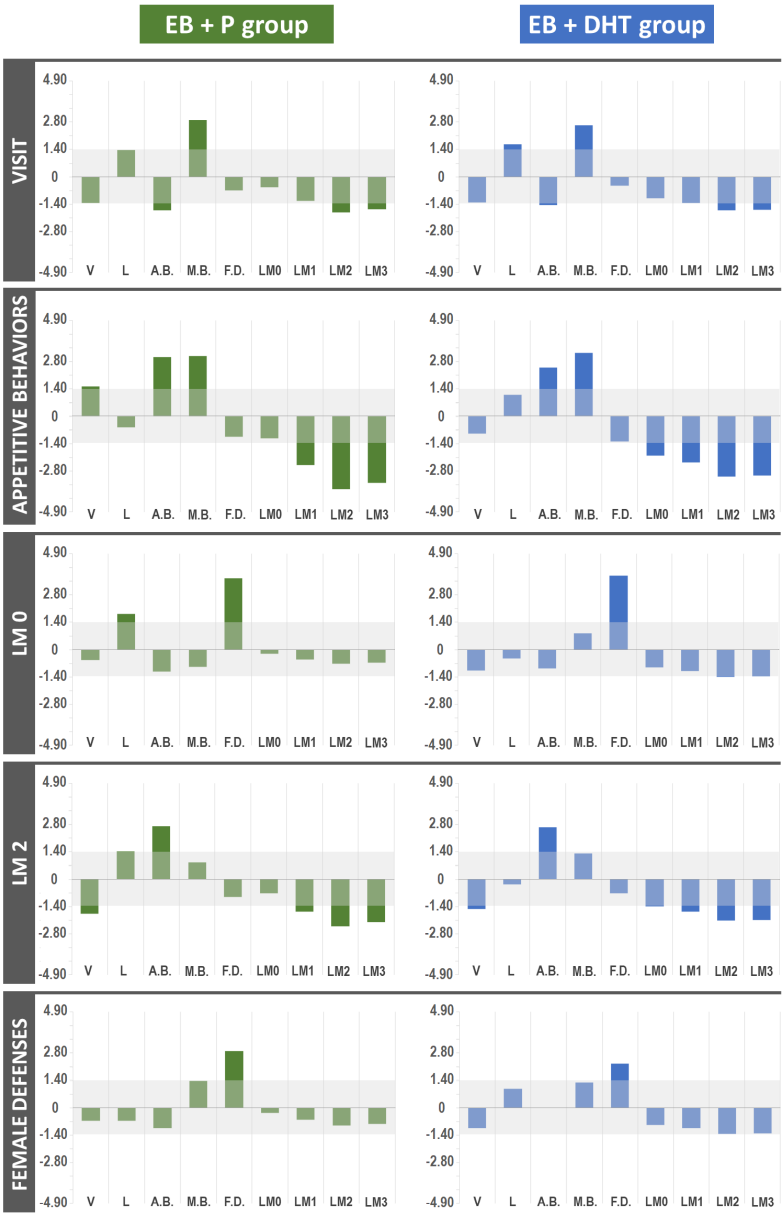
Behavioral transition			Kruskal-Wallis Test (α = 0.003, Bonferroni's correction)		Dunn's post-hoc test (α = 0.008, Bonferroni's correction)					
					column A	B	C	D	E	F
			χ^2 (3)	*p*	Oil+Oil vs Oil+DHT	Oil+Oil vs EB+DHT	Oil+Oil vs EB+P	Oil+DHT vs EB+DHT	Oil+DHT vs EB+P	EB+DHT vs EB+P
H/D	*to*	**H/D**	38.86	**< 0.003**	*p* = 1.000	***p* < 0.008**	***p* < 0.008**	*p* = 0.010	***p* < 0.008**	*p* = 0.761
H/D	*to*	**V**	20.29	**< 0.003**	*p* = 1.000	*p* = 0.040	***p* < 0.008**	*p* = 0.927	*p* = 0.016	*p* = 0.675
H/D	*to*	**L**	13.03	= 0.005	n/a	n/a	n/a	n/a	n/a	n/a
H/D	*to*	**M/I/E**	34.40	**< 0.003**	*p* = 1.000	***p* < 0.008**	***p* < 0.008**	***p* < 0.008**	***p* < 0.008**	*p* = 1.000
LM1	*to*	**H/D**	26.21	**< 0.003**	*p* = 1.000	***p* < 0.008**	*p* = 0.012	***p* < 0.008**	*p* = 0.009	*p* = 1.000
LM2	*to*	**H/D**	34.53	**< 0.003**	*p* = 1.000	***p* < 0.008**	***p* < 0.008**	***p* < 0.008**	***p* < 0.008**	*p* = 1.000
LM2	*to*	**M/I/E**	36.66	**< 0.003**	*p* = 1.000	***p* < 0.008**	***p* < 0.008**	***p* < 0.008**	***p* < 0.008**	*p* = 1.000
LM3	*to*	**H/D**	27.60	**< 0.003**	*p* = 1.000	*p* = 0.003	***p* < 0.008**	*p* = 0.002	***p* < 0.008**	*p* = 1.000
LM3	*to*	**M/I/E**	25.50	**< 0.003**	*p* = 1.000	*p* = 0.004	***p* < 0.008**	*p* = 0.003	***p* < 0.008**	*p* = 1.000
V	*to*	**M/I/E**	16.82	**< 0.003**	*p* = 1.000	*p* = 0.014	*p* = 0.003	*p* = 0.172	*p* = 0.054	*p* = 1.000
L	*to*	**H/D**	16.06	**< 0.003**	*p* = 1.000	*p* = 0.339	***p* < 0.008**	*p* = 1.000	*p* = 0.017	*p* = 0.345
L	*to*	**V**	8.29	= 0.040	n/a	n/a	n/a	n/a	n/a	n/a
M/I/E	*to*	**LM1**	29.10	**< 0.003**	*p* = 1.000	***p* < 0.008**	*p* = 0.015	***p* < 0.008**	*p* = 0.029	*p* = 0.813
M/I/E	*to*	**LM2**	36.62	**< 0.003**	*p* = 1.000	*p* = 0.002	***p* < 0.008**	***p* < 0.008**	***p* < 0.008**	*p* = 1.000
M/I/E	*to*	**LM3**	29.82	**< 0.003**	*p* = 1.000	***p* < 0.008**	***p* < 0.008**	***p* < 0.008**	***p* < 0.008**	*p* = 1.000

Table 1. Statistical differences among the behavioral transitions illustrated in Fig. 2. Non-parametric Kruskal-Wallis test was followed by Dunn's post-hoc to test differences among groups. Bonferroni correction was applied to reduce the risk of type-I error.

In order to refine comparison between EB + DHT and EB + P rats, we also performed an analysis based on Adjusted Residual Matrices (ARMs; Vanderschuren et al., 1996), which allows for a qualitative assessment of sexual behavior. Histograms in Fig. 3 are a graphical representation of ARMs: positive bars represent transitions occurring significantly more often than expected; negative bars represent transitions occurring significantly less often than expected. The vast majority of behavioral transitions (75 over 81) occurred with similar residuals in EB + DHT and EB + P subgroups. In fact, in both groups, appetitive behaviors appear to be followed more often than expected by appetitive behaviors themselves or by male behaviors, which in turn are followed by lordosis. Lordoses showed different residuals depending on their magnitude. In both groups, LM0 showed more frequent than expected transitions towards female defenses, whereas LM1-3

showed towards appetitive behaviors. In addition, only LM3 displayed positive residuals in the direction of Leaving (L; exit from the male's side of the cage).

Only slight differences were found between the residuals of the EB + DHT as compared to EB + P rats. Only EB + DHT rats exhibited a lower-than-expected transition from the receipt of male M/I/E to defensive behaviors (FD), while EB + P did not (Fig. 3). EB + P rats specifically showed a higher-than-expected transition from lordosis posture of the lowest magnitude (LM0) to leaving the male's side of the cage (L) and a higher frequency of transition from lordosis of magnitude (LM3) to female defensive (FD) behaviors (Fig. 3).



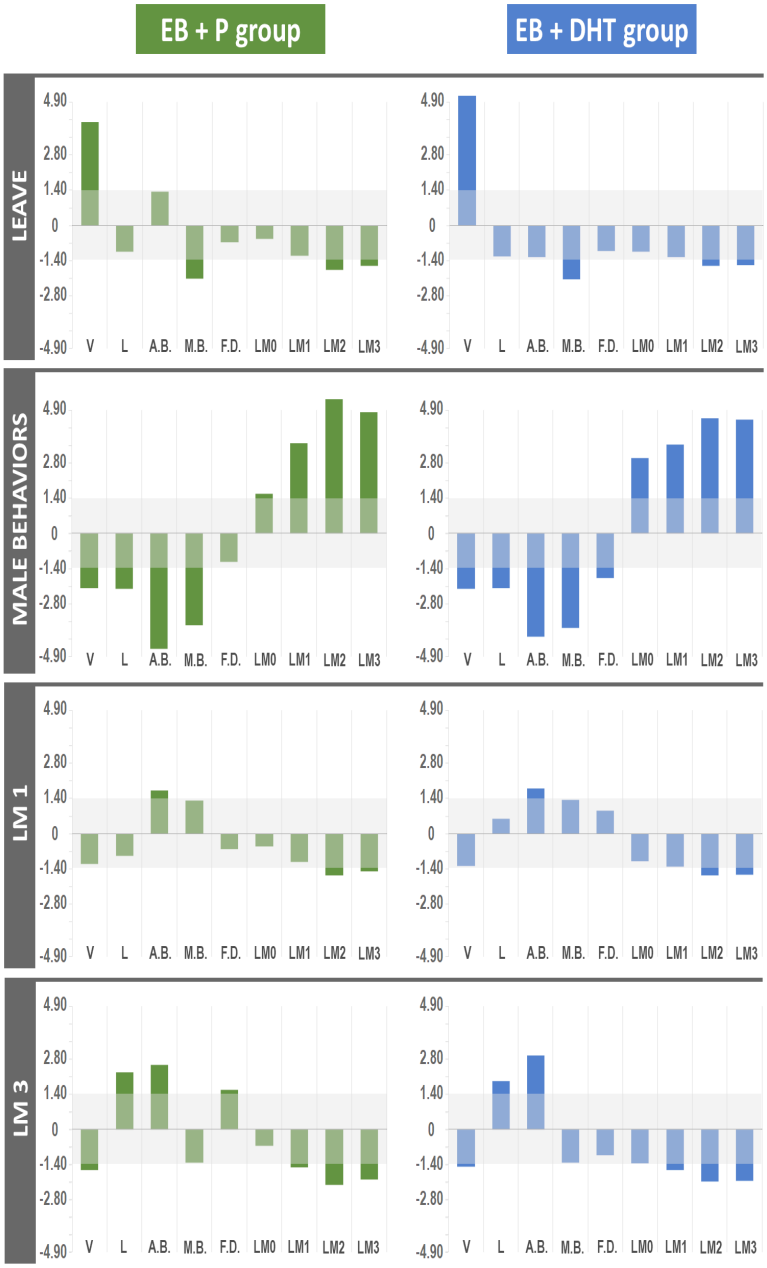


Figure 3. Adjusted residuals representing the association strength of transitions among the behavioral components for the EB + P group (n = 12) and the EB + DHT group (n = 12). The behaviors on the left of each box (white letters in dark background) represent behavioral components that occurred in sequence before the components indicated at bottom of each X-axis. Y-axes: values of adjusted residuals. Positive bars: transitions occurring more often than expected. Negative bars: transitions occurring less often than expected. According to the Z-table the bars outreaching grey areas indicate a statistical significance ($p < 0.05$).). "V" = Visiting and "L" = Leaving the male's compartment of the chamber; "A.B." = Appetitive Behaviors such Hops/Darts and Solicitations; "M.B." = Male copulatory Behaviors (Mounts/Intromissions/Ejaculations); "FD" = Female Defences; "LM 0 – 3" = Lordosis Magnitude, from 0 to 3.

3.7.2 T-Pattern analysis

Multivariate T-Pattern analysis has been employed to detect the temporal structure of behavior. Fig. 4 illustrates, for each group, the behavioral sequences as terminal strings (that is, the textual expression of the events in sequences). For each sequence, length (Fig. 4, "Length" column) and number of occurrences (Fig. 4, "Occs" column) are indicated as well. Appetitive components of female sexual behavior ("H/D", hops/darts and "Solicit.", solicitations) are highlighted in red. In the two oil groups (O + O; O + DHT) the only behavioral sequences detected were characterized by the visit-leave pattern (Fig. 4; "Visit Leave" string). On the other hand, both in EB + DHT and EB + P, several hierarchically higher-order patterns have been detected, thus suggesting a more complex temporal organization of sexual behavior. EB + DHT rats performed 33 different T-patterns distributed and composed as follows: 17 encompassing two events in their structure, 9 with three events, 3 with four events, and 4 with five events. In contrast, EB + P showed 43 different patterns with even a higher number of events in their structure as compared to EB + DHT (15 with two events, 6 with three events, 9 with four events, 4 with five events, 4 with six events, 3 with seven events and 2 with eight events; Fig. 4). Fig. 5 illustrates the percent distribution of T-Patterns containing specific behavioral components detected in EB + P and in EB + DHT groups. As assessed by means of Chi-square test, significant ($p < 0.0005$) differences have been detected for T-patterns containing female behaviors only (64.9% vs. 57.9%), male behavior only (15.1% vs. 21.8%), Appetitive behaviors (47.4% vs. 35.8%), Appetitive behaviors + other male behaviors (13.2% vs. 3.7%). Finally, significant differences have been detected in higher order patterns (i.e. T-patterns containing 3 or more events in sequence) containing appetitive behaviors (65.9% vs. 38.8%).

Interestingly, in the EB + P group more than half of the different types of T-patterns included LM3, while this behavior was present only in one type of T-pattern in the

EB + DHT group, namely the one coupled with male ejaculation ("Ejac. LM3") (Fig. 4). In contrast, solicitation was present exclusively in T-patterns of the EB + DHT group: "Solicit. LM2", "Solicit. (H/D H/D)", "(H/D H/D) Solicit", "[(H/D H/D) Solicit.] (H/D H/D)", "(H/D H/D) [Solicit. (H/D H/D)]", "Mount Solicit.", "[Solicit. (Intro. Intro.)]", "[Solicit. (Intro. Intro.) (H/D H/D)]", "[LM2 (Mount Mount) (H/D H/D)]". On the other hand, solicitation was completely absent in T-patterns of the EB + P group. EB + DHT also specifically displayed T-patterns of higher order encompassing appetitive sexual behavior-only events: "[(H/D H/D) Solicit.] (H/D H/D)" and "(H/D H/D) [Solicit. (H/D H/D)]" (Fig. 4).

