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Prenatal exposure to testosterone masculinises the female gerbil and promotes the development of lesions in the prostate (Skene's gland)

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Abstract. Androgenic imbalance may disrupt prostate development, leading to morphological alterations in adulthood and predisposing this gland to develop diseases during ageing. However, little is known about the endocrine disruption of the prostate that is caused by androgenic compounds, especially in female experimental models. Therefore, this study aimed to evaluate the prostates of aged female gerbils exposed to testosterone at certain periods in intrauterine and postnatal life, to determine whether exposure at a particular age increases susceptibility to prostatic lesions in these animals. To this end, morphological, stereological, immunohistochemical and immunofluorescence analyses were employed. It was found that females exposed to testosterone during intrauterine life were masculinised, showing increased anogenital distance, absence of the vaginal opening and ectopic development of prostatic tissue. Several areas of adenomatous hyperplasia, generally associated with inflammatory foci and mainly located in the ectopic prostatic tissue around the vaginal wall, were also observed. In conclusion, the results showed that abnormal prenatal exposure to testosterone severely affects the reproductive systems of female animals by disrupting normal prostate morphogenesis and increasing susceptibility to the development of prostatic diseases during ageing.

Additional keywords: androgens, endocrine-disrupting chemicals.

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Introduction

Prostate morphogenesis is a process highly dependent on androgenic action during both the prenatal and postnatal periods. Steroids such as testosterone, the principal androgen secreted by the fetal and adult testes, play a central role during prostate formation (Wilson 2011). Early events in prostate development are very sensitive to testosterone imbalance, since this androgen, acting through its receptor (AR), is responsible for the expression of more than a hundred genes (Black and Pashal 2004). The first manifestation of the presence of ARs appears during the prenatal period, when testosterone produced by the Leydig cells acts in the periurethral mesenchyme of the urogenital sinus (UGS; Takeda *et al.* 1985), leading to a cascade of paracrine signalling from the mesenchymal cells to the epithelial compartment.

The UGS is an undifferentiated embryonic structure with an endodermic origin (Staack *et al.* 2003). It is found in male and female mammals of several species during an undifferentiated stage of embryonic life; in humans, this stage occurs during the 7th week of gestation (Wilhelm and Koopman 2006). The UGS is in charge of prostate development, a process that begins with determination of the cells, followed by initiation and budding, branching morphogenesis, differentiation and maturation of the gland (Prins and Putz 2008). Together, these events are responsible for normal prostate morphogenesis. The process of prostate formation encompasses complex cascades of signalling (Thomson 2008; Timms 2008; Meeks and Schaeffer 2011; Wilson 2011) that are precisely regulated spatially and temporally, so that certain kinds of interference with this homeostasis may be detrimental to normal prostate organogenesis,

predisposing the gland to develop lesions with ageing (Schaeffer *et al.* 2008; Timms and Hofkamp 2011; Biancardi *et al.* 2012).

Lately, a growing body of evidence has shown a close relationship between abnormal processes in prostate development and susceptibility to the development of prostate disorders during ageing (Schaeffer *et al.* 2008; Perez *et al.* 2011; Timms and Hofkamp 2011; Biancardi *et al.* 2012). Taken together, this evidence has drawn researchers' attention to the initial stages of prostate development, considering that this period of life has a profound effect on the formation of this gland. Furthermore, a better understanding of these events may be helpful since the early events of prostate organogenesis are very similar to events seen in disease development in adult or aged patients (Schaeffer *et al.* 2008; Timms and Hofkamp 2011).

Although several studies have shown the influence of endocrine-disrupting chemicals (EDCs) with oestrogenic potential on the reproductive system (Söder 2005; Timms *et al.* 2005; Prins *et al.* 2008; Perez *et al.* 2011), little is known about the roles of EDCs with regard to androgenic potential and their mechanisms of action on the reproductive tract and specifically on the prostate (Biancardi *et al.* 2012; Perez *et al.* 2012). Besides, several conditions such as polycystic ovary syndrome (PCOS), adrenal hyperplasia and exposure to certain drugs may increase the serological concentrations of androgenic compounds, which may be harmful during critical periods of prenatal development. This changed hormonal environment may cause irreversible interference during prostate development, increasing the likelihood that the individual will develop prostatic lesions as an adult or aged person (Schaeffer *et al.* 2008; Biancardi *et al.* 2012).

Female gerbils (*Meriones unguiculatus*) have been employed as an experimental model with increasing frequency lately, mainly because most of them develop a functional prostate (Skene's gland; Santos and Taboga 2006; Santos *et al.* 2006) similar to that found in humans (Zaviačič 1999). In addition, these females furnish valuable data in experiments concerning drug administration (Perez *et al.* 2011; Biancardi *et al.* 2012; Zanatelli *et al.* 2013). These factors have alerted us to the usefulness of the female gerbil as a model for the early stages of prostate development, since new evidence regarding these stages may be very helpful to our understanding of the complex events that regulate both normal and abnormal prostate growth. Based on this evidence, and considering that normal prostatic morphogenesis is highly dependent on precise androgenic regulation, our hypothesis was that abnormal exposure to testosterone would affect prostate development in females, predisposing them to developing lesions in old age. Thus, the aim of this study was to evaluate the interferential effects of exogenous testosterone exposure during the prenatal and pubertal periods on the morphophysiology of the prostate in aged female gerbils.

Materials and methods

Animals and experimental design

The animals were provided by the São Paulo State University (UNESP; São José do Rio Preto, Brazil), maintained in polyethylene cages under controlled conditions of light and temperature, and provided with filtered water and rodent food *ad libitum*. Animal handling and experiments were performed according to the ethical guidelines of the São Paulo State University (ethical committee number 021/09 CEUA) and in keeping with the Guide for Care and Use of Laboratory Animals. During all experiments we provided filtered water in glass bottles to avoid exposing the animals to endocrine-disrupting chemicals such as bisphenol A from plastic bottles.