Oil + Oil group

	#	T-pattern string	Length	Occs.
	1	(Visit Leave)	2	11

Oil + DHT group

	#	T-pattern string	Length	Occs.
	1	(Visit Leave)	2	11

EB + P group

	#	T-pattern string	Length	Occs.
TP containing male OR female behaviors only (female)	1	(H/D H/D)	2	695
	2	(LM2 LM2)	2	182
	3	(LM3 LM3)	2	114
	4	(Visit Leave)	2	64
	5	(Leave Visit)	2	61
	6	(LM3 Leave)	2	37
	7	(LM1 LM1)	2	36
	8	(LM1 LM2)	2	31
	9	(LM2 Leave)	2	22
	10	(LM3 (Leave Visit))	3	33
	11	((Leave Visit) LM3)	3	27
	12	((LM2 LM2) LM3)	3	17
	13	((LM1 LM2)(H/D H/D))	4	24
	14	((Leave Visit)(LM3 Leave))	4	22
TP containing male OR female behaviors only (male)	15	(Intro. Intro.)	2	310
	16	((Mount Mount)(Intro. Intro.))	4	7
TP containing male AND female behaviors	17	(Mount LM3)	2	36
	18	(Mount LM1)	2	32
	19	(Ejac. LM3)	2	11
	20	(Ejac. LM2)	2	7
	21	(LM3 Mount)	2	7
	22	((H/D H/D) Mount)	3	40
	23	(Mount (LM3 LM3))	3	14
	24	((Mount LM3) Ejac.)	3	10
	25	((H/D H/D)(Mount LM3))	4	31
	26	(((H/D H/D) Mount) LM3)	4	31
	27	((Mount LM3)(H/D H/D))	4	21
	28	(Mount ((LM2 LM2) LM3))	4	13
	29	(((H/D H/D) Mount) LM1)	4	13
	30	(LM3 ((H/D H/D) Mount))	4	10
	31	((Intro. Intro.)((LM2 LM2) LM3))	5	13
	32	(((H/D H/D) Mount) LM3) Ejac.)	5	12
	33	(((Mount LM3)(H/D H/D)) LM3)	5	11
	34	((((H/D H/D) Mount) LM1) LM3)	5	10
	35	((H/D H/D)((Mount LM3)(H/D H/D)))	6	18
	36	(((Mount LM3)(H/D H/D))(Intro. Intro.))	6	18
	37	((LM3 ((H/D H/D) Mount))(LM2 LM2))	6	9
	38	((Intro. Intro.)(LM3 ((H/D H/D) Mount)))	6	8
	39	(((((Mount LM3)(H/D H/D))(Intro. Intro.)) LM3)	7	9
	40	(((LM3 ((H/D H/D) Mount))(LM2 LM2)) Ejac.)	7	8
	41	(((H/D H/D)((Mount LM3)(H/D H/D))) LM3)	7	7
	42	((H/D H/D)(((Mount LM3)(H/D H/D))(Intro. Intro.)))	8	15
	43	((Intro. Intro.)((LM3 ((H/D H/D) Mount))(LM2 LM2)))	8	7

EB + DHT group

	#	T-pattern string	Length	Occs.
TP containing male OR female behaviors only (female)	1	(H/D H/D)	2	381
	2	(LM2 LM2)	2	107
	3	(Visit Leave)	2	54
	4	(Leave Visit)	2	46
	5	(Leave Leave)	2	41
	6	(LM1 LM1)	2	33
	7	(LM1 LM2)	2	20
	8	(LM2 LM1)	2	17
	9	(Solicit. LM2)	2	12
	10	(Leave (Visit Leave))	3	42
	11	(Solicit. (H/D H/D))	3	17
	12	((H/D H/D) Solicit.)	3	16
	13	(((H/D H/D) Solicit.)(H/D H/D))	5	12
	14	((H/D H/D)(Solicit. (H/D H/D)))	5	11
TP containing male OR female behaviors only (male)	15	(Intro. Intro.)	2	241
	16	(Mount Mount)	2	63
TP containing male AND female behaviors	17	(Mount LM2)	2	53
	18	(Mount LM1)	2	43
	19	(Mount Solicit.)	2	15
	20	(LM2 Mount)	2	13
	21	(LM1 Mount)	2	11
	22	(Ejac. LM3)	2	10
	23	((Mount LM1) Mount)	3	25
	24	(LM1 (Mount LM1))	3	19
	25	(Mount (LM1 LM2))	3	18
	26	(Solicit. (Intro. Intro.))	3	18
	27	(LM2 (Mount Mount))	3	10
	28	((LM1 Mount) LM1)	3	8
	29	((Intro. Intro.)(LM2 LM1))	4	9
	30	((LM2 Mount)(Intro. Intro.))	4	8
	31	((Intro. Intro.)(LM2 Mount))	4	6
	32	((Solicit. (Intro. Intro.))(H/D H/D))	5	11
	33	((LM2 (Mount Mount))(H/D H/D))	5	7

Figure 4. See next page for caption.

Figure 4. Results from T-pattern analysis for each group of the study. T-pattern string: textual representation of T-patterns of different composition. Numbers on the right of each string indicate their length (i.e. the number of behavioral components included in that sequence) and the overall occurrences (Occs.). Parentheses indicate the hierarchical organization of T-patterns. TP = T-pattern. "H/D" = Hops/Darts and "Solicit." = Solicitations indicate appetitive components of female sexual behavior and are highlighted in red. "Intro." = Intromission; "Ejac." = Ejaculation. "LM 0 – 3" = Lordosis Magnitude, from 0 to 3. Top - Left: Oil + Oil group, n = 11. Top - Right: Oil + DHT group, n = 12. Bottom - Left: EB + P group, n = 12. Bottom - Right: EB + DHT group, n = 12.

Figure 5. Percent distribution of T-patterns detected in EB + P group (n = 12) and EB + DHT group (n = 12). TP: T-pattern. Chi-square test, * = $p < 0.0005$.

4. Discussion

This study demonstrates that the administration of the non-aromatizable androgen DHT enhanced female sexual activity in OVX, EB-primed rats by significantly increasing both appetitive and receptive behaviors when compared to negative controls (O + O and O + DHT), at comparable levels with the positive control (EB + P); furthermore, DHT enhanced appetitive behaviors when administered alone, compared to O + O. The current study was also uniquely designed to examine sexual behavior dynamics by analyzing the various stages of sexual intercourse as they unfolded over time. By using sequential analyses related to the elaboration of transition matrices we found that the administration of 10 μg EB followed by 500 μg DHT 4 hours before testing significantly increased the probability of transition from either female appetitive or receptive behaviors to successful copulation, expressed by the receipt of male mounts/intromissions/ejaculations. These facilitatory effects of EB + DHT were as prominent as those observed after the canonical stimulatory treatment EB + P, used as a positive control. However, when we assessed hidden repetitive behavioral sequences through T-pattern analysis, some significant differences emerged between EB + DHT and EB + P groups: EB + DHT was the only treatment to enhance repetitive behaviors characterized by Solicitation, used by females to entice vigorous copulatory activity from males (Erskine, 1989). Our data finally confirm (Baum et al., 1974; Gladue, 1984) that DHT significantly triggers the emergence of both appetitive and receptive behaviors, suggesting that aromatization is not a mandatory prerequisite for the positive action of androgens on sexual activity.

OVX Long-Evans rats treated with EB + DHT displayed a significant increase in hops/darts and solicitations, which are commonly chosen as the main indicator of sexual motivation and appetitive behavior in this animal setting (Jones et al., 2015, 2017). In particular, these behaviors are aimed at enforcing the rate of sexual contact, thus reflecting the willingness of the female to initiate and engage in sexual interaction. Interestingly, in

our study the positive effect of DHT on these measures was as potent as that observed with P over the EB pretreatment. Jones and colleagues, by using a different testing apparatus (bilevel pacing chamber), recently showed that 200 μg T propionate administered 4 h prior to testing in OVX, EB-primed rats facilitated hops/darts and lordosis ratings (Jones et al., 2017). These findings confirmed earlier studies demonstrating a positive involvement of aromatizable androgens in female rat sexual behavior, and specifically in appetitive behaviors (Gladkova and Karpenko, 1986; Jones et al., 2012). Hereby, we demonstrated for the first time that also a non-aromatizable androgen, DHT, exerts a potent facilitatory effect on the full array of female sexual behavior in the EB primed rat, and is able to trigger appetitive measures when given alone. A previous study on the effects of DHT in rodents was also promising, demonstrating that 500 μg DHT added to EB increased masculine sexual behaviors (female mounting of a receptive female) in OVX Wistar rats (Baum et al., 1974). In our study we did not assess female-female mounting, because it has been considered more an expression of dominance in social hierarchy than a display of sexual motivation (Fang and Clemens, 1999).

Similar to the effects for appetitive behaviors, OVX rats treated with EB + DHT received more mounts, intromissions and ejaculations compared to both oil control groups, and up to similar levels to those undergoing EB + P replacement. As already described in another experimental setting (Jones et al., 2017), this increase in males' responsiveness might be a direct consequence of females displaying more hops/darts and solicitations and thus being more attractive to the males who then are enticed to chase and mount them accordingly. In the present study, we also evaluated female-male mounting, failing to observe a significant stimulation by DHT alone or EB + DHT. However, this parameter has been strongly linked to sexually sluggish or non-copulating males, as females mount a male when he is nonresponsive (Afonso and Pfaus, 2006).

Administration of DHT in rats primed with EB also resulted in both enhanced lordosis magnitudes (expressed by LR) and the lordosis to mount ratio (expressed by LQ) relative to negative controls. Lordosis, a stationary dorsiflexion of the spine that allows the male to mount and intromit, is the hallmark of receptive behavior in female rats (Beach, 1976). The specific involvement of DHT in facilitating not only appetitive behaviors but also lordosis emphasizes its central effects and supports the view that the neural pathways underlying appetitive and receptive phases of rat sexual intercourse are deeply intertwined (Pfaus, 2006). Indeed, earlier works hypothesized that receptive behavior was specifically stimulated by P administration (Pfaff et al., 1994). In particular, as reviewed by Pfaus, treating OVX females with EB alone produces only a moderate activation of lordosis in response to male stimulation, whereas its full expression normally depends on additional activation by P (Pfaus, 2006). Therefore, our finding that LR and LQ were increased by EB + DHT up to levels equivalent to the EB + P regimen is of particular interest. Even though the possibility of a direct action of DHT on its receptor might be an interpretation of the present findings, we cannot exclude a cross reactivity between DHT and the P receptor, due to the high sequence homology between their respective receptor ligand-binding domains (Gao et al., 2005).

Another important novelty of the present study was the employment of Matrix and T-pattern analyses to deeply investigate the effect of DHT on the dynamics of sexual behavior. Indeed, even though such methodologies are considered valuable tools for behavioural testing in rodent models (Casarrubea et al., 2018; Santangelo et al., 2018), they have never been applied in studies focusing on sexuality. Interestingly, Matrix and T-pattern analyses were able to reveal several analogies and some divergences in the sexual behavior dynamics observed after EB + DHT or EB + P administration. By using Matrix analysis we found that both groups performed frequent transitions from hops/darts to copulation (receipt of mounts/intromissions/ejaculations together with lordosis), and vice-

versa. This finding confirms the presence of a recurring behavioral loop in the performance of sexual intercourse in rats (Pfaus et al., 2006). Moreover, the adjusted residuals plot highlighted some aspects related to the influence of lordosis magnitude on the organization of sexual behavior which were identical in the EB + P and EB + DHT groups. In both groups, the absence of lordosis (LM0) was associated with female defenses, indicating a stop signal on sexual intercourse. In contrast, lordosis of every magnitude (LM1 to LM3) was more frequently-than-expected followed by appetitive behaviors, thus reinforcing the sexual activity cycle. Noteworthy, only LM3 displayed positive residuals in the direction of leaving, indicating an alternative behavioral negative feedback. Although both LM0 and LM3 were associated with a negative feedback on sexual behavior, the underlying motivational mechanism might be divergent. In fact, as proposed in Fig. 6, LM0 might be reasonably related to a rejection of sexual activity (low desire), whilst LM3 could mirror the achievement of sexual satisfaction. Expanding this concept, not only the magnitude of lordosis reflex appears as a marker of the female rat disposition within the sexual activity cycle, as previously described in bilevel chambers (Pfaus et al., 1999), but its systematic evaluation from a behavioural dynamic perspective allowed us to suggest a formalization of its significance in the unilevel bisected testing apparatus. Indeed, while LM0 terminates sexual interaction, LM3 alternatively leads to appetitive behaviors (i.e. hops/darts), thus reinforcing the stimulus towards the male and starting a new copulation bout, or is followed by the female leaving the male's side (Fig. 6). This can be interpreted as a display of contentment following male's ejaculation, in response to the male's post-ejaculatory refractory period, preceding a new ejaculatory series. However, if ejaculation did not occur, leaving the male's side also allows the female to put as much distance as possible between herself and the male, to eventually make a full solicitation.

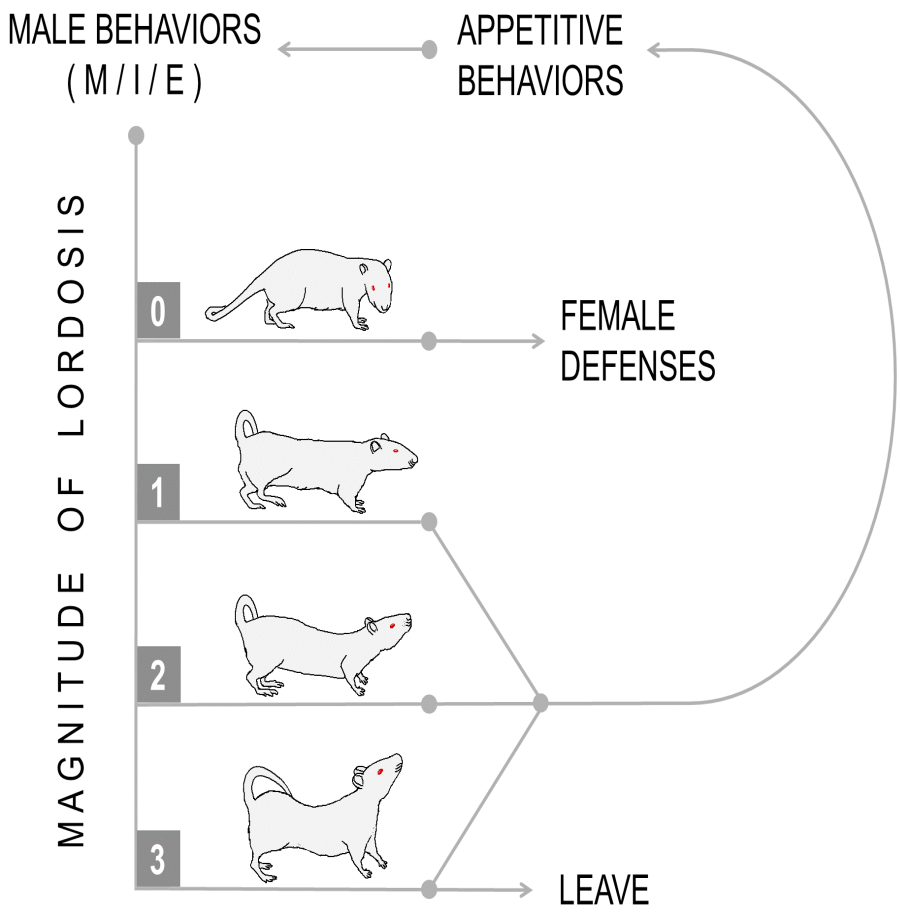


Figure 6. Graphical representation of lordosis magnitude (LM) in the behavioral loop of rat sexual intercourse. Both LM0 and LM3 were associated with a negative feedback on sexual behavior; however, LM0 might be reasonably related to a rejection of sexual activity (low desire), whilst LM3 could mirror the achievement of sexual satisfaction.

Additionally, T-pattern analysis revealed the recurrence of hidden sequences of sexual-related behaviors only in EB + DHT and EB + P groups, and not in the two oil groups (O + O and O + DHT). T-pattern analysis is indeed a technique that allows for the assessment of the structure of behavior with a more complete representation, while encompassing sequences of behaviors that would be invisible to conventional analysis. In particular, T pattern-analysis demonstrated that EB + DHT was associated with hidden sequences containing Solicitation, that appears as a wider, longer and more robust movement than hops/darts and may therefore be considered as the strongest proxy of appetitive behavior. Interestingly, in the EB + DHT group hidden appetitive behaviors were not only present in strings containing male behaviors (mounts, intromissions or

ejaculations), but they also appeared as standing-alone in complex (length ≥3) strings. In contrast, in the EB + P group complex hidden sequences included only appetitive behaviors associated with male mounts, intromissions, or ejaculations. Therefore, T-pattern analyses of hidden behavioral activity suggest that DHT modifies male-female sexual interplay by triggering appetitive behaviors either immediately linked or not to the copulatory act. Ultimately, T-pattern analyses revealed subtle peculiarities of sexual behavior in rats treated with EB + DHT, suggesting that these statistical techniques might be employed to thoroughly explore the effect of hormonal manipulations in this and other animal models.

Although evidence exists suggesting that DHT may exert its effects on sexual response by acting on the brain, the complex underlying mechanisms have not been clarified. ARs have a similar, broad distribution in the brain of rodents, primates and adult humans (Pelletier, 2000) with the highest levels described in the hypothalamic nuclei involved in modulating neuroendocrine and reproductive functions, including the ventromedial hypothalamus (VMH), arcuate nucleus, periventricular nucleus and medial preoptic area (mPOA) (Handa and McGivern, 2002). In these and other regions, such as the mesolimbic reward system, androgens may modulate sexual function mainly acting on dopaminergic neurotransmission (Sanderson et al., 2008; Hermans et al., 2010). It is worth emphasizing that, beyond a central action, several lines of evidence from preclinical and clinical studies demonstrate that a peripheral role of androgens in genitourinary physiology is also crucial. It has been widely reported that androgens, via their actions on AR, are critical for modulating hemodynamics and maintaining structural and functional integrity in female genital tissues (reviewed by Traish et al., 2018). In particular, in vitro animal studies showed that androgens positively regulate the relaxant machinery in smooth muscle cells, both in the clitoris (Comeglio et al., 2016) and in the vagina (Kim et al., 2004). Although these mechanisms are related to the peripheral arousal response, their potential

involvement in the present experiments has to be taken into account. Future studies on similar animal models should address histological and functional changes of genital tissues following the administration of different hormone regimens, including DHT.

A few limitations of the present work have to be recognized. First, rats treated with E + O were not included in the investigation of sexual behavior dynamics, as they were dropped from further analyses once significant differences with the O + O group on the main outcome measure (appetitive behaviors) had been excluded. Second, the lack of a naturally cycling, sexually receptive group may be considered as a limitation. Finally, while hypothesizing a central action of DHT on the brain, we cannot rule out a peripheral stimulating effect, which could have been blunted, for example, by anesthetization of the clitoris and external vagina. In this regard, in the previously mentioned study by Baum et al. (1974), 200 mcg DHT administered daily for 17 days was found to increase both clitoral size and the growth of cornified papillae on the glans clitoris in Wistar OVX rats. In this study, lidocaine paste was applied on the clitoris and external vagina prior to behavioral tests in order to avoid peripheral stimulating effects, and compared with vaseline; interestingly, local anesthetization did not change the findings on the facilitation of mounting behavior in EB + DHT vs. EB only rats (Baum et al. 1974).