We selected 20 adult (3–4 months old) female and 20 adult male gerbils (*Meriones unguiculatus*, Muridae: Gerbillinae) for mating. We paired each male with one female at random to form independent families. We assigned five couples to each group. The pregnant females of these couples underwent different manipulations and their offspring formed the experimental groups as follows: C (control) group, offspring from non-treated pregnant females; C + T (testosterone during puberty) group, offspring from non-manipulated pregnant females (littermates of the C group) treated with subcutaneous injections of 100 μg of T (testosterone cypionate, Deposteron; EMS, Hortolândia, São Paulo, Brazil) diluted in 100 μL of mineral oil during the 6th, 7th and 8th weeks of life; TG (testosterone during gestation) group, offspring from mothers exposed to subcutaneous injections of 500 μg of T during gestation and TG + T (testosterone during gestation plus puberty) group, offspring from mothers exposed to subcutaneous injections of 500 μg of T during gestation that were also exposed to subcutaneous injections of 100 μg of T during the 6th, 7th and 8th weeks of life. Of the pups exposed to prenatal testosterone, only those born four days after exposure were employed in this study. All animals utilised in this study were killed at one year of age. The protocol of T treatment was adapted from that of Wolf *et al.* (2002). The overall experiment design, including details of T treatment and the age at which the animals were killed, is shown in Fig. 1.

All animals were killed by CO_2 inhalation followed by decapitation. According to the method of Nishino and Totsukawa (1996), we killed the females in the C and C + T groups at the proestrus phase. The females in the TG and TG + T groups never cycled due to their lack of a vaginal opening. Body, prostatic complex (PrC – urethra, vagina and prostate structures), ovaries and adrenals were weighed. These fragments were dissected out using a Leica stereoscopic microscope (Leica, Wetzlar, Germany) to remove adipose tissues and isolate the urethral segment plus the associated prostatic tissue. The anogenital distance (AGD) was measured with a digital caliper rule. For the characterisation and confirmation of the female prostate lesions we consulted a pathologist, who helped us with the pathological diagnoses of the prostate disorders.

Light microscopy

PrC from female gerbils were fixed by immersion in 4% paraformaldehyde (buffered in 0.1 M phosphate, pH 7.2) or in methacarn (proportions: 60% methanol, 30% chloroform and 10% acetic acid) for three hours. After fixation, the tissue was washed in water, dehydrated in ethanol, clarified in xylene and embedded in paraffin (Histosec; Merck, Darmstadt, Germany). All tissue fragments employed in this study were serially sectioned into 5-μm slices with an automatic rotator microtome

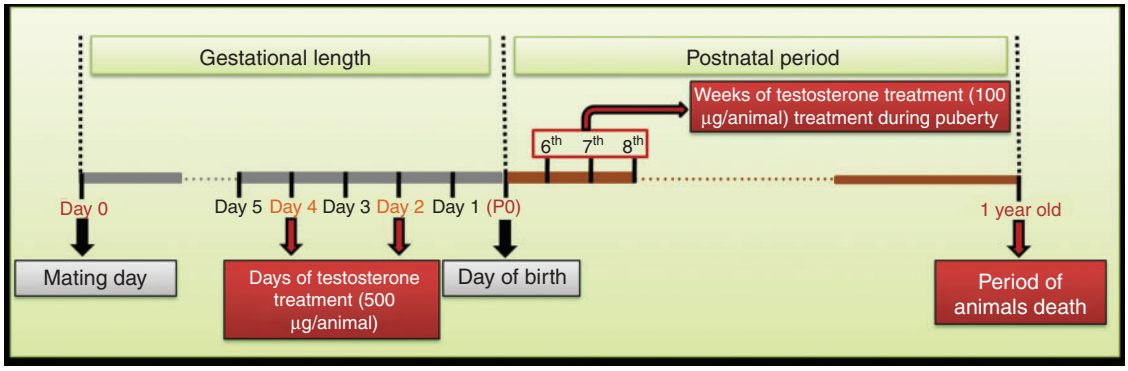

Fig. 1. Schematic representation of the experimental protocol employed in this study. The interval between Day 0 (day of mating) and Day P0 (day of birth) represents the gerbil gestational period. Days 2 and 4 represent the prenatal days of testosterone treatment (groups TG and TG + T). 6th, 7th and 8th represent the pubertal weeks in which the animals were treated once per week with testosterone (groups C + T and TG + T). The end of the timeline (one year old) represents the animals' age at death.

(Leica RM2155; Leica, Nussloch, Germany). The sections were stained with haematoxylin–eosin (HE) and picrosirius for general morphological analysis. Prostatic reticular fibres and elastic fibres were identified, respectively, by the Gömöri's reticulin and resorcin–fuchsin techniques. The specimens were analysed with an Olympus BX60 light microscope (Olympus, Tokyo, Japan) and the images were digitalised using DP-BSW software Version 3.1 (Olympus) and a virtual slide system (BX 61VS; Olympus).

Stereology

The stereological analyses were carried out using Weibel's multipurpose graticulate with 130 points and 10 test lines (Weibel 1963) to compare the relative proportion (relative volume) of each component of prostatic tissue (epithelium, lumen and muscle and non-muscle stroma) as described by Huttunen *et al.* (1981). We chose thirty microscopic fields at random from each experimental group (six fields per animal; $n = 5$). Briefly, we determined the relative values by counting the coincident points in the test grid and dividing them by the total number of points. Stereological analysis was performed using Image-Pro Plus software Version 6.1 for Windows (Media Cybernetics Inc., Silver Spring, MD, USA).

Immunohistochemistry

Tissue sections were subjected to immunohistochemistry for the detection of androgen receptor (AR), as described in protocols applied to the prostate (adapted from Cordeiro *et al.* 2008), oestrogen receptor-α (ERα) and p63 protein. Primary antibodies reactive to AR (rabbit polyclonal IgG; Santa Cruz Biotechnology, Santa Cruz, CA, USA), ER-α (rabbit polyclonal IgG; Santa Cruz Biotechnology) and p63 (mouse monoclonal IgG_{2a}; Santa Cruz Biotechnology) were employed at a dilution of 1 : 100. Polymers (Post-Primary Block and Polymer; Novocastra, Newcastle Upon Tyne, UK or DAKO Envision + Dual-link system-HRP; DAKO, North America, Inc., Carpinteria, CA, USA) were used as secondary antibodies, according to the procedures described by the manufacturers. The sections were stained with diaminobenzidine and counterstained with Harris's haematoxylin. The histological sections were analysed using an Olympus BX60 light microscope (Olympus).

Immunofluorescence

Tissue sections were subjected to immunofluorescence for the detection of smooth-muscle α-actin by means of incubation with mouse monoclonal IgG_{2a} (Santa Cruz Biotechnology) at a dilution of 1 : 100 overnight. The next morning, fluorochrome-conjugated specific second antibodies (anti-mouse IgG-FITC; Santa Cruz Biotechnology) were used as secondary antibodies over two hours at room temperature. DAPI (4',6-diamidino-2-phenylindol) was employed to allow the visualisation of the cells' nuclei. The histological sections were analysed with a Zeiss Imager M2 fluorescence microscope (Zeiss, Göttingen, Germany), and laser-scanning microscope (LSM 710; Zeiss, Jena, Germany).

Statistical analyses

The hypothesis tests employed to determine statistical significance were the Kruskal–Wallis test for non-parametric distributions and ANOVA for parametric distributions. Further localisation of the statistically significant differences between experimental groups was performed using Student–Newman–Keuls's test for non-parametric distributions and Tukey's test for parametric distributions. The data were analysed using Statistica 6.0 (StarSoft, Inc., Tulsa, OK, USA) and BioEstat 5.0 (http://www.mamiraua.org.br/pt-br/downloads/programas/) software. The level of significance was set at 5% ($P \leq 0.05$). Values are presented as mean ± standard error of mean (s.e.m.).

Results

Biometry

Both the TG group and the TG + T group had enlarged PrCs. The TG group exhibited a mean PrC weight significantly different from those of the C and C + T groups, whereas the TG + T group exhibited a mean PrC weight significantly different from that of the C group only (Table 1). In addition, total bodyweight and the anogenital distance (AGD) were significantly increased in the TG and TG + T groups when compared with the C and C

Table 1. Biometrical data of female gerbils

$n = 6$ animals per group; mean ± s.e.m. [a,b,c]Superscript letters represent statistically significant differences ($P \leq 0.05$) between the experimental groups; values labelled with the same letters are not significantly different

Variable	Group			
	C	C + T	TG	TG + T
Bodyweight (g)	62 ± 2.37	65.67 ± 2.03	68.67 ± 1.61	60.80 ± 2.58
Prostatic complex (PrC) weight (g)	0.103 ± 0.01[a]	0.122 ± 0.01[a,c]	0.243 ± 0.03[b]	0.223 ± 0.06[c,b]
Relative weight[A] ($\times 10^{-3}$)	1.6 ± 0.10[a]	1.9 ± 0.14[a]	3.6 ± 0.49[b]	3.7 ± 1.10[b]
Ovary weight (g)	0.04 ± 0.050	0.045 ± 0.004	0.046 ± 0.009	0.037 ± 0.011
Adrenal weight (g)	0.037 ± 0.003	0.038 ± 0.002	0.039 ± 0.003	0.041 ± 0.002
Anogenital distance (AGD; mm)	1.96 ± 0.15[a]	2.22 ± 0.07[a]	4.05 ± 0.35[b]	4.06 ± 0.27[b]

[A]Relative weight corresponds to the ratio between the weight of the prostate and that of the whole body.

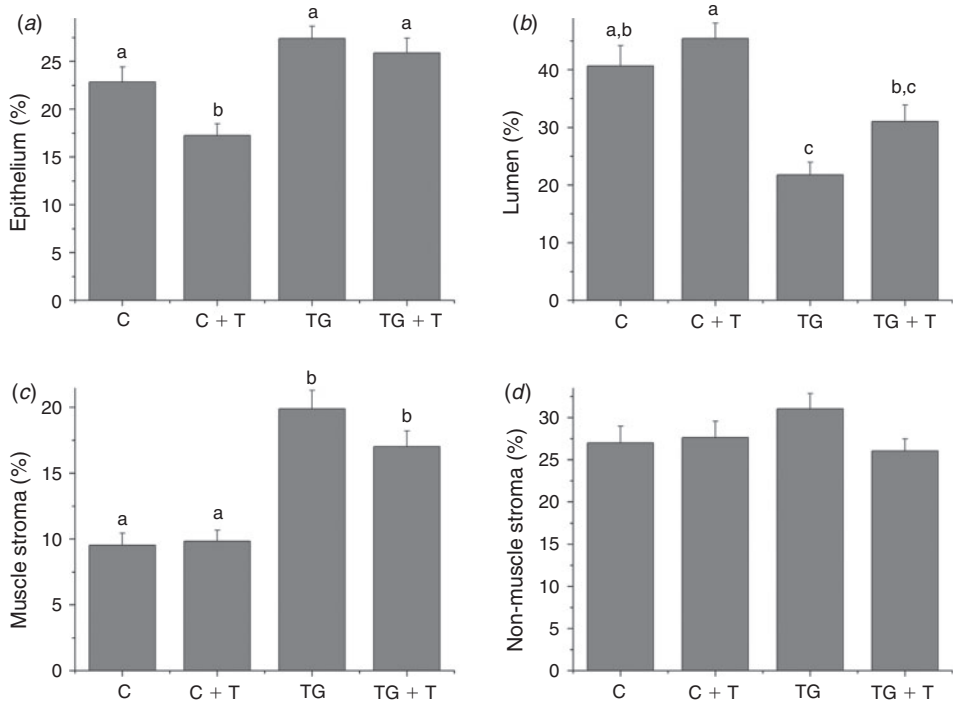

Fig. 2. Stereological analysis of prostate compartments (epithelium, lumen, muscle stroma and non-muscle stroma). Values represent the mean ± standard error of mean (s.e.m.). Superscript letters ([a,b,c]) represent statistically significant differences ($P \leq 0.05$). Values labelled with the same letters are not significantly different. The values are expressed as percentages (%) and represent the relative volume of prostate compartments in each experimental group. $n = 5$ animals per group.

+ T groups. The other variables studied were not significantly different between the experimental groups.

Stereology

The epithelial relative volume was decreased in the prostates of C + T females (Fig. 2*a*). The luminal relative volume, on the other hand, was decreased in the prostates of the TG and TG + T groups, although the statistical analysis revealed that only the TG group was significantly different from the others (Fig. 2*b*). The muscle relative volume was significantly increased in the prostates of TG and TG + T females compared with C and C + T (Fig. 2*c*). We did not observe any statistically significant difference in non-muscle stromal compartments between the groups (Fig. 2*d*).