A strength of the study is the methodological design. First, by using the uni-level chamber, an apparatus specifically designed to allow the female to pace the copulatory contact, we selectively focused on female sexual behavior. Secondly, the other main novelty of our data lies in the study of behavioral dynamics, and in particular T-patterns, used for the first time to investigate qualitative effects of hormonal stimulation in this animal model. From a clinical perspective, our findings can provide useful input for developing new treatments for women with Hypoactive Sexual Desire Disorder (HSDD) targeting selective androgen signaling, in particular in women undergoing surgical or iatrogenic menopause. Indeed, the complications of surgical or iatrogenic menopause often

affect young women and represent an important risk factor for HSDD, with a strong negative impact on quality of life (West et al., 2008). As reported by the recent Global Consensus Position Statement (Davis et al., 2019), there is an unmet need for the approval of testosterone treatments in postmenopausal women. It has also to be considered that testosterone therapy is aggravated by the risk of estrogen-dependent adverse events, due to aromatization; it follows that menopausal hormone-dependent cancers survivors, representing a population enriched in HSDD, are completely out of the indication for testosterone therapy. In this context, if a favorable action of DHT on sexual behavior is confirmed, it could be considered as the base for safe therapeutic options for HSDD in these populations.

5. Conclusions

In conclusion, in the present work we observed a significant facilitative effect of DHT on appetitive sexual behaviors (hops/darts and solicitations) and indices of sexual receptivity (lordosis rating, lordosis quotient, received mounts, intromissions, and ejaculations) in OVX, EB-primed Long-Evans rats. Most importantly, the array of sexual behaviors displayed by the EB + DHT group reached levels equivalent to that of the positive control (EB + P). Therefore, a direct stimulation by androgens on the female sexual response, independent of aromatization, has to be hypothesized. The study of behavioral dynamics, in particular T-patterns, demonstrated a specific stimulus of DHT both on appetitive behaviors linked to copulation and on repeated partner stimulations, independent of the stimulation–copulation loop. Future studies should address the molecular mechanism underpinning these effects.

Part 2

Anti-inflammatory effects of dihydrotestosterone (DHT) in rat and human vaginal smooth muscle cells

1. Introduction and aim

Genitourinary syndrome of menopause (GSM) is a definition introduced in 2014 by the International Society for the Study of Women's Sexual Health (ISSWSH) and the North American Menopause Society (NAMS) (Portman and Gass, 2014), replacing older, limiting diagnoses such as "atrophic vaginitis". GSM is a complex chronic, progressive condition which encompasses physiological and anatomical alterations that affect the labia majora/minora, vestibule/introitus, clitoris, vagina and the lower urinary tract tissue, as a result of the decrease in sex hormones levels. GSM affects approximately 50% of middle-aged and elderly women and has been reported to exert detrimental effects on body image, interpersonal relations, sexual health and overall quality of life (Sturdee and Panay, 2010; Nappi and Kokot-Kierepa, 2012).

Clinical manifestations of GSM consist of decreased turgor and elasticity of the vagina, shrinking of labia minora, loss of vaginal *rugae*, pallor, erythema, increased vaginal friability with ecchymosis and petechiae (Portman and Gass, 2014; Simon et al., 2018). Urological symptoms ("lower urinary tract symptoms", LUTS) include urgency, frequency, urinary incontinence, dysuria, *nocturia* and recurrent urinary tract infections (UTIs) (Portman and Gass, 2014; Simon et al., 2018). Women affected by GSM often report dryness, decreased lubrication, discomfort or pain with sexual activity, post-coital bleeding, irritation/burning/itching of the vulva and/or vagina and pelvic pain (Portman and Gass, 2014; Simon et al., 2018).

Sex steroids are critical modulators of the development and maintenance of a healthy genital tissue. Despite the longstanding perspective that low estrogens levels after menopause can lead to GSM symptoms and signs, several lines of evidence from clinical and preclinical studies suggest that androgens also are important in human genitourinary physiology (Traish et al., 2018; Simon et al., 2018). Indeed, recent studies have shown that the androgenic receptor is already expressed in structures deriving from the urogenital sinus, including the vagina, starting from the first weeks of gestation (Cunha et al., 2017). Interestingly, it has been hypothesized that also chronic inflammation plays an important role in the onset of the GSM and other genitourinary tract disorders characterized by chronic pain in women, including vulvar pain syndrome, Genitopelvic pain/penetration disorder (GPPPD), Chronic pelvic pain syndrome (CPPS) and Bladder Pain Syndrome (BPS) (Thomson, 2005; Grover et al., 2011).

The innate immunity system in the lower female mucosal tract represents the first barrier to pathogen entry and provides a broadly specific recognition of invading bacteria, fungi, parasites, and viruses. Epithelial vaginal cells, along with professional, resident immune cells (macrophages, neutrophils, dendritic cells), recognize specific patterns in conserved key molecules on the surface of pathogens (microbe-associated molecular patterns, MAMPs), which include lipopolysaccharide (LPS), lipoproteins, and peptidoglycan (Farage et al., 2011). Among pattern recognition receptors (PRRs), the toll-like receptors (TLRs) system plays a pivotal in the recognition of microbial antigens and in the rapid response against them (Fazeli et al., 2005). TLRs (TLR1-TLR9) have been described at different levels in the lower genital tract epithelium (Sonnex, 2010). Activation of TLRs leads to the release of pro-inflammatory cytokines and chemokines, such as IL1β (interleukin 1β), IL-6, CXCL8 [chemokine (C-X-C motif) ligand 8] and CCL21 [Chemokine (C-C motif) ligand 21], normally expressed at low basal levels but nevertheless upregulated by the onset of inflammatory processes (Pivarcsi et al., 2005;

Herbst-Kralovetz and Pyles, 2006). This acute response to infectious agents, to which the lower female genitourinary tract is particularly exposed, normally acts by removing the pathogens and initiating the healing process, leading to the restoration of tissue homeostasis and resolution of the inflammation. However, acute inflammatory processes, including vascular permeability changes, leukocyte recruitment, and inflammatory mediator release, must be spatially- and temporally-controlled. Conversely, uncontrolled acute inflammation may become chronic, contributing to tissue damage and to a variety of inflammation-associated diseases (Chen et al., 2017).

Few effective therapies have shown immunomodulatory and protective effects in experimental models of chronic inflammation, including the selective androgen receptor (AR) agonist dihydrotestosterone (DHT). The anti-inflammatory effect of androgens has been investigated in previous studies by our group, in which we employed an experimental model of stromal cells isolated by BPH (Benign Prostatic Hyperplasia) patients (Vignozzi et al., 2012; Vignozzi et al., 2013). In these studies, the expression and the secretion of the main pro-inflammatory cytokines and the activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) signalling, induced by proinflammatory stimuli including TNFα (Tumor necrosis factor α) and LPS, was significantly counteracted by DHT treatment.

The decline in sex steroids levels probably contributes to the high prevalence of GSM and other inflammatory conditions of the lower genitourinary tract in menopausal women. In placebo-controlled trials, an intravaginal formulation of dehydroepiandrosterone (DHEA; prasterone) has been shown to decrease vaginal pH and restore vaginal epithelial maturation index and epithelial thickness, with an improvement in dyspareunia and in all domains of sexual function (Elraiyah et al., 2014). Therefore, prasterone intravaginal ovules have been approved by the FDA (U.S. Food and Drug Administration) and the EMA (European Medicines Agency) for the management of

moderate to severe dyspareunia in menopausal women. It has to be noted that, when applied intravaginally, DHEA is converted not only into estrogens, but also into androgens such as androstenedione, testosterone and DHT. Nevertheless, the potential role of androgen-based therapies in the modulation of chronic genital inflammation in women has not been explored in literature.

In the present study, we aimed to investigate, in primary cell lines derived from intact rat distal vaginal tissues (rSMCs) and in human distal vagina smooth muscle cells (hSMC) derived from peri- and postmenopausal women, a) the expression of sex steroids receptors, in particular the AR, b) the potential of the cells to be involved in the inflammatory response, acting as non-professional antigen presenting cells (APCs) and expressing a variety of proinflammatory mediators, and c) the eventual anti-inflammatory in vitro effect of the selective AR agonist DHT. Our hypothesis is that, similarly to what previously demonstrated in the prostate in men with BPH, androgens negatively modulate the inflammatory response in the vagina.

2. Material and methods

2.1 Surgical procedures and collection of biological samples

Intact Sprague-Dawley rats (Envigo, San Pietro al Natisone, Udine, Italy), weighting approximately 250 gr, were individually caged under standard conditions in a temperature- and humidity-controlled room on a 12-hour light and dark cycle, with free access to water and food throughout the study. After 8 weeks, the animals were sacrificed by cervical dislocation, and the distal vagina and other tissues were collected for subsequent analysis. Animal handling complied with the Institutional Animal Care and Use Committee (IACUC) of the University of Florence, Italy, in accordance to the Italian Ministerial Law, n. 326/2013-B.

Human vaginal tissue was obtained from biopsies of peri- or postmenopausal patients undergoing vaginal hysterectomy for benign gynecological diseases, specifically uterine prolapse, enrolled at the Obstetrics and Gynecology Unit of AOU Careggi (protocol HUMVAGDHT, authorization 12903/OSS, 13.11.2018). Thanks to the benign nature of the treated disease and the rarity of tumors of the vaginal mucosa, histological analysis is not routinely performed. Distal vaginal specimens were preserved in a 4°C saline solution immediately after being acquired. Then, the fragments were partly processed for smooth muscle cell isolation and partly maintained at -80° C for subsequent analyses in our facility (Andrology and Gynecological Endocrinology Lab, Department of Biomedical, Experimental and Clinical Sciences, University of Florence).

2.2 Rat and human vagina smooth muscle cell cultures

Rat smooth muscle cells (rSMCs) were isolated from distal vagina obtained from intact control female rats. The tissues biopsies were processed by mechanical and enzymatic dissection as previously described (Granchi et al., 2002; Comeglio et al., 2016). Similarly, human smooth muscle cells (hSMCs) were isolated from vagina tissues.

Briefly, single vagina tissue samples were digested with bacterial collagenase type IV (2 mg/mL; Worthington Biochemical Corporation, Lakewood, NJ) overnight at 37°C, the fragments were washed in phosphate buffered saline (PBS) and centrifuged at 1500 rpm for 10 minutes; the pellet was recovered and cultured in growth medium (GM) Dulbecco's Modified Eagle Medium and Ham F-12 medium (DMEM/F12 1:1; Sigma-Aldrich, St Louis, MO) red phenol free supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), antibiotics (penicillin 100 IU/mL, streptomycin 100 mg/mL; Sigma-Aldrich), and amphotericin B (1 mg/mL; Sigma-Aldrich) in a fully humidified atmosphere of 95% air and 5% CO_2. SMCs began to emerge within 48 hours and were used until the seventh/eighth passage.

For mRNA expression analyses of cytokines and chemokines, rSMCs and hSMCs were seeded onto 6-well plates ($1.5x10^5$ cells/well) in GM. After overnight serum starvation, the cells were left untreated (NT) or pre-treated with dihydrotestosterone (DHT, 30 nM; Sigma-Aldrich) with or without the non-steroid anti-androgen bicalutamide (BICA, 1μM; Sigma-Aldrich) for 24 h and then stimulated with LPS (100 ng/ml) or interferon γ (IFNγ 1000 UI/ml; R&D systems, Minneapolis, MN) for 24 hrs. The effect of lipopolysaccharide (LPS) treatment was previously tested on SMCs by a preliminary time course experiments (24 h and 48 h) to identify the highest inflammatory effect, using IL6 and IL8 expression as a readout (data not shown). Concentration of DHT (30 nM) was selected as it has been previously reported as the physiological concentration of DHT within the human prostate (Salerno et al., 1988; Marks et al., 2006) able to completely block TNF (Tumor Necrosis Factor)α-, LPS-, or activated CD4+ T-cell-induced inflammatory response in human Benign Prostatic Hyperplasia (hBPH) myofibroblasts (Vignozzi et al., 2012). The experiments were performed in triplicate, using at least three different cell preparations.

2.3 Immunohistochemistry cell characterization

The cells were characterized as previously described (Comeglio et al., 2016) by immunostaining for specific smooth muscle markers myosin heavy chain 11 mouse monoclonal antibody (MHC11, 1:200 vol/vol; Abcam, Cambridge, UK) and α smooth muscle actin (αSMA, 1:100 vol/vol; Sigma Aldrich). The staining for the epithelial marker cytokeratin C (pre-diluted mouse monoclonal antibody; Ventana Medical System, Oro Valley, AZ) was considered as a negative control.

Immunocytochemistry studies were performed for androgen receptor (AR) detection. Briefly, cells were fixed in 2% paraformaldehyde, followed by permeabilization with 0.1% Triton X-100 and blocking with 1% BSA. In rSMCs immunostaining was

performed with anti-AR (1:50 vol/vol; Santa Cruz Biotechnology; Dallas, TX) mouse monoclonal antibodies followed by Alexa Fluor 488 goat anti-mouse IgG (H + L) (1:200 vol/vol; Invitrogen Molecular Probes, Eugene, OR). In hSMCs, anti-AR rabbit polyclonal antibody (1:50 vol/vol; Santa Cruz Biotechnology) was used and followed by Alexa Fluor 568 goat anti-rabbit IgG (H + L) (1:200 vol/vol; Invitrogen Molecular Probes). Antibody specificity was verified by omitting the primary antibody.

2.4 Real-Time Quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Isolation of total RNA from tissues and cells was performed using TRIzol reagent (Life Technologies, Paisley, UK) and/or RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. cDNA synthesis was carried out using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Quantitative real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR) amplification and detection were carried out using SsoAdvanced Universal Probes Supermix or a SsoAdvanced Universal SYBR Green Supermix, and a CFX96 Real-Time PCR Detection System (all from Bio-Rad Laboratories). PCR probes and primers were purchased from Life Technologies. Specific PCR primers for rat target genes were designed on sequences available at the National Center for Biotechnology Information GenBank (http://www.ncbi.nlm.nih.gov) or Ensemble Genome (http://www.ensembl.org). The expression of 18S ribosomal subunit was used as the reference gene for the relative quantization of the target genes based on the comparative threshold cycle $2^{-\Delta\Delta Ct}$ method.

2.5 Immunofluorescence microscopy

rSMCs and hSMCs were seeded at the density of $1x10^4$ cells on glass coverslips in GM. After overnight serum starvation, cells were left untreated (NT) or incubated with DHT (30 nM) in presence or absence of bicalutamide (1 μM) for 24 h, then stimulated

with LPS (100 ng/ml) for 5 h. Immunostaining was performed as previously described (Penna et al., 2009) using a primary mouse monoclonal antibody against NF-κB ("nuclear factor kappa-light-chain-enhancer of activated B cells") p65 (1:100 vol/vol; Santa Cruz Biotechnology) followed by Alexa Fluor 488 goat anti-mouse conjugated secondary antibody (1:200 vol/vol; Molecular Probes). Slides were then examined with a phase contrast microscope (Nikon Microphot-FX microscope, Nikon, Tokyo, Japan). Experiments were performed three times with different cell preparations.

2.6 Analysis of cytokines and chemokines secretion by hSMCs cells

The hSMCs were seeded onto 6-well plates in 1 ml of GM ($1.5x10^5$ cells/well), after one night of serum starvation they were left untreated (NT) or were pre-treated for 24 h with DHT (30 nM) in presence or absence of bicalutamide (1 μM) and then stimulated with LPS (100 ng/ml) or IFNγ (1000 UI/ml) for 24 h. Each experiment was performed in triplicate using at least three different cell preparations. Cell culture supernatants were collected and analysed according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA).

A bead-based multiplex immunoassay (Bio-Rad Laboratories) and a Bioplex 200 system (Luminex Map Technology, Bio-Rad Laboratoires,) were used to measure simultaneously the concentrations of the following cytokines and chemokines in cell culture supernatants: interleukin1β (IL1β), IL1RA (interleukin-1 receptor antagonist), IL2, IL4, IL5, IL6, IL8, IL9, IL10, IL12, IL13, IL15, IL17A, IFNγ, TNFα, GCSF (Granulocyte-colony stimulating factor), GMCSF (Granulocyte-macrophage colony-stimulating factor), VEGF (Vascular endothelial growth factor), PDGF (Platelet-derived growth factor), FGF (Fibroblast growth factor), IP10 (IFNγ-induced-Protein), MCP1 (Monocyte Chemoattractant Protein-1), RANTES (Regulated on Activation, Normal Y Expressed and Secreted), MIP1A (Macrophage Inflammatory Protein 1A), and MIP1B.

Briefly, 50 µl of supernatant was added to 50 µl of antibody-conjugated beads directed against the analytes listed above in a 96-well filter plate. After a 30-min incubation, the plate was washed and the biotinylated anti-cytokine antibody solution was added to each well. The plate was then washed and streptavidin-conjugated phycoerythrin (PE) was added to each well. After a final wash, the plate was read with the Bioplex 200 system. Standard curves were derived from various concentrations of the different cytokine standards in the assay and followed the same protocol as the supernatant samples. The concentration of each cytokine (pg/ml) in each supernatant was calculated by Bioplex software and has been normalized by the number of cells. The assay sensitivity for all proteins tested was <1 pg/ml.

2.7 Flow cytometry

Vaginal hSMCs were seeded onto 60 mm plates ($2x10^5$ cells) in 3 ml of GM; after serum starvation, cells were pre-treated for 24 h with DHT (30 nM) and stimulated with IFNγ (1000 UI/ml) for 48 h. Untreated cells (NT) were taken as control. hSMCs were detached by trypsin (Sigma Aldrich), and after centrifugation $1x10^5$/ml cells for each experimental point were counted and immunolabelled with HLA-DR (PE) (1:10 vol/vol; BD Biosciences PharMingen, San Jose, CA) for 15 minutes at RT; unlabelled cells were used as negative control. Flow cytometric acquisition was performed by collecting $1x10^4$ events on a FACScalibur (BD Biosciences) or an Epics XL-System II flow cytometer (Beckman Coulter, Brea, CA), and data were analysed as previously described (Urbani et al., 2006).

3. Results

3.1 mRNA expression of sex steroid receptors in rat and human vaginal tissues

In order to investigate how the activation of the androgen receptor (AR) could mediate anti-inflammatory effects we first analyzed, by RT-PCR, the mRNA expression of sex steroid receptors in rat (Fig. 7, panel A) and human (Fig. 8, panel A) distal vagina tissues.

In the distal vaginal tissue of both rat (Fig. 7, panel A) and human (Fig. 8, panel A) distal vagina tissues, the AR displayed a significantly higher mRNA expression compared to estrogen receptor β (ERβ, $p<0.001$) and G-protein coupled estrogen receptor 1 (GPER1, $p<0.001$). On the contrary, in rat vaginal tissue AR expression was significantly lower if compared to estrogenic receptor α (ERα) and progesterone receptor (PR) (both $p<0.05$; Fig. 7, panel A), whereas in human vaginal tissue AR mRNA was less represented than ERα ($p<0.01$), but more represented than PR ($p<0.001$; Fig. 8, panel A). In the rat, ERα showed a significantly higher mRNA expression compared to ERβ and GPER1 (both $p<0.01$), whereas we did not observed any difference compared to PR mRNA expression (Fig. 7, panel A). In both rat and human vaginal tissues, ERβ and GPER1 were the two less expressed receptors, with GPER1 significantly more expressed than ERβ ($p<0.001$; Fig. 7, panel A and Fig. 8, panel A).

The main difference between the human and rat vagina tissue lies in PR mRNA expression, which in the human was significantly lower than AR and ERα (both $p<0.001$).

3.2 Characterization of vaginal rat (SMCs) and human smooth muscle cells (hSMCs) and sex steroid receptors mRNA expression

Rat smooth muscle cells (rSMCs) isolated from rat distal vagina tissue showed a high positive immunostaining for the specific smooth muscle markers, myosin heavy chain 11 (MHC11: 94.5±4.4%; Fig. 9, panel A) and α smooth muscle actin (αSMA: 90.2±4.3%,

Fig. 9, panel B), and negative staining for cytokeratin (Fig. 9, panel C). Similarly, human smooth muscle cells (hSMCs) isolated from human vagina specimens showed a high positive staining for smooth muscle markers MHC11 (91.5±6.2%; Fig. 10, panel A) and αSMA (100%; Fig. 10, panel B), whereas they resulted negative for epithelial marker cytokeratin (Fig. 10, panel C).

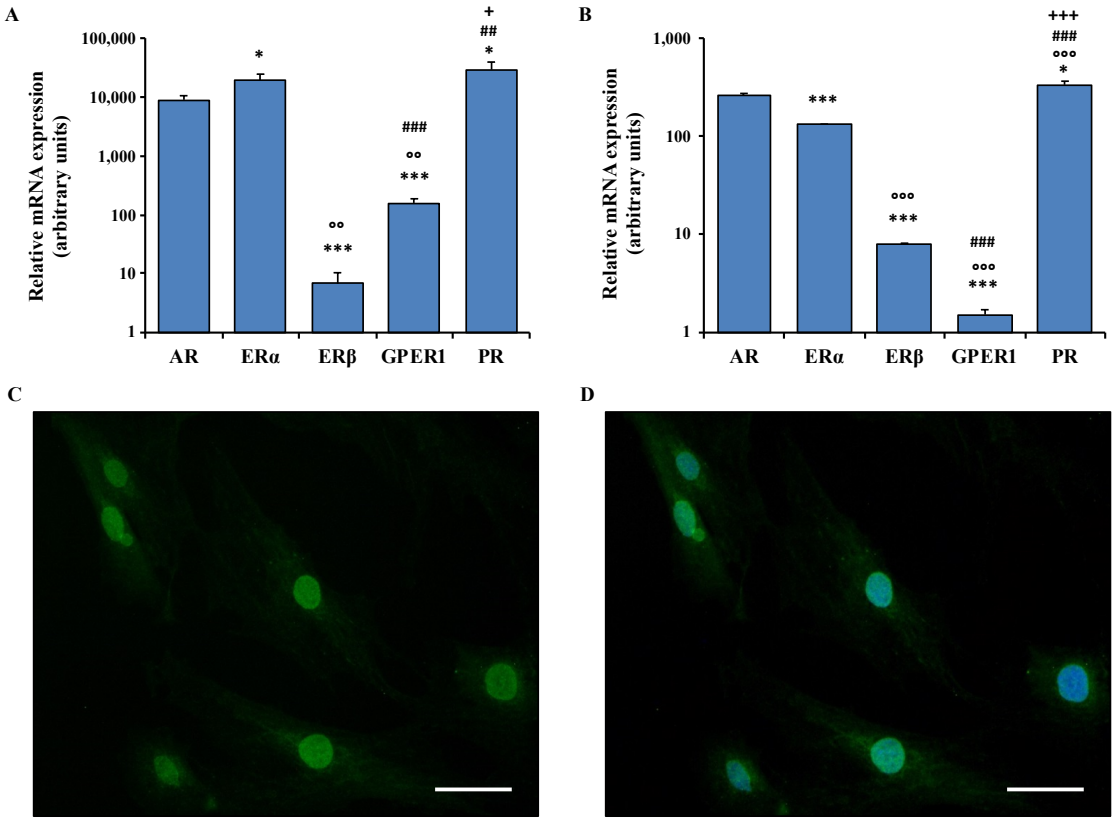


Figure 7. *mRNA expression of sex steroid receptors in rat distal vagina tissues and cells.* Panels A and B show the mRNA expression of sex steroid receptors AR, ERα, ERβ, GPER1 and PR in rat distal vagina samples from intact animals and in smooth muscle cells (rSMCs) isolated from vagina tissues, respectively. Data were calculated according to the 2-ΔΔCt comparative method, using ribosomal subunit 18S as the reference gene for normalization (* $p<0.05$, *** $p<0.001$ vs. AR; °° $p<0.01$, °°° $p<0.001$ vs. ERα; ## $p<0.01$, ### $p<0.001$ vs. ERβ; + $p<0.05$, +++ $p<0.001$ vs. GPER1). Panels C and D show two representative images of nuclear immunostaining for AR receptor and the corresponding nuclear DAPI counterstaining, respectively (scale bar=50 μm).

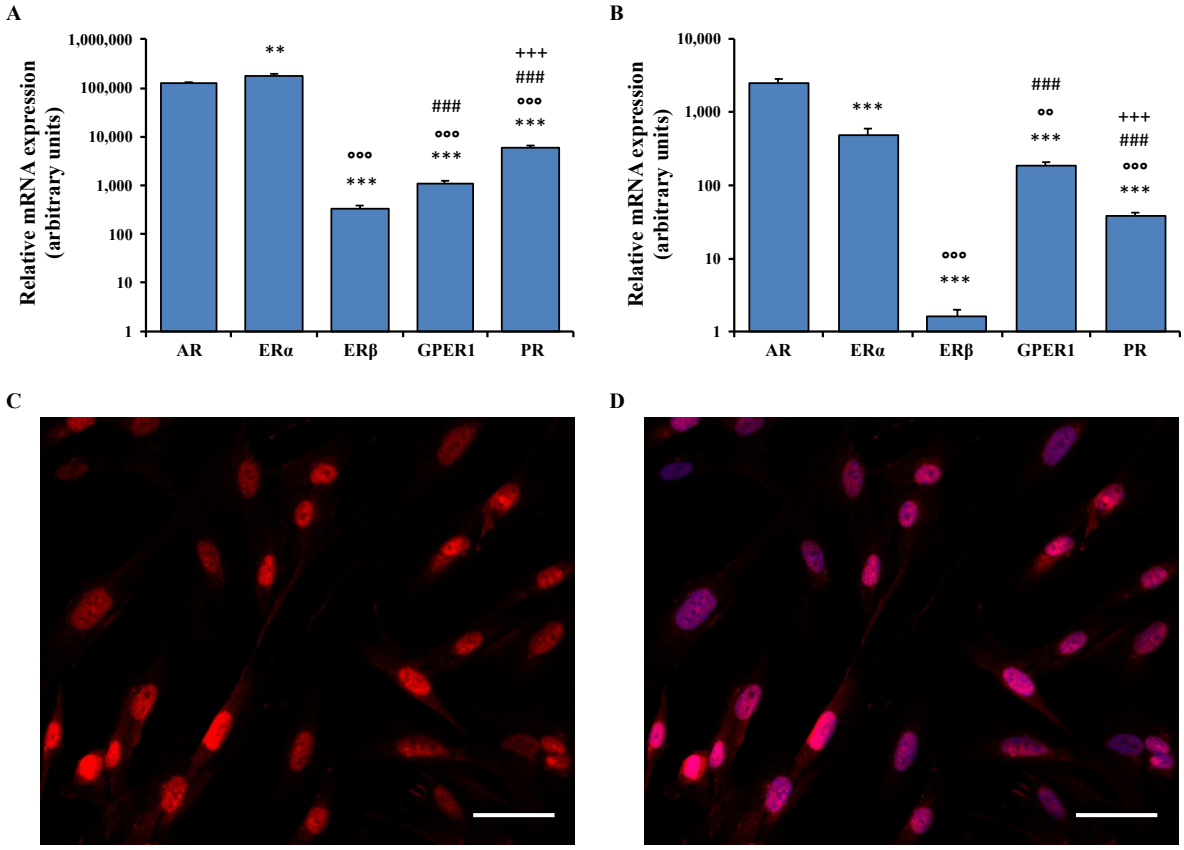


Figure 8. *mRNA expression of sex steroid receptors in human distal vagina tissues and cells.* Panels A and B show the mRNA expression of sex steroid receptors AR, ERα, ERβ, GPER1 and PR in human vagina tissues derived from laparoscopic biopsies and in smooth muscle cells (hSMCs) isolated from the corresponding vaginal samples, respectively. Data were calculated according to the 2-ΔΔCt comparative method, using ribosomal subunit 18S as the reference gene for normalization (** $p<0.01$, *** $p<0.001$ vs. AR; °° $p<0.01$, °°° $p<0.001$ vs. ERα; ### $p<0.001$ vs. ERβ; +++ $p<0.001$ vs. GPER1). Panels C and D show two representative images of nuclear immunostaining for AR receptor and the corresponding nuclear DAPI double labeling, respectively (scale bar=50 μm).

In rSMCs, AR showed a significantly higher expression compared to ERα, ERβ and GPER1 (all $p<0.001$), but significantly lower compared to PR ($p<0.05$; Fig. 7, panel B). The mRNA expression of ERα was significantly higher than ERβ and GPER1 but lower than PR (all $p<0.001$; Fig. 7, panel B). Similarly to what was observed in the rat tissue samples, ERβ and GPER1 mRNA expression resulted lower compared to other receptors, with ERβ mRNA expression significantly higher than GPER1 ($p<0.001$; Fig. 7, panel B). Representative images for nuclear immunostaining of AR (Fig. 7, panel C) and the corresponding nuclear DAPI counterstaining (Fig. 7, panel D) in rSMCs are shown; the

quantification of cellular nuclear positivity was performed by nuclei count and the amount of AR positive cells was observed as 100%.

In hSMCs, AR resulted the most expressed receptor, with mRNA expression levels significantly higher compared to ERα, ERβ, GPER1 and PR (all $p<0.001$; Fig. 8, panel B). ERα mRNA expression was significantly higher compared to ERβ, GPER1 and PR ($p<0.01$ vs. GPER1, $p<0.001$ vs. both ERβ and PR). ERβ showed the lowest level of mRNA expression while GPER1, conversely to what observed in the tissue, resulted significantly more expressed than PR ($p<0.001$). Fig. 8 shows nuclear immunostaining for AR (panel C) and the corresponding nuclear DAPI counterstaining (panel D) in hSMCs. Cellular nuclear positivity for AR was 100%.

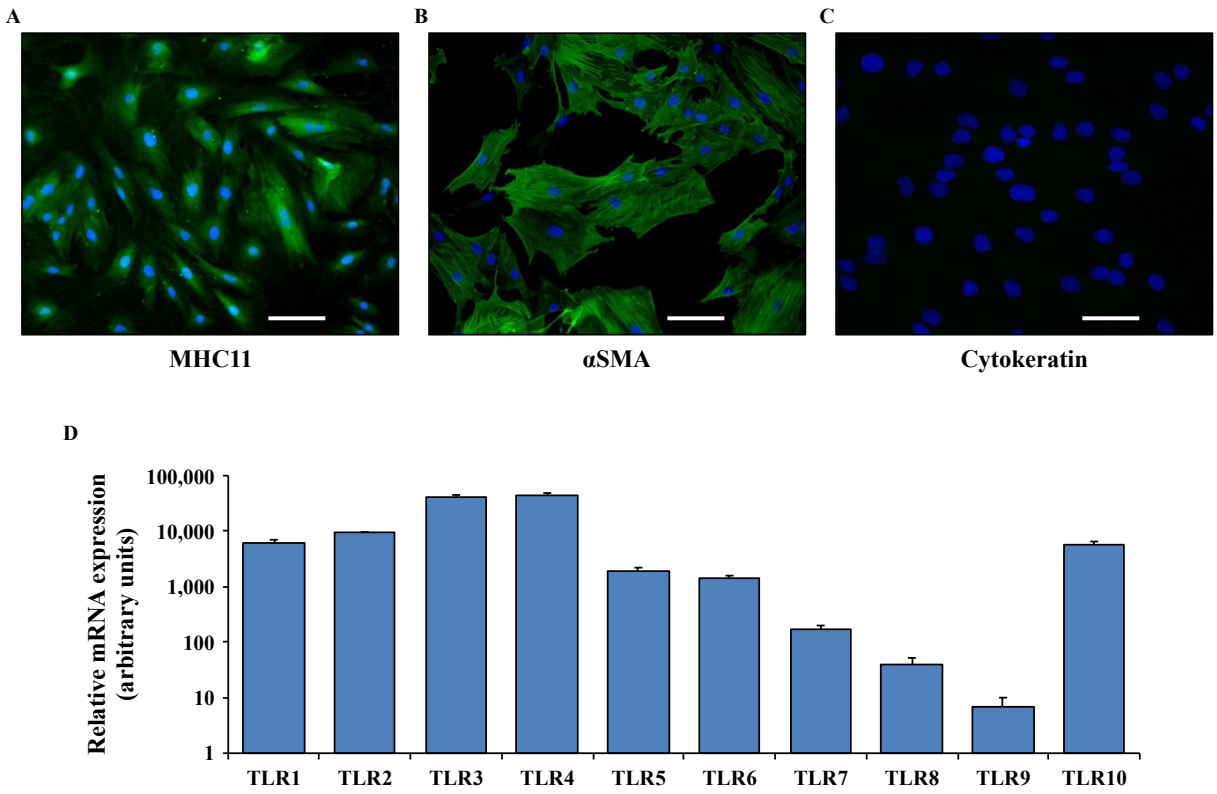


Figure 9. *Characterization of rSMCs isolated from rat vagina and toll-like receptors (TLRs) mRNA expression pattern.* Immunostaining for the specific smooth muscle markers MHC11 (panel A) and αSMA (panel B) and for the epithelial marker cytokeratin (panel C), taken as negative control, in rSMCs. The quantitative analysis was performed by counting positive cells in at least 10 fields per slide of three different cell preparations (scale bar=100 μm). Panel D shows the complete panel of TLRs mRNA expression in rSMCs. Data were calculated according to the 2-ΔΔCt comparative method, using the ribosomal subunit 18S as the reference gene for normalization and are represented as the mean±SEM of three independent experiments.

3.3 Toll-like receptors (TLRs) mRNA expression pattern in vaginal rSMCs and hSMCs

To understand the role of vagina SMCs in the inflammatory process we evaluated the membrane profile of toll-like receptors (TLRs), by using real-time RT-PCR. We observed that rSMCs expressed all TLRs (Fig. 9, panel D), with a relatively higher expression of transcripts encoding TLR1, TLR2, TLR3, TLR4 and TLR10.