General anatomical aspects and morphology of the PrC

In addition to the absence of a vaginal opening (data not shown) and an increase in the AGD, we also observed hydrometrocolpos in the PrC of the TG and TG + T groups. In such cases, the PrC contained a large vaginal cavity filled with an inflammatory secretion inside the uterus and vagina (Fig. 3*c*, *d*).

The prostates of the C and C + T groups showed normal anatomical localisation, being located in a lateral position

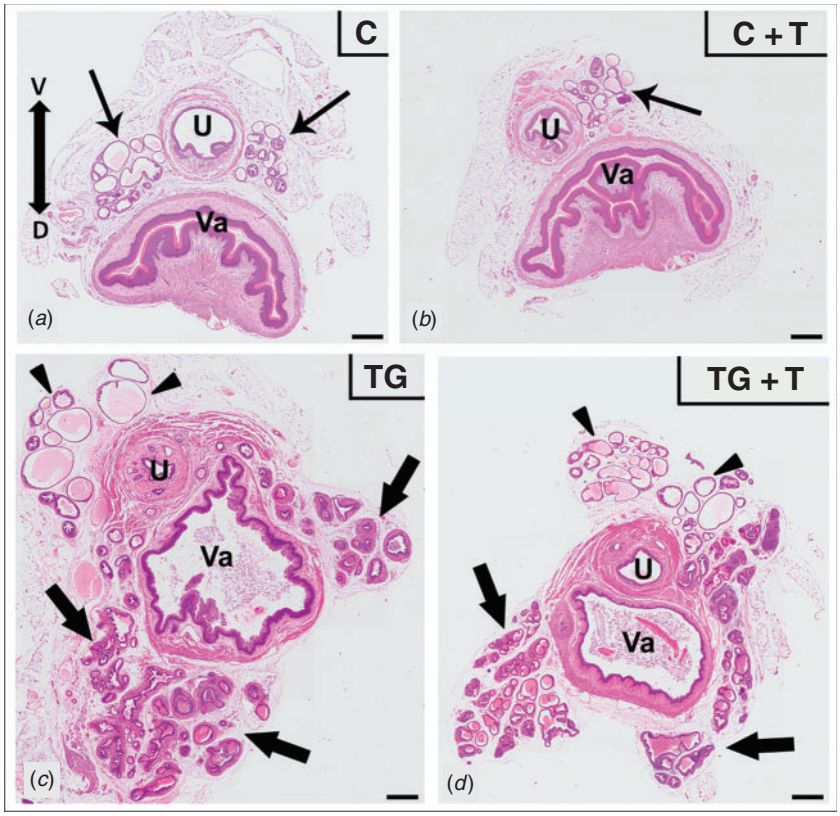

Fig. 3. General histological view of the female urogenital tract in all experimental groups. (*a*, *b*) Representative example of the female urogenital tract in the (*a*) C and (*b*) C + T groups, showing normal paraurethral localisation of the prostate in the female gerbil. (*c*, *d*) In the (*c*) TG and (*d*) TG + T groups, in contrast, we observed the formation of a dorsolateral prostate around the vaginal wall, in addition to the formation of abnormal prostate acini above the urethra. V, ventral; D, dorsal; U, urethra; Va, vagina; arrows, paraurethral prostatic acini; large arrows, ectopic prostate around the vaginal wall; arrowhead, abnormal prostate acini at the ventral localisation.

relative to the urethra (Fig. 3*a*, *b*). In TG and TG + T females, on the other hand, in addition to the normal paraurethral glands, the urogenital tract exhibited ectopic formation of prostate tissue surrounding the vaginal wall (Fig. 3*c*, *d*). We could observe a distinct difference between the glands located in the normal paraurethral positions and the ectopic glands located around the vaginal wall (Fig. 3*a*–*d*). The prostates of all experimental groups showed glands with large paraurethral acini, flattened epithelia and normal stromal compartments (Fig. 3*a*–*d*). In the TG and TG + T groups, however, the ectopic glands surrounding the vaginal wall had distinct histological aspects (Fig. 3*c*, *d*). These glands presented ectopic acini, normally composed of a multilayered epithelium, and an unusually thick stromal compartment (Fig. 3*c*, *d*). Moreover, we observed the formation of abnormal prostate tissue above the urethra in females from the TG and TG + T groups (Fig. 3*c*, *d*).

Morphological aspects of the prostate and the association with lesions

Prostates in the C and C + T groups showed normal histological aspects, being characterised by acini composed of a simple columnar epithelium, rich in secretions, and surrounded by a stromal compartment composed mainly of fibroblasts, smooth-muscle cells and fibres such as collagen and elastin (Fig. 4*a*–*h*). In addition, we also found some foci of prostatic disorders, such as hyperplasia, in the glands of the C and C + T groups (Fig. 4*c*, *g*). On the other hand, in addition to the normal paraurethral glands, the urogenital tracts of the TG and TG + T groups presented ectopic glands severely affected by prostatic lesions characterised by adenomatous hyperplasia (Fig. 4*i*–*o*). These glands showed abnormal acinar architecture, normally with an invasive aspect, atypical cell nuclei and generally associated with several inflammatory foci (Fig. 4*i*–*o*). Along with these aspects, prostates in the TG and TG + T groups presented increased stromal compartments and greatly increased fibromuscle compartments (Figs 3*c*, *d* and 4*i*, *m*). All experimental groups contained prostates with normal secretory function, as determined according to the Periodic Acid-Shiff (PAS) technique (Fig. 4*d*, *h*, *l*, *o*).