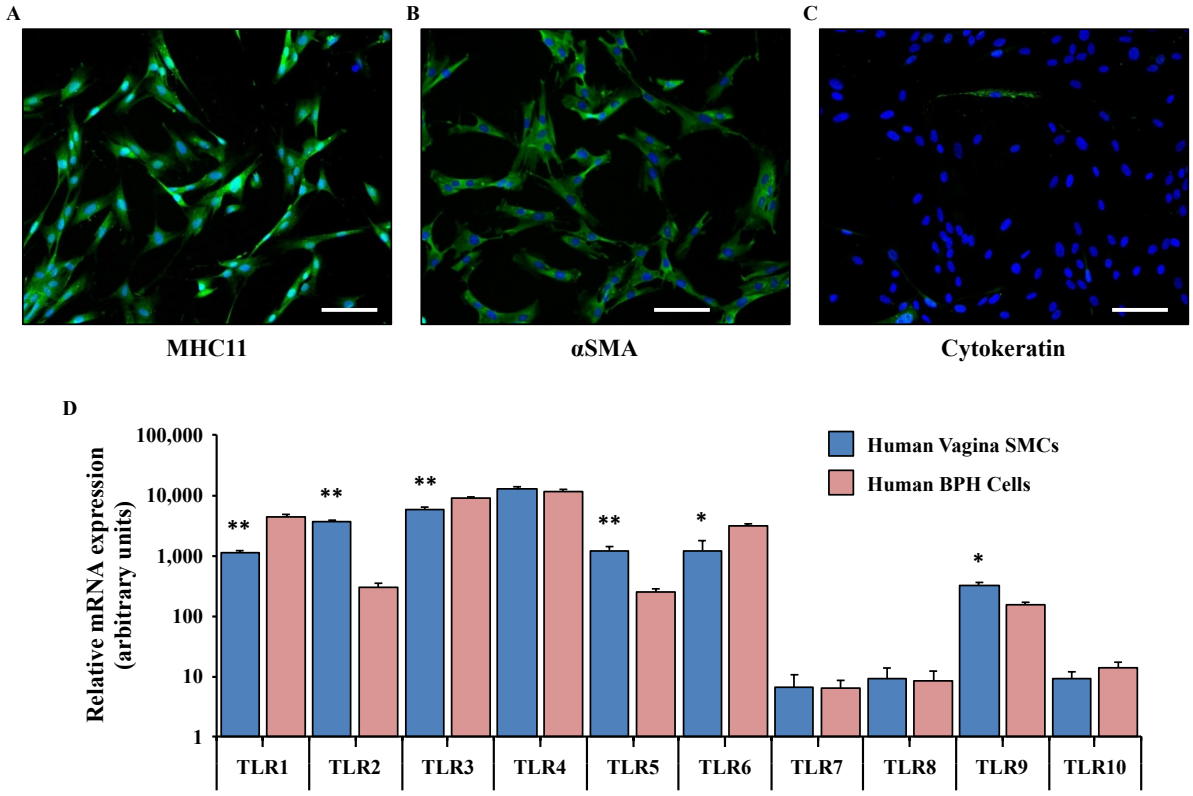


Figure 10. *Characterization of hSMCs isolated from human vagina and toll-like receptors (TLRs) mRNA expression pattern.*
Immunostaining for the specific smooth muscle markers MHC11 (panel A) and αSMA (panel B) and for the epithelial marker cytokeratin (panel C), taken as negative control, in hSMCs. The quantitative analysis was performed by counting positive cells in at least 10 fields per slide of three different cell preparations (scale bar=100 μm). Panel D shows the complete panel of the TLRs mRNA expression in hSMCs, with the relative TLRs gene expression in human BPH cells taken as positive controls. The results were calculated according to the 2-ΔΔCt comparative method, using the ribosomal subunit 18S as the reference gene for normalization, and data are represented as the mean±SEM of three independent experiments (* $p<0.01$, ** $p<0.001$ vs. BPH). BPH = benign prostate hyperplasia.

TLRs mRNA expression was evaluated also in hSMCs, comparing the results to the pattern of primary cell lines isolated from benign prostatic hyperplasia (hBPH) samples, a

cell model previously validated in the literature (Penna et al., 2009) and taken as a positive control (Fig. 10, panel D). hSMCs expressed all TLR receptors, with particular abundance of TLR1, TLR2, TLR3, TLR4, and the mRNA expression profile presented was similar to that of hBPH cells. However, compared to hBPH cells, hSMCs showed a significantly higher expression of TLR2 ($p<0.001$), TRL5 ($p<0.001$) and TLR9 ($p<0.01$) receptors and a significantly lower expression of TLR1 ($p<0.001$), TLR3 ($p<0.001$) and TLR6 ($p<0.01$) (Fig. 10, panel D).

3.4 DHT stimulation inhibits basal and LPS-induced mRNA expression of pro-inflammatory cytokines/chemokines in vaginal rSMCs

We next stimulated vaginal SMCs with the selective AR ligand DHT, in presence or absence of the non-steroid anti-androgen bicalutamide (Fig. 11).

Treatment with DHT significantly inhibited the basal mRNA expression of proinflammatory markers IL6 (interleukin 6; $p<0.01$ vs. NT), CXCL1 [chemokine (C-X-C motif) ligand 1; $p<0.001$ vs. NT], MCP1 (Monocyte Chemoattractant Protein-1; $p<0.05$ vs. NT) and COX2 (Cyclo-oxygenase 2) (Fig. 11, panels A, B, D, and E respectively), while significantly increasing the mRNA expression of STAMP2 (Six Transmembrane Protein of Prostate 2; $p<0.01$ vs. NT; Fig. 11, panel F), an androgen-dependent counter regulator of inflammation. These DHT-induced effects were mostly blunted by co-treatment with bicalutamide ($p<0.05$ vs. DHT for IL6 and MCP; $p<0.001$ vs. DHT for COX2).

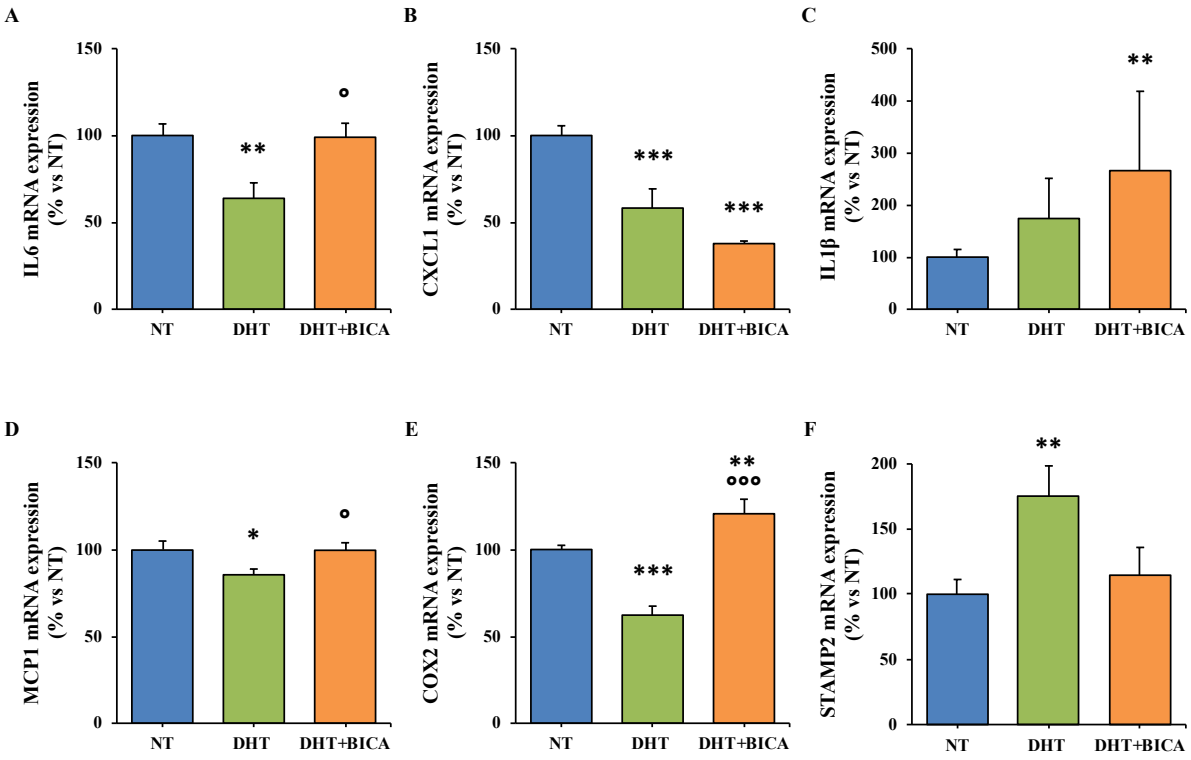


Figure 11. *DHT effects on basal mRNA expression of pro-inflammatory markers in rSMCs.* Vaginal rSMCs were cultured for 24 h in serum-free medium alone (untreated cells, NT) or DHT (30 nM), with or without anti-androgen bicalutamide (BICA, 1 μM). The mRNA expression of pro-inflammatory markers (IL6, CXCL1, IL1β, MCP1, COX2 and STAMP2) is showed in panels A-F. Data were calculated according to the 2-ΔΔCt comparative method, using the 18S ribosomal subunit as the reference gene for normalization. Data are displayed as mean±SEM of five independent experiments using different cell preparations, performed in triplicate and reported as percentage vs. NT (** $p<0.01$, *** $p<0.001$ vs. NT; ° $p<0.05$, °°° $p<0.001$ vs. DHT).

The rSMCs were then stimulated by TLRs agonist lipopolysaccharide (LPS), with or without co-treatment with DHT, in the presence or absence of bicalutamide. Increasing concentration of DHT (ranging from 10 pM to 1 μM) significantly blunted LPS-induced IL6 and CXCL1 mRNA expression in a dose dependent manner (EC_{50} 5.6 x 10^{-10} M and 5.1 x 10^{-10} M, respectively) (Fig. 12, panel A). In addition, LPS significantly increased mRNA expression of pro-inflammatory mediators, namely IL1β ($p<0.001$ vs. NT), MCP1 ($p<0.001$ vs. NT) and COX2 ($p<0.01$ vs. NT; Fig. 12, panels B-D). These effects were significantly reduced by pre-treatment with DHT (all $p<0.05$ vs. LPS), whose action was completely counteracted by bicalutamide ($p<0.01$ vs. LPS+DHT for IL1β, $p<0.05$ vs. LPS+DHT for MCP1 and COX2; Fig. 12, panels B-D).

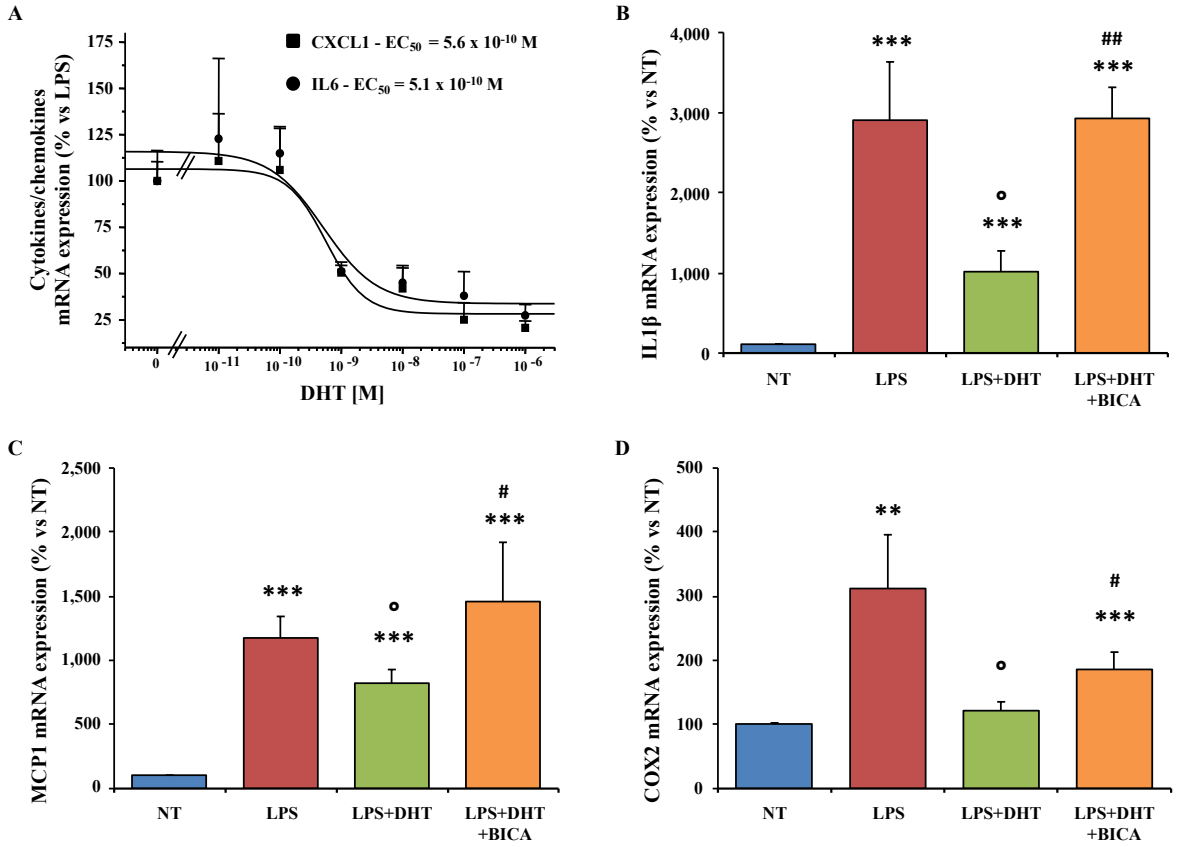


Figure 12. *DHT effects on LPS-induced mRNA expression of pro-inflammatory cytokines/chemokines in rSMCs.*
Vaginal rSMCs were stimulated with LPS (100 ng/ml for 24 h), with pre-incubation with DHT (30 nM for 24 h) in the presence or absence of BICA (1 μM for 24 h). Panel A shows the inhibitory effect of increasing concentrations of DHT on LPS-induced mRNA expression of CXCL1 and IL6 in rSMCs. Ordinate: mRNA expression of the indicated pro-inflammatory factors (black square: CXCL1; black circle: IL6) induced by LPS (100 ng/ml for 24 h) after DHT pre-incubation. Data are expressed as percentage of the effect of LPS alone. Abscissa: molar concentrations of DHT (10 pM - 1 μM). Data are represented as the mean±SEM of three independent experiments performed in triplicate. The relative half-maximal response (EC50) value is reported in the graph. Panels B-E show the mRNA expression of pro-inflammatory markers IL1β, MCP1 and COX2 induced by LPS stimulation alone and after pre-incubation with DHT, in combination or not with BICA. Data were calculated according to the 2-ΔΔCt comparative method, using the 18S ribosomal subunit as the reference gene for normalization. The results represent the mean±SEM of three independent experiments, performed in triplicate and reported as percentage vs. untreated cells (NT) (** $p<0.01$, *** $p<0.001$ vs. NT; ° $p<0.05$ vs. LPS; # $p<0.05$, ## $p<0.01$ vs. LPS+DHT).

3.5 DHT inhibits NF-κB p65 nuclear translocation in LPS-stimulated vaginal rSMCs and hSMCs

To better characterize the effect of DHT on the inflammatory response of LPS-stimulated rSMCs and hSMCs, nuclear translocation of NF-κB p65 was evaluated. As assessed by immunofluorescence (Fig. 13, panels A-E), in NT rSMCs, NF-κB p65 was only detected in the cells cytoplasm (Fig. 13, panels A and E). LPS stimulation (100 ng/ml for 5 h) induced a partial but significant translocation of NF-κB p65 to the nucleus (Fig. 13, panels B and E; 40.2±2.8%; $p<0.001$ vs. NT), which was significantly inhibited (20.8±1.8%; $p<0.0001$ vs. LPS), albeit not normalized ($p<0.001$ vs. NT), by DHT treatment (Fig. 13, panels C and E). Co-treatment with bicalutamide (1 μM for 24 h) partially contrasted DHT-induced effect (Fig. 13, panels D and E; 31.3±2.3%; $p<0.05$ vs. LPS; $p<0.01$ vs. LPS+DHT).

The immunofluorescence analyses for NF-κB p65 nuclear translocation in hSMCs are shown in Fig. 14 (panels A-E). Similarly to rSCMs, we observed that, compared to NT cells (Fig. 14, panel A), LPS treatment induced a significant increase in nuclear translocation of NF-κB p65 (26.5±2.6%; $p<0.001$ vs. NT; Fig. 14, panels B and E), which was significantly blunted by DHT (13.7±1.8%; $p<0.0001$ vs. LPS), although not normalized ($p<0.001$ vs. NT; Fig. 14, panels C and E). Co-treatment with bicalutamide (1 μM for 24 h) counteracted this effect (Fig. 14, panels D and E; 31.3±2.3%; $p<0.01$ vs. LPS+DHT).

3.6 DHT stimulation up-regulates the mRNA expression of STAMP2 in hSMCs

DHT treatment massively up-regulated the mRNA expression of androgen-sensitive gene STAMP2 (10939.15±1996.91%, $p<0.001$ vs. both NT and LPS), which was also stimulated by LPS, but to a much lower extent (168.73±29.62%, $p<0.05$ vs. NT) (Fig. 15).

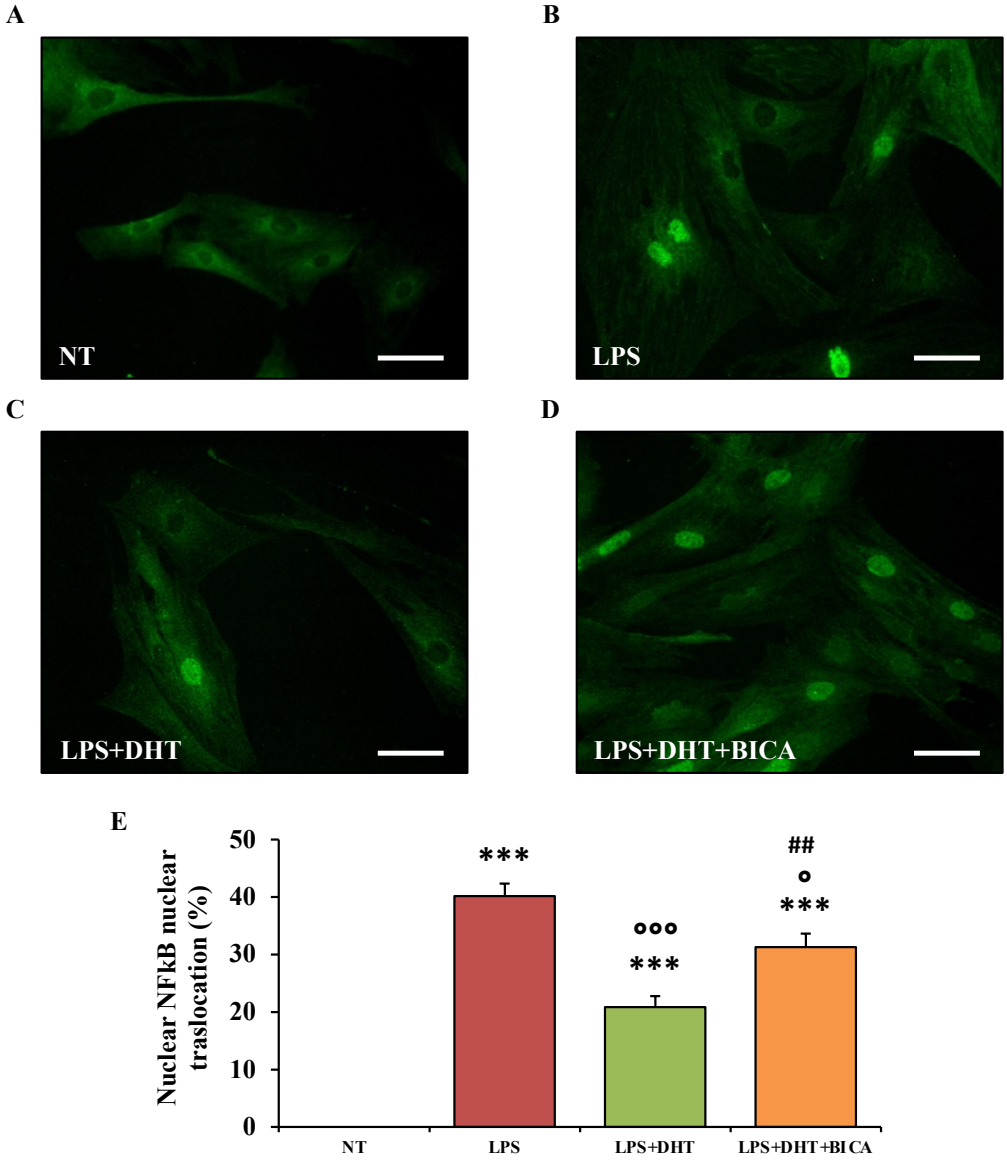


Figure 13. *DHT effects on LPS-induced NF-κB p65 nuclear translocation in rSMCs*
Vaginal rSMC cells were left untreated (NT) or stimulated with LPS (100 ng/ml for 5 h) with or without pre-treatment with DHT (30 nM for 24 h), in combination or not with BICA (1 μM for 24 h). The NF-κB p65 nuclear translocation was analyzed by immunofluorescence and representative images of NF-κB immunostaining are shown in panels A, B, C and D for NT, LPS, LPS+DHT and LPS+DHT+BICA experimental groups, respectively (scale bar=50 μm). Panel E bar graph shows the quantitative analysis of NF-κB nuclear translocation, expressed as the number of positive cells as percentage of total cells. The data represent the mean±SEM of three independent experiments performed in triplicate (*** $p<0.001$ vs. NT; ° $p<0.05$, °°° $p<0.001$ vs. LPS; ## $p<0.01$ vs. LPS+DHT).

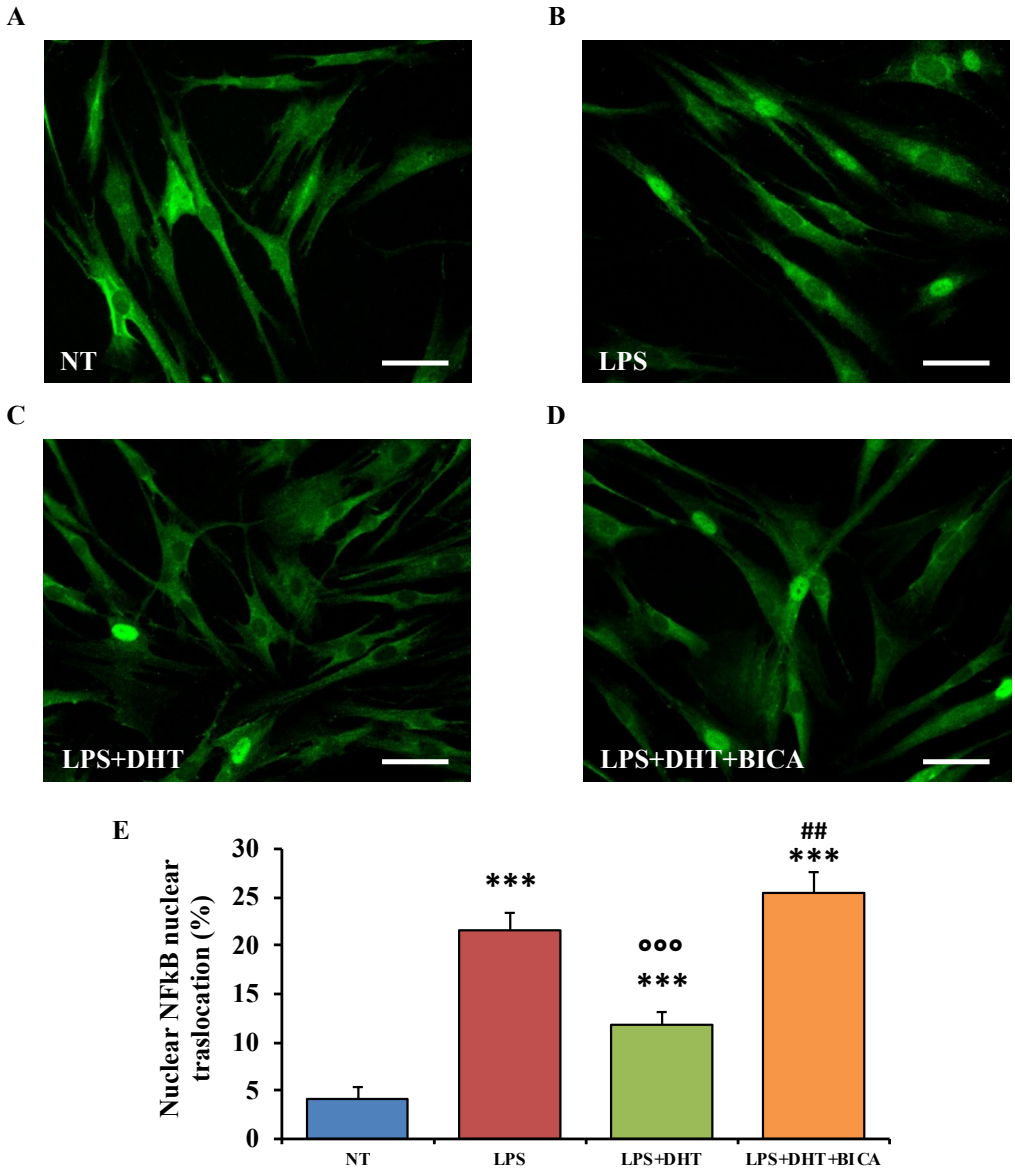


Figure 14. *DHT effects on LPS-induced NF-κB p65 nuclear translocation in hSMCs*
Vaginal hSMCs were left untreated (NT) or stimulated with LPS (100 ng/ml for 5 h) with or without pre-treatment with DHT (30 nM for 24 h), in combination or not with BICA (1 μM for 24 h). The NF-κB p65 nuclear translocation was analyzed by immunofluorescence and representative images of NF-κB immunostaining are shown in panels A, B, C and D for NT, LPS, LPS+DHT and LPS+DHT+BICA experimental groups, respectively (scale bar=100 μm). Panel E bar graph shows the quantitative analysis of NF-κB nuclear translocation, expressed as the number of positive cells as percentage of total cells. The data represent the mean±SEM of three independent experiments performed in triplicate (*** $p<0.001$ vs. NT; °°° $p<0.001$ vs. LPS; ## $p<0.01$ vs. LPS+DHT).

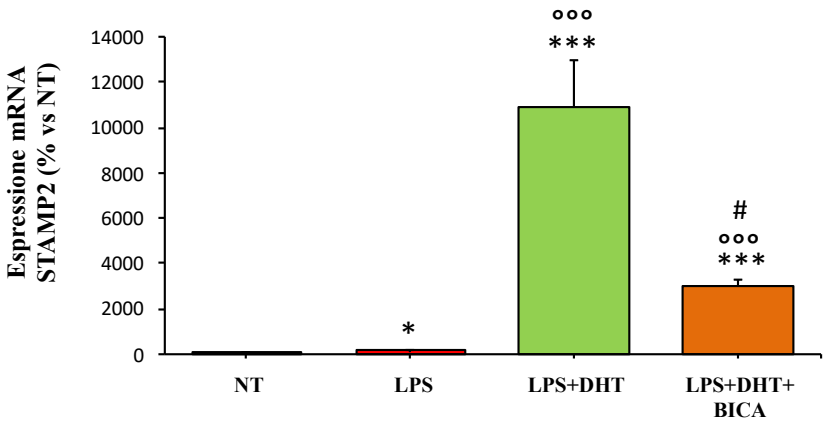


Figure 15. *DHT effects on STAMP2 mRNA expression in hSMCs*
Vaginal hSMCs were left untreated (NT) or stimulated with LPS (100 ng/ml for 5 h) with or without pre-treatment with DHT (30 nM for 24 h), in combination or not with BICA (1 μM for 24 h). The mRNA expression of STAMP2 was evaluated. Data were calculated according to the 2-ΔΔCt comparative method, using the 18S ribosomal subunit as the reference gene for normalization. Data are displayed as mean±SEM of five independent experiments using different cell preparations, performed in triplicate and reported as percentage vs. NT (* $p<0.05$, *** $p<0.001$ vs. NT; °°° $p<0.001$ vs. DHT; # $p<0.05$ vs. LPS+DHT).

3.7 DHT stimulation inhibits the LPS-induced mRNA expression and secretion of pro-inflammatory mediators in vaginal hSMCs

As shown in Fig. 16, LPS stimulation (100 ng/ml for 24 h) significantly increased the mRNA expression of COX2 ($p<0.001$ vs. NT; panel A), IL6 (interleukin 6, $p<0.01$ vs. NT; panel B), IL12A ($p<0.05$ vs. NT; panel C), and IFNγ (interferon-γ, $p<0.05$ vs. NT; panel D). Pre-treatment with DHT significantly counteracted LPS-induced COX2, IL6, IL12A and IFNγ mRNA upregulation ($p<0.05$ vs. LPS for all mediators; Fig. 16, panels A-D), whereas co-treament with bicalutamide blunted the DHT-induced effects ($p<0.01$ vs. LPS+DHT for COX2 and IL6, $p<0.05$ vs. LPS+DHT for IL12A and IFNγ; Fig. 16, panels A-D).

We next evaluated the effect of DHT, in combination or not with bicalutamide, on LPS-stimulated cytokine production in the supernatant of hSMC. We found that LPS (100 ng/ml, 24 h) significantly increased the secretion of several pro-inflammatory protein,

including IL1RA (interleukin-1 receptor antagonist), IL2, IL4, IL5, VEGF (vascular endothelial growth factor), and TNFα (Tumor necrosis factor-α) ($p<0.05$ vs. NT), IL15, FGF (fibroblast growth factor) ($p<0.01$ vs. NT) and, most importantly, IFNγ ($p<0.001$ vs. NT) (Fig. 17, panels A-I).

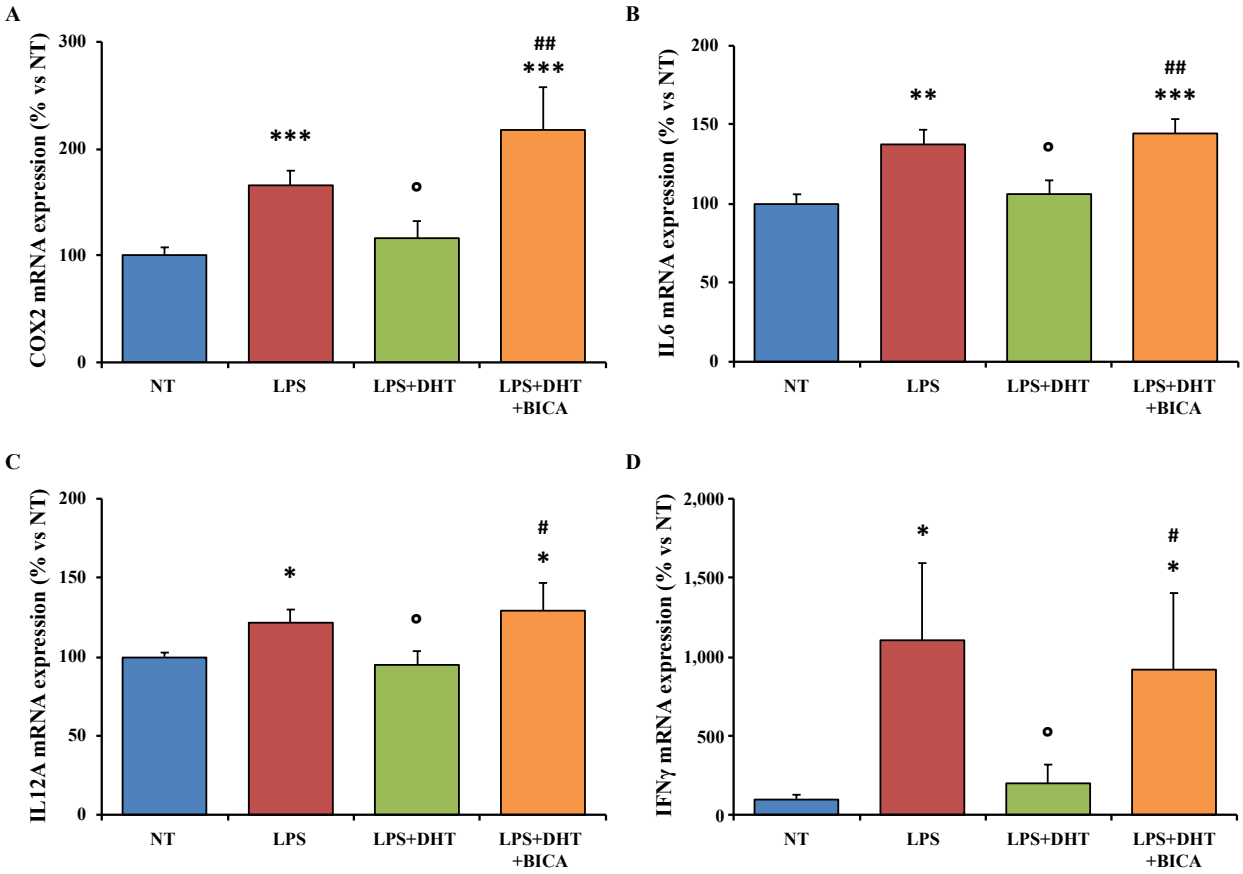


Figure 16. *DHT effects on the mRNA expression of pro-inflammatory mediators in LPS-stimulated vaginal hSMCs*
Vaginal hSMCs were left untreated (NT) or stimulated with LPS, in presence or absence of DHT pre-incubation (30 nM for 24 h), in combination or not with BICA (1 μM for 24 h). The mRNA expression of pro-inflammatory markers COX2, IL6, IL12 and IFNγ are shown in panels A, B, C and D, respectively. Data were calculated according to 2-ΔΔCt comparative method, using 18S ribosomal subunit as reference gene for normalization. Results are expressed as percentage of NT and are reported as mean±SEM of three independent experiments performed in triplicate (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. NT; ° $p<0.05$ vs. LPS; # $p<0.05$, ## $p<0.01$ vs. LPS+DHT).