The C and C + T groups showed normal reticular, general collagen and elastic fibre components of the stromal compartments (Fig. 5*a*, *b*, *e*, *f*, *i*, *j*). The TG and TG + T groups, on the other hand, showed abnormal stromal compartments,

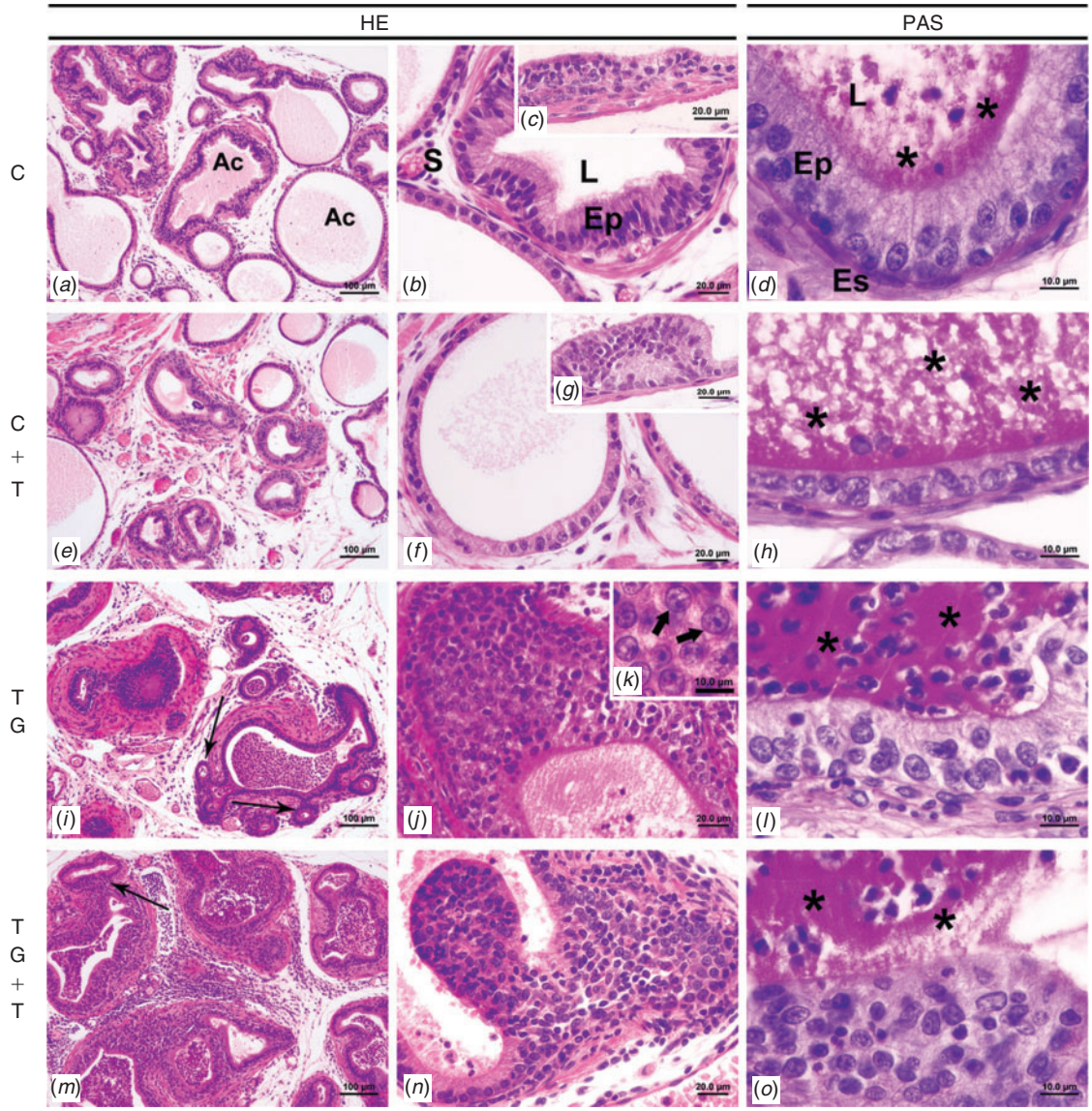

Fig. 4. Morphological characterisation of the prostate by the HE and PAS techniques. (*a–h*) Morphological aspects of C and C + T prostates, showing (*c*, *g*) some hyperplasic areas. (*i–o*) Morphological aspects of prostates from TG and TG + T groups; these show more severely injured glands affected by adenomatous hyperplasia. We observed several invasive prostatic acini in addition to the large number of areas with inflammation foci in prostates in the TG and TG + T groups. (*d*, *h*, *l*, *o*) The pattern of secretion was similar between all experimental groups. Ac, acini; Ep, epithelium; L, lumen; S, stroma; arrows, invasive acini characteristic of adenomatous hyperplasia; large arrows, vesicular nuclei present in adenomatous hyperplasia; asterisk (*), glycoprotein secretion stained by PAS.

mainly in the form of glands with ectopic localisation around the vaginal wall. Gömöri's reticulin technique showed an intense stromal reshuffling of collagen III around the affected acini, with some regions lacking these elements, mainly in invasive acini (Fig. 5*c*, *d*). The general collagen, in addition to an intense architectural reshuffling, had undergone a considerable increase in the stromal compartment (Fig. 5*g*, *h*). We also observed an abnormal increase in the thickness of the elastic components surrounding the acini in the TG and TG + T prostates (Fig. 5*k*, *l*) in comparison with prostates in the C and C + T groups (Fig. 5*i*, *j*).

Immunohistochemical analyses

AR identification was similar for all experimental groups, showing up as an intense mark in both the epithelial and stromal compartments (Fig. 6*a–d*). AR immunolocalisation was strong in the nucleus for both epithelial and stromal cells (Fig. 6*a–d*). Some foci of prostatic lesions appeared as multilayered epithelia in which most cells positively expressed AR (Fig. 6*c*, *d*).

Regarding ERα immunolocalisation, we observed these receptors to have similar localisations in the prostatic stromal compartment in all experimental groups (Fig. 6*e–h*). We did, however, observe an abnormal immunolocalisation of p63

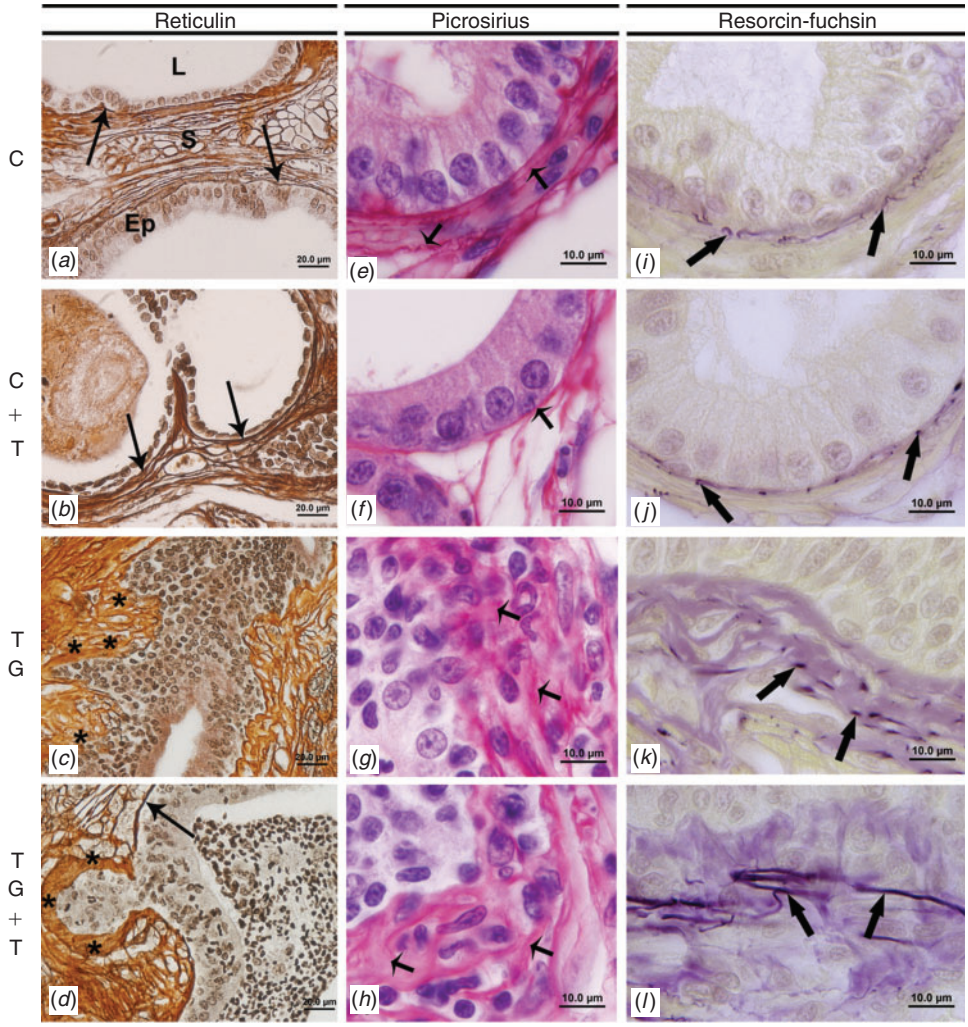

Fig. 5. Gömöri's reticulin, picrosirius and resorcin–fuchsin techniques for identification of (*a–d*) reticular fibres (collagen III), (*e–h*) general collagen and (*i–l*) elastic fibres. (*a*, *b*) Regular localisation of reticular fibres adjacent to prostatic epithelium in the (*a*) C and (*b*) C + T groups. (*c*, *d*) The TG and TG + T groups, on the other hand, showed a intense reshuffling of these fibres, mainly in regions affected by lesions. (*g*, *h*) Regarding general collagen, prostates in the TG and TG + T groups exhibited a drastic reshuffling of these fibres, especially in injured regions. (*k*, *l*) Regarding the elastic system, we observed several regions with a changed pattern of these stromal components in both the TG and TG + T groups, showing an increase in elastic-fibre thickness. Ep, epithelium; L, lumen; S, stroma; arrows, reticular fibres; short arrows, general collagen; large arrows, elastic fibres; asterisk (*), regions lacking reticular layer.

protein (Fig. 6*i–l*) in some regions of the prostate in the TG and TG + T groups, mainly in the acini that had developed ectopically around the vaginal wall (Fig. 6*k*, *l*). Between the abnormalities, we observed a changed pattern of disposal in the basal layer (Fig. 6*k*, *l*), making it possible for p63-positive cells to be localised inside lesions (Fig. 6*k*).

Immunofluorescence analyses

Immunofluorescence analyses for smooth-muscle α-actin confirmed what we had observed through cytochemical approaches (Fig. 7*a–h*). Besides the increase in the smooth-muscle layer (Fig. 7*e–h*), the glands in the TG and TG + T groups exhibited invasive acini with an interrupted smooth-muscle layer in some proliferative regions affected by lesions (Fig. 7*f*, *h*).

Discussion

This study showed that prenatal exposure to exogenous testosterone may severely disturb the normal process of urogenital development in female gerbils, leading to permanent masculinisation, hydrometrocolpos and the formation of ectopic prostate tissue around the vaginal wall. In addition, the ectopic tissue found in females of the TG and TG + T groups presented several foci of inflammation, which were generally associated with proliferative areas often characterised by adenomatous