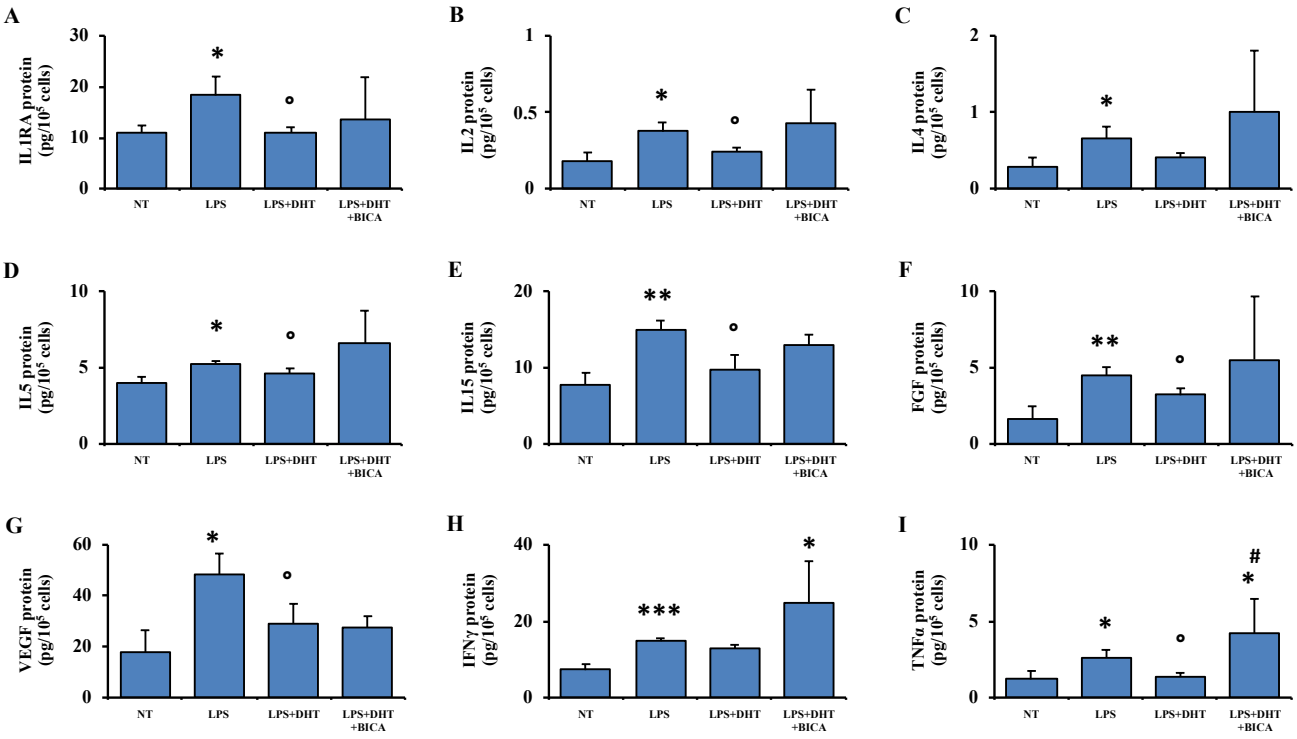


Figure 17. *DHT effects on the secretion of pro-inflammatory mediators in LPS-stimulated vaginal hSMCs*
Vaginal hSMCs were left untreated (NT) or stimulated with LPS, in presence or absence of DHT pre-incubation (30 nM for 24 h), in combination or not with BICA (1 μM for 24 h). Cell culture supernatants from each experimental point were analyzed for the production of the indicated pro-inflammatory cytokines, chemokines, and growth factors (panels A-I). The data represent the mean±SEM of three independent experiments performed in triplicate (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. NT; ° $p<0.05$, °°° $p<0.001$ vs. LPS; # $p<0.05$ vs. LPS+DHT).

Pre-treatment with DHT significantly counteracted the secretion of IL1RA, IL2, IL5, IL15, FGF, VEGF and TNFα (all $p<0.05$ vs. LPS), also reducing the secretion of IL4 and IFNγ, albeit without reaching statistical significance (Fig. 17, panels A-I). In addition, the secreted levels of IL6, IL8, IP10, GCSF, GMCSF, MCP1 and MIP1B were also significantly increased by LPS, but no relevant effect of DHT was observed on their secretion (not shown).

The co-treatment of DHT with bicalutamide (1 μM for 24 h) tended to contrast the effects of DHT, bringing most of the secreted protein to the levels observed with LPS (Fig. 17, panels A to F) or significantly higher ($p<0.05$) than NT cells for IFNγ (panel H) or

TNFα (panel I). Conversely, co-treatment with bicalutamide did not exert any significant effect on VEGF secretion (Fig. 17, panel G), compared to the effect induced by DHT.

3.8 Evaluation of HLA-DR expression and effects of IFNγ and DHT on the mRNA expression and secretion of pro-inflammatory mediators in vaginal hSMCs

We next evaluated the effect of stimulation of hSMCs with IFNγ (1000 UI/ml for 48 h) on the cell-surface expression of HLA-DR and the expression and secretion of pro-inflammatory mediators. DHT 30 nM treatment, in combination or not with bicalutamide, was also tested. As expected, HLA-DR was not expressed in NT and DHT only-treated cells, whereas its expression was significantly increased (47.5±5.5%; $p<0.001$ vs. NT) after IFNγ stimulation (Fig. 18, panels A and B). We also observed that DHT pre-treatment significantly decreased IFNγ-induced HLA-DR expression (37.6±1.3%; $p<0.01$ vs. IFNγ), albeit without normalizing it ($p<0.001$ vs. NT) (Fig. 18, panels A and B).

Furthermore, stimulation with IFNγ (24 h) significantly increased the mRNA expression of iNOS (inducible Nitric Oxide synthase), COX2 and MCP1 (599.7±128.5, 183.85±34.32, 879.07±191.30, respectively; $p<0.001$ vs. NT, Fig. 18, panels C-E). This effect was significantly counteracted, although not normalized, by DHT pre-treatment (281.34±49.82, 108.85±20.73, 444.91±53.23 for iNOS, COX2 and MCP1, respectively; all $p<0.05$ vs. IFNγ; Fig 18, panels C-E). In addition, the mRNA expression of IL12A and IP10 (IFNγ-induced-Protein) was also significantly increased by IFNγ stimulation (722.9±111.6% and 245978.1±28479.2%, respectively; both $p<0.001$ vs. NT; Fig. 18, panels F and G). This effect was significantly blunted, although not normalized, by DHT (IL12A: 436.3±50.5%, $p<0.05$ vs. IFNγ; IP10: 179,840.3±13,744.3%, $p<0.05$ vs. IFNγ; Fig. 18, panels F and G).

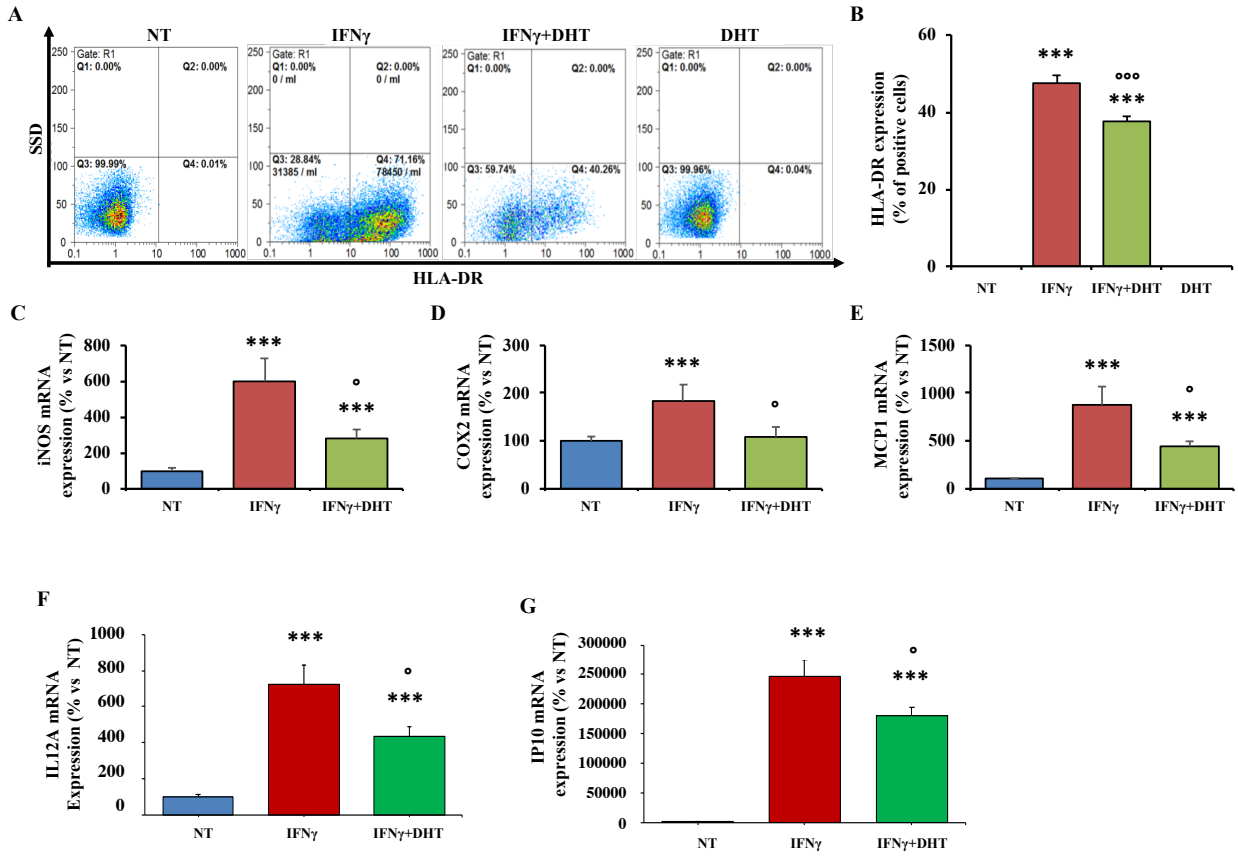


Figure 18. *DHT effects on the cell-surface HLA-DR expression and the mRNA expression of pro-inflammatory mediators in IFNγ-stimulated vaginal hSMCs*
Panel A reports the results of a representative experiment of flow cytometry showing the expression of HLA-DR in hSMCs stimulated with IFNγ (1x103 IU/ml) for 48 h and/or treated with DHT (30 nM, for 24 h). Panel B shows the quantification of cell positivity for HLA-DR, expressed as percentage of positive cells compared to the total number of cells. Data are reported as mean±SEM of three independent experiments performed in triplicate (*** $p<0.001$ vs. NT; °°° $p<0.001$ vs. IFNγ).
For mRNA expression analysis and detection of pro-inflammatory cytokines and chemokines, hSMC cells were stimulated with IFNγ (1x103 IU/ml) for 24 h, with or without DHT incubation (30 nM, 24 h). The mRNA expression of iNOS, COX2, MCP1, IL12A and IP10 genes are shown in panels C-G. Data were calculated according to 2-ΔΔCt comparative method, using 18S ribosomal subunit as reference gene for normalization. Results are expressed as percentage of untreated cells (NT) and are reported as mean±SEM of three independent experiments performed in triplicate (*** $p<0.001$ vs. NT; ° $p<0.05$ vs. IFNγ).

Fig. 19 (panels A-F) shows the secretion of several pro-inflammatory markers after IFNγ stimulation (1000 IU/ml, 24 h) and the effect of DHT pre-treatment (30 nM for 24 h) with or without bicalutamide (1 μM for 24 h). Similarly to the findings previously obtained with LPS (Fig. 17), IFNγ significantly increased the secretion of many pro-inflammatory mediators, including IL1β, IL2, IL5, IL6, MCP1 and GCSF (Granulocyte-colony

stimulating factor) ($p<0.001$ vs. NT for all proteins; Fig. 19, panels A-F). DHT pre-treatment significantly blunted this effect ($p<0.05$ vs. IFNγ for all preteins); however, it was not able to restore the secretion to the levels observed in NT cells ($p<0.01$ vs. NT for IL2, IL5, IL6 and MCP1; $p<0.001$ vs. NT for IL1β and GCSF). Co-treatment with bicalutamide (1 μM for 24 h) in combination with DHT significantly increased the secretion of MCP1 ($p<0.05$ vs. IFNγ+DHT; Fig. 19, panel E). As for the other proteins, no statistically significant differences between IFNγ+DHT vs. IFNγ+DHT+BICA was found (Fig. 19).

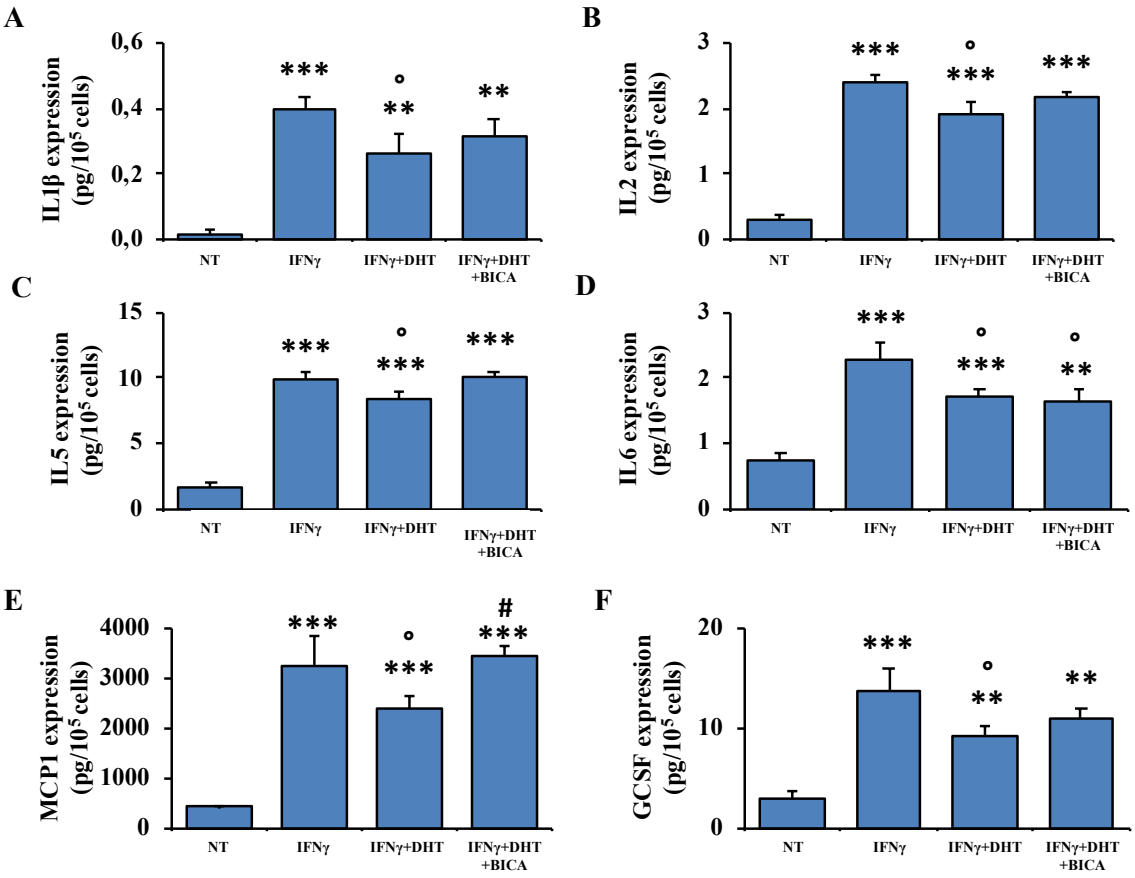


Figure 19. *DHT effects on the secretion of pro-inflammatory mediators in IFNγ-stimulated vaginal hSMCs*
Vaginal hSMCs were left untreated (NT) or stimulated with LPS, in presence or absence of DHT pre-incubation (30 nM for 24 h), in combination or not with BICA (1 μM for 24 h). Cell culture supernatants from each experimental point were analyzed for the secretion of pro-inflammatory cytokines, chemokines and growth factors (panels A-F). Data are represented as the mean±SEM of three independent experiments performed in triplicate (** $p<0.01$, *** $p<0.001$ vs. NT; ° $p<0.05$ vs. IFNγ; # $p<0.05$ vs. IFNγ+DHT).

Interestingly, DHT pre-treatment reduced, even though not significantly, IFNγ-stimulated secretion of TNFα, VEGF, FGF and IL17 (not shown). Finally, IFNγ induced a significant increase in the secretion of IL1RA, IL4, IL7, IL9, IL12, IL13, IL15, IP10, MIP1A (Macrophage Inflammatory Protein 1A, MIP1B and RANTES (Regulated on Activation, Normal Y Expressed and Secreted); however, no significant effects were observed in their levels with DHT pre-treatment (not shown).

4. Discussion

The present study demonstrates, for the first time, that androgen signaling plays a suppressive role in the inflammatory response in rat (rSMCs) and human smooth muscle vagina cells (hSMCs), reducing their potential to be involved in the initiation and maintaining of inflammation.