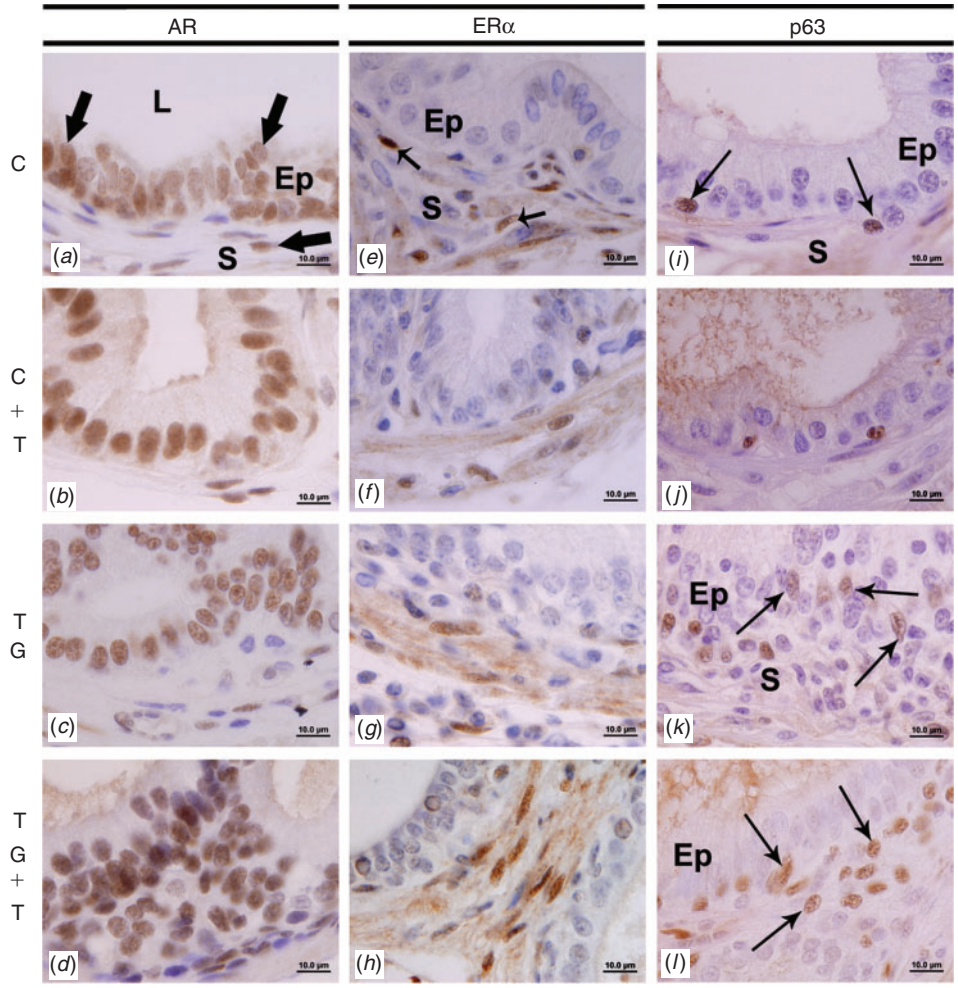

Fig. 6. Immunohistochemical analysis of AR, ERα and p63 protein in the female prostate. (*a–d*) AR immunolocalisation in both epithelial and stromal compartments of the prostate in all experimental groups. (*e–h*) Stromal expression of ERα, which was similar in all experimental groups. (*i–l*) Immunolocalisation of p63 protein in the basal-cell population in the prostate epithelium. We observed that the regularity of this cellular layer was lost in some injured areas of the prostate in the (*k*) TG and (*l*) TG + T groups. Ep, epithelium; L, lumen; S, stroma; arrows, p63-positive cells; large arrows, AR-positive cells, small arrows, ERα-positive cells.

hyperplasia. These findings show that the prenatal period of urogenital development is a critical period for female prostate morphogenesis in gerbils, in which development is highly sensitive to external androgenic interference. Additionally, the study showed that gerbil urogenital development in puberty is less sensitive to external interference by androgenic stimulation, demonstrating that the potential for the prostate to develop differently in response to external factors is lost with ageing.

Although a previous study by Welsh *et al.* (2008) has already demonstrated the appearance of prostates in female rats prenatally exposed to testosterone, the present study, which is a continuation of previous work by our research group (Biancardi *et al.* 2012; Perez *et al.* 2012), focussed on the effects of *in utero* exposure to testosterone on the prostate gland. In this sense, the present work, in addition to showing the development of ectopic prostate tissue in female gerbils exposed to testosterone *in utero*, analysed other important aspects, such as the manifestation of adenomatous hyperplasia, a novel aspect that has not been demonstrated in other studies employing similar methodologies.

Through biometrical analyses, our data showed that the TG and TG + T experimental groups were significantly different from the others only in PrC weight, relative weight and AGD. The increase in prostatic-complex weight in the reproductive tracts of the TG and TG + T groups occurred mainly due to the development of hydrometrocolpos, an anomaly characterised by the retention of secretions inside the uterus and vagina (Wolf *et al.* 2002). The formation of new prostatic tissue surrounding the vaginal wall also contributed to the increase in prostatic-complex weight in TG and TG + T females. Although prostatic-complex weight was not significantly different between the TG + T and C + T groups, there was an notable increase in this value in TG + T females, whereby the value came close to that observed in the TG group. This fact was confirmed by comparing the relative weight between the groups, which showed a

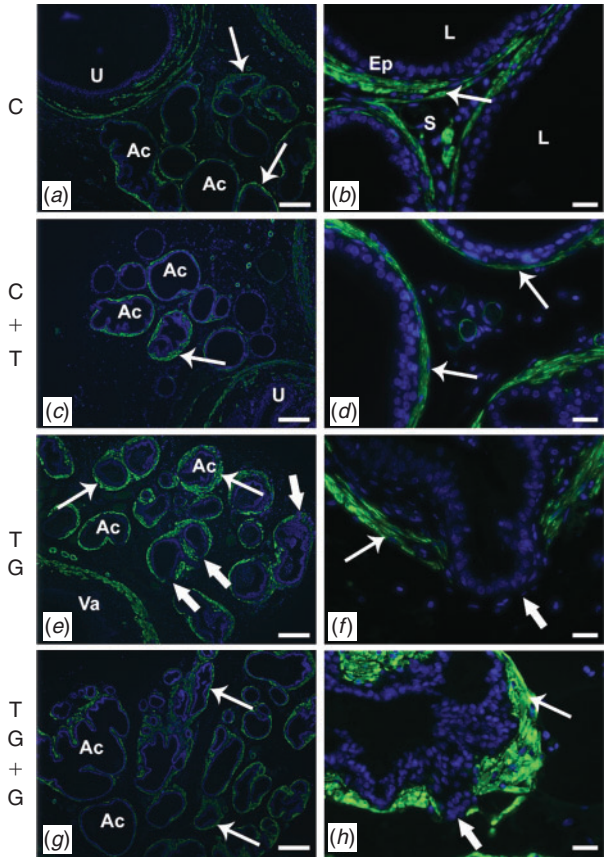

Fig. 7. Immunofluorescence for smooth-muscle α-actin. (*a–d*) Normal pattern of smooth muscle surrounding the epithelium in prostates from the (*a*, *b*) C and (*c*, *d*) C + T groups. (*e–h*) Note the increased thickness of the smooth-muscle layer (SML) in prostates in the (*e*, *f*) TG and (*g*, *h*) TG + T groups as well as the proliferative regions lacking adjacent SML. Ac, acini; Ep, epithelium; L, lumen; S, stroma; U, urethra; arrows, smooth-muscle layer; large arrows, regions lacking smooth muscle. (*a*, *c*, *e*, *g*) Scale bar = 200 μm. (*b*, *d*, *f*, *h*) Scale bar = 20 μm.

statistically significant increase in the TG and TG + T groups in comparison with C and C + T.