Inflammation is vital in that it represents the main immune system's defense to harmful stimuli such as external pathogens, and usually acts by removing pathogenic *noxae* and restoring tissue homeostasis (Chen et al., 2018). However, it is well recognized that exacerbated or uncontrolled inflammation may progress to a persistent, aberrant condition, contributing to chronic diseases that affect a variety of systems, including the female genitourinary tract (Chen et al., 2018). Both animal and human studies have been extensively documenting that androgens act as negative regulators of inflammation (see Gilliver, 2010 for a review): with regard to genitourinary tissue in males, we previously demonstrated that *in vitro* DHT treatment inhibits the potential of prostatic stromal cells derived from BPH (Benign Prostatic Hyperplasia) patients to induce and sustain immune and inflammatory responses (Vignozzi et al., 2012). Although emerging literature highlights that androgen-mediated processes might be involved in the resolution of genitourinary conditions in women, such as the GSM (Traish et al., 2018), immunomodulatory effects of androgen signaling in the vagina have not been investigated.

In the first set of experiments we aimed at confirming previous preclinical (Pelletier et al., 2004) and clinical (Baldassarre et al., 2013) observations indicating that the vagina is an androgen target organ. Expression of sex steroids receptors was assessed both in vagina tissue samples collected from rats, human and in SMCs derived thereof, revealing a similar pattern and an overall abundant expression of AR. The main difference between rat and human data consisted in the expression of PR, which was the most represented receptor in both rat tissue and cells, but not in the human counterpart; this discrepancy is likely due to the fact that the analysis was performed in vagina derived from intact rats, whereas the human vagina samples were collected from peri- and postmenopausal women, characterized by a decrease in estrogen levels and, therefore, of estrogen-induced PR expression in genital tissues. In the whole human tissue, AR was abundantly expressed, being second only to ERα. In hSMCs, not only AR was significantly more expressed than PR and all three investigated estrogen receptors (ERα, β and GPER1), but it was by far the most detected receptor, exceeding ERα and GPER1 by approximately half log unit, PR by one log unit and ERβ by three log units. Although most studies on AR localization in the vagina have focused on the epithelium, our data point toward a particularly rich representation in the muscle layer, both in the animal and in the human model. This is consistent with immunohistochemistry analyses in the monkey vagina highlighting that AR positive cells were not only present in the epithelium and *lamina propria*, but also in the *muscularis* (Bertin et al., 2014). Similarly, a study on female Sprague-Dawley rats found that the highest proportion of AR-immunoreactive cells occurred in the *muscularis*, whereas ERα and PR were most heavily concentrated in the epithelium (Pessina et al., 2006).

To investigate whether androgens could suppress inflammatory responses in the vagina, as hypothesized following previous results in prostatic stromal cell cultures of BPH patients, we performed *in vitro* studies in rSMCs and hSMCs using the potent and selective

AR ligand DHT. Existing literature indicates that the inflammation response to invading microbes in the female reproductive tract is initiated and sustained by non-professional host epithelial cells (Darville and Hiltke, 2010), which act as sentinel components of innate immunity (Farage et al., 2011). In the present study, we confirmed that, in the rat and in the human vagina, SMCs share with the epithelium the ability to initiate and coordinate an acute reaction to pathogens by expressing all identified TLRs. Specifically, in hSMCs TLR1-4 emerged among the most expressed, with TLR2 being found at higher levels than in BPH stromal cells, used as positive controls of non-professional APCs. A systematic investigation of TLR2 mRNA levels in human female reproductive tissues previously revealed the highest expression in fallopian tube and cervical tissues, followed by endometrium and ectocervix (Pioli et al., 2004). However, immunohistochemistry studies in the vagina showed that TLR2 was highly represented in the epithelium, whereas only a weak staining of stromal muscle cells and no staining in the endothelium were reported (Fazeli et al., 2005). Our finding relative to the high expression of TLR2 in hSMCs appears of great importance, since this molecule is considered as the primary Gram-positive TLR and is known to form heterodimers with other members of the family (TLR1/TLR2 and TLR2/TLR6), thus displaying a particularly wide repertoire of recognition of microorganism-derived substances (Sonnex, 2010). In human epithelial cell lines derived from embryonic kidney, TLR2 has been identified as a signal transducer for Gram-positives' atypical LPS, soluble peptidoglycan (sPGN) and lipoteichoic acid (LTA) (Schwandner, 1999), as well as for *Chlamydia trachomatis'* ligands, following recruitment to its intracellular inclusions [O'Connell et al., 2006). In other studies examining common genital tract pathogens, TLR2 has been described to mediate the *Neisseria gonorrhoeae* lipoprotein-induced release of inflammatory mediators (Fisette et al., 2003), to trigger NF-kB signaling in response to *Mycoplasma genitalium* infections (McGowin et al., 2009) and to recognize *Candida albicans'* phospholipomannan (Li et al., 2009) in epithelial cells. On

the other hand, there is a paucity of data relative to the role of TLR2 expressed by SMCs in the immune response. Interestingly, in 2000 Watari and colleagues reported that cervical SMCs expressed TLR2 and responded to LPS with the production of pro-inflammatory cytokines, that in turn were able to induce TLR2 expression in the same cells (Watari et al., 2000).

Along with TLR2, TLR4 is considered the other crucial defense against bacteria thanks to its binding to LPS of Gram-negative species (Sonnex, 2010), in addition to some protozoa components and viral envelope proteins (Nasu and Narahara, 2010). As far as genital infections are concerned, *C. trachomatis* antigen heat-shock protein (HSP60) has been described as a common TLR4 ligand (Vabulas et al., 2001). Evidence regarding the presence of TLR4 in the epithelial cells of the lower female genital tract, in particular in the vagina, is debated since some authors failed to detect it (Fazeli et al., 2005; Fichorova et al., 2002), hypothesizing a role of TLR4 in preventing ascending bacterial infections and, at the same time, in preventing the activation of inflammation in response to resident commensal flora. Interestingly, TLR4 levels were found to be higher in stromal cells than in epithelial cells in the endometrium (Hirata et al., 2007). Our experiments are somehow consistent with these results, showing a significant expression of TLR4 in hSMC, similar to that detected in stromal BPH cells. Finally, our data indicate the possible involvement of hSMCs in the response to viral insults, including herpes simplex virus (HSV) through TLR3- (Hirata et al., 2007), and to atypical *mycobacteria* through TLR9- mediated pathways (Jo et al., 2007).

In light of our findings, vaginal SMCs seem to contribute to provide protection against infections and to modulate local immunological tolerance. It is important to note that hSMCs in our study were derived from perimenopausal women, a specific population that may display an initial alteration of the vaginal environment and microbiota composition due to aging and hormonal changes; this may influence the representation of

TLRs in SMCs. In this regard, some data have been supporting a modulation of TLRs' expression by sex steroids, with a general higher representation during the secretory phase with respect to the menstrual and proliferative phases, thus suggesting an inhibitory effect of estrogens and/or a stimulating influence of progesterone (Aflatoonian and Fazeli, 2008). However, Pioli and colleagues failed to find significant correlations between TLR1-6 expression in the female reproductive tract and the cycle phase or menopausal state (Pioli et al., 2004).

An important finding of our study is that in rSMCs DHT-induced activation of AR, confirmed by the up-regulation of androgen-dependent gene STAMP2 and by the opposing effect of co-treatment with bicalutamide, inhibited the basal expression of pro-inflammatory markers IL6, CXCL1, MCP1 and COX2. After engaging with specific antigenic stimuli, TLRs mediate the initiation of a signaling cascade leading to the production of various proinflammatory effector molecules (Fazeli et al., 2005). In our experiments we were able to demonstrate that LPS, typical of Gram-negative bacteria, can have a direct action on vaginal rSMCs and hSMCs, causing them to produce important inflammation mediators. In humans, COX-2 has been extensively associated with mucosal inflammation and its transcriptional and translational induction, accompanied by the synthesis of prostaglandins, has been previously demonstrated in vaginal epithelial cells stimulated with TLR ligands, surrogates of bacterial, mycoplasmal and viral infections (Joseph et al., 2011). As for interleukins, IL12A is an important cytokine produced mainly by APCs, which has been reported to bridge the innate immune response and the following adaptive immunity by inducing IFNγ production and promoting Th1-cells differentiation (Chehimi and Trinchieri,1994). Interestingly, in women susceptible to recurrent urinary tract infections (UTIs), IL-12 production was found to be aberrantly enhanced, suggesting a predisposing immunological defect in APCs function (Kirjavainen et al., 2009). IL6, in addition to eliciting the production of acute phase proteins, is known to dictate the

transition from acute to chronic inflammation by changing the nature of leucocyte infiltrate from neutrophils to monocyte/macrophages thanks to a shift in the chemokines produced by stromal and professional immune cells (i.e., MCP1 rather than IL8; M1/M2 shift) (Gabay, 2006). Consistent with this, LPS-stimulated hSMCs also produced IFNγ, the principal Th-1 cytokine and a key regulator of macrophage activation well known to activate macrophages toward the M1 phenotype, characterized by high levels of inflammatory cytokines (i.e. IL1β, IL12, and TNFα), and sustain the inflammatory response by maintaining Th1 activation and inhibiting differentiation of regulatory T cells (Th2 and Th17) (Green et al., 2017). Therefore, these data point toward the participation of stimulated vaginal SMCs not only in the initiation of inflammation, but also in favoring chronic inflammatory responses.

However, the most striking finding of the present study is that activation of AR by DHT markedly suppresses the inflammatory response in vaginal SMCs in both rat and human vagina, and that this effect is counteracted by the co-administration of AR antagonist bicalutamide, thus confirming that androgen signaling plays an important role in maintaining adult vagina homeostasis. As demonstrated in rSMCs, the blunting effect of DHT on LPS-induced cytokines and chemokines' expression was dose-dependent. Specifically, in hSMCs, pre-treatment with DHT significantly decreased LPS-induced expression of COX2, IL6, IL12A and IFNγ, albeit without normalizing it. Also in this human model, the activation of AR was confirmed by the pronounced up-regulation of anti-inflammatory androgen-sensitive gene STAMP2, which was also stimulated by LPS, but to a much lower extent. Sex steroids and aging have a profound influence in regulating the immune and inflammatory system (Gubbels Bupp, 2015). Despite the fact that one of the most relevant attributes of immunosenescence is an aberrant chronic low-grade pro-inflammatory state (called "Inflamm-Aging"), which occurs to a greater extent in females, menopause is associated with a decline in certain lymphocyte subsets, indicating an

extremely complex scenario (Klein and Flanagan, 2016). Systemic chronic inflammation (Gameiro et al., 2010), changes in the immune response (Ghosh et al., 2014) and low sex steroids levels (Traish et al., 2018) probably contribute to vulvo-vaginal atrophy (VVA) and lower urinary tract symptoms (LUTS) which are the hallmarks of the Genitourinary Syndrome of Menopause (GSM). Due to the fact that young women are at a higher risk of developing autoimmune diseases and that multiple immune parameters have long been known to be estrogen-responsive, until now menopause research has focused primarily on the effects of estrogen deprivation and replacement on the immune system (Ghosh et al., 2014). Surgical menopause was found to alter immune cells profile in the peripheral blood, whereas estrogen replacement therapy reversed these alteration inducing a decrease in the CD8+ cells and increase in CD19+ cells and IFNγ concentration (Kumru et al., 2004). On the other hand, estrogen replacement reduced the elevated plasma levels of the pro-inflammatory cytokines TNFα, IFNγ and IL6 detected in blood from postmenopausal women (Vural et al., 2006). In animal models, SERM treatment has been found to exert a direct effect on the secretion of antimicrobial chemokines by mouse uterine epithelial cells (Fahey et al., 2011). Conversely, the potential immunoregulatory effect of androgens in women, in particular in the genital tract, has not been investigated so far.

In our study, a significant inhibitory effect of DHT was observed in the expression of many inflammatory mediators in the human vagina following IFNγ-triggered pathways. In fact, pre-treatment with DHT counteracted the expression of iNOS, COX2, MCP1, IL12A and IP10, which are involved in amplifying and sustaining inflammatory states. In particular, a dramatic induction in the expression of IP10 (IFNγ-induced Protein) was observed with IFNγ treatment, which was significantly reduced by DHT. IP10 is a chemokine involved not only in T-lymphocyte, monocyte and neutrophil chemotaxis, T-cell adhesion to endothelial cells, enhancement of NK cell migration and cytolysis, but also in chronic inflammatory conditions in vascular disease (Neville et al., 1997). In this

regard, while acute inflammation is a limited beneficial response, particularly useful during infectious challenge, chronic inflammation is a persistent phenomenon that can lead to tissue damage, fibrosis, hyperalgesia and allodynia (Gabay et al., 2006). At some point, development of adequate anti-inflammatory mechanisms is necessary to promote remodeling and retain homeostasis; otherwise, non-resolving inflammatory processes may develop and induce pathological consequences, contributing to a variety of systemic and local diseases such as atherosclerosis, cancer, autoimmune disorders (Atri et al., 2018) and genitourinary tract diseases, including Chronic pelvic pain syndrome (CPPS) and bladder pain syndrome/interstitial cystitis (BPS/IC) (Grover et al., 2011; Thompson, 2005). As abovementioned, pleiotropic immunosuppressive role of androgens on key components of both innate and adaptive immunity has been consistently described in recent literature in preclinical and clinical models in males (see Trigunaite et al., 2015 for a review). For example, *in vitro* T treatment of isolated mouse macrophages elicited a decrease in TLR4 expression and sensitivity to a specific ligand; furthermore, macrophages isolated from orchiectomized mice had significantly higher TLR4 cell surface expression than those derived from controls, and most importantly, these effects were reverted by *in vivo* T treatment (Rettew et al., 2008). With regard to the effect of androgen on the peripheral T cell population, treatment with androgens *in vitro* resulted in inhibition of Th1 differentiation and less IFNγ production in mouse models and prostate cancer patients undergoing androgen deprivation therapy (Kissick et al., 2014). Noteworthy, in experimental autoimmune encephalitis (EAE), a mouse model of multiple sclerosis, T directly promoted Th2 differentiation and production of IL10 in order to suppress the overactive Th1 response associated with this disease (Hepworth et al., 2010). Since Th2 response (including the M1/M2 switch) has a modulator purpose, negatively regulating proinflammatory cytokines, and inducing production of anti-inflammatory mediators such

as IL10 and TGFβ, this suggests that androgens' action converge to the resolution of inflammation.

Our data are consistent this view and appear innovative in that, for the first time, they expand the paradigm of the immunosuppressive effects of androgens to females and specifically to the vagina, an organ which is constantly exposed to infections. Confirming the results observed with LPS stimulation, that mimicked the first phases of the host reaction, DHT treatment in hSMCs significantly counteracted IFNγ-induced secretion of cytokines, chemokines and growth factors (IL1β, IL2, IL5, IL6, MCP1 and GCSF). DHT also significantly reduced the surface expression of HLA-DR, an MHC class II receptor that acts presenting peptide antigens to professional immune cells with the aim of eliciting eventual production of antibodies. The contrasting effect exerted by DHT on IFNγ-activated pathways is of major importance, since IFNγ is the key effector cytokine of Th1 immunity.

The mechanisms by which androgen signaling exerts its anti-inflammatory effects are not completely understood. In previous studies in human BPH cells, we demonstrated that DHT inhibits NF-kB activation, a master transcription factor in inflammation, as evidenced by its reduced nuclear translocation (Vignozzi et al., 2012). The same effect was subsequently described in a modified adipocyte chronic inflammation model, in which DHT-AR binding suppressed TNFα-induced MCP1 and IL6 expression via a mechanism involving NF-kB (Morooka et al., 2016). Similarly, in the present study, inhibition of NF-kB nuclear translocation by DHT was detected in LPS-stimulated vaginal rSMCs and hSMCs.

5. Conclusions

In conclusion, our data demonstrate that DHT exerts an immune regulatory role on vaginal SMCs, inhibiting their potential to actively induce and/or sustain immune and inflammatory responses. The vagina is a unique immunocompetent organ that must maintain a vigilant and vigorous immunity against pathogenic intruders, but also an environment favorable to the reproductive process. This delicate balance is accomplished by a complex interplay of local humoral, cell-mediated, and innate immunity. The epithelium, vaginal secretions rich in antimicrobial molecules, and the commensal microbiota have traditionally been considered as the main components of vaginal innate immunity (Farage et al., 2011); hereby, we demonstrate that vaginal SMCs have also the ability to be involved in the immune response, secreting several proinflammatory cytokines and recruiting and activating cell-mediated immunity. Although beneficial to the host under most conditions, this immune competence might result in non-resolving processes that, with the contribution of aging and sex steroids deprivation, leads to the development of VVA and LUTS, the hallmarks of GSM. Interestingly, our data indicate that androgen signaling is able to restrain inflammation in the vagina, representing a potential therapeutic strategy in these conditions.

The positive role of androgens in the maintenance of vaginal health in terms of trophism, vascularization, nociception and general functionality has been receiving increasing attention in the literature (Traish et al., 2018); their immunomodulatory effects seem also promising and should be further investigated.

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