The tendency towards hydrometrocolpos in female TG and TG + T gerbils is indicative of the effects of exogenous testosterone upon urogenital development. According to the literature, hydrometrocolpos is a rare condition characterised by the retention of liquid inside the vagina and uterus due to congenital vaginal obstruction (Khan *et al.* 2007). Our data showed that exogenous testosterone impaired the vaginal opening process, which led to the retention of secretions inside the vagina and uterus, leading to a chronic inflammatory response. It is likely that this inflammation reached the prostatic tissue around the vaginal wall, inducing proliferation and contributing to the development of adenomatous hyperplasia of the prostate.

Regarding the AGD, some previous studies employing female rats have shown that prenatal exposure to testosterone promotes increase of the AGD (Wolf *et al.* 2002; Hotchkiss *et al.* 2007*a*, 2007*b*), which is a parameter that indicates masculinisation in the female. Indeed, the employment of AGD could provide a non-invasive method of predicting neonatal and adult reproductive disorders (Welsh *et al.* 2008), being a useful technique to assess suspected effects of masculinisation during gestation. Although prenatal exposure to testosterone did promote an increase in AGD in the TG and TG + T groups, we did not observe any variation in this parameter in the C + T group compared with the C group, which suggests that the prenatal period is more sensitive to the masculinising effects of testosterone than the pubertal period.

Regarding the time-window for masculinisation in gerbils we are unable to precisely define the events of this programming since there is a lack of information in the literature for the gerbil. Thus, for the present study we adapted the treatment protocol based on previous studies reported in literature on the time-window for masculinisation described for rats (Wolf *et al.* 2002; Hotchkiss *et al.* 2007*a*, 2007*b*; Welsh *et al.* 2008).

Our stereological analyses showed that females exposed to testosterone during the prenatal period (TG and TG + T) exhibited the most significant alterations of the prostate, mainly related to the lumen and stromal compartments. These animals typically presented glands with reduced lumina and increased smooth-muscle layers. In fact, these characteristics were predominant in the ectopic tissues (dorsolateral prostate) around the vaginal wall. These results were very similar to those of previous studies by our research group, which showed the same tendency towards luminal reduction and increased smooth muscle in the prostate stromal compartment of adult female gerbils exposed to testosterone cypionate during the prenatal period (Biancardi *et al.* 2012).

Recent studies have shown that one of the events that occurs during benign prostatic hyperplasia (BPH) is the epithelial–mesenchymal transition (EMT), in which epithelial cells differentiate into mesenchymal-like cells and further into myofibroblasts and smooth-muscle cells, resulting in a considerable increase of the area of the glandular stroma (Alonso-Magdalena *et al.* 2009). Given this, the results of our study suggest that the increase of muscle stroma in the prostates of TG and TG + T gerbils may have occurred due to the activation of the same EMT pathways. These findings may contribute to the discovery of new roles played by androgens during the differentiation of smooth-muscle cells and their relationship to the development of BPH during ageing. Furthermore, they support the hypothesis that prostate development is a determinant of future prostate health when it comes to lesions.

Another question concerns the role of adjacent mesenchymal tissues with the potential to induce prostate developmental processes. Some studies have demonstrated the role of the ventral mesenchymal pad (VMP) during prostate development in male rodents (Timms *et al.* 1994, 1995; Thomson *et al.* 2002; Thomson 2008; Timms 2008). However, little is known about the role of other mesenchymal tissues responsible for the development of the lateral, dorsal and anterior prostate in the male. In the female, there is a lack of data showing the role of mesenchymal tissue and its relationship with prostate development. In female gerbils, the development of a prostate suggests the presence of paraurethral mesenchymal tissues with the potential to induce yearly prostatic development as well as to maintain this tissue in the condition of the stromal compartment.

The present study, however, suggests that other mesenchymal tissues may be present in the UGS of the female gerbil, allowing the development of dorsolateral prostate-like glands around the vaginal wall. These observations show that the development of a female prostate is highly influenced by androgen activity.

Morphological analyses revealed that females in the C and C + T groups presented hyperplasic glands. Previous studies made by our research group have already related this phenomenon to the development of spontaneous prostatic lesions in aged female gerbils (Custódio *et al.* 2010). Custódio *et al.* (2010) also showed that aged female gerbils have a propensity to develop prostatic lesions such as hyperplasia and prostatic intraepithelial neoplasia (PIN).

Regarding the stromal components, we observed an intense reshuffling of the collagen and elastic fibres in TG and TG + T prostates. In both groups, the general collagen was intensely changed, mainly in regions affected by lesions; this phenomenon is characteristic of a reactive stroma. In addition, we observed an increase in elastic components, as evidenced by the thickness of the elastic fibres. The reasons why most of the stromal alterations occurred in glands surrounding the vaginal wall (dorsolateral prostate), despite the paraurethral glands, are still unknown to our research group, although their proximity to the inflamed vagina could possibly be a cause. Alternatively, during organogenesis, an impairment of the signalling by the adjacent mesenchymes could cause an imprint predisposing the future gland to develop lesions with ageing. These hypotheses need to be more thoroughly explored in future studies.

In our immunohistochemical analyses, AR immunostaining showed a close similarity between all the experimental groups, presenting epithelial and stromal localisations, suggesting that the gland is functional and active in terms of AR signalling. In addition, ERα showed a predominantly stromal localisation, which was very similar between all the experimental groups. However, we observed a disrupted pattern of p63 localisation in several areas in the prostates of TG and TG + T gerbils, as some p63-positive cells occurred inside the multilayered epithelial compartment, mainly in altered glands. This evidence confirms other published findings that have shown the progressive loss of the basal layer in advanced prostatic lesions, such as high-grade prostatic intraepithelial neoplasia (HGPIN; Grisanzio and Signoretti 2008).

Immunofluorescence techniques for smooth-muscle α-actin confirmed the increase of muscle stroma in the prostates of TG and TG + T animals, as shown through stereological analyses. In addition, several prostatic areas affected by lesions in these two groups showed an absence of the smooth-muscle layer in regions of intense proliferation. This suggests an intense stromal reshuffling of the smooth-muscle cells during epithelial–stromal interactions in the development of prostatic lesions.

Altogether, our new evidence reinforces other findings indicating the risk associated with androgenic exposure in the prenatal period, especially for females. This study also shows that testosterone exposure *in utero* may be involved in predisposing the gland towards the development of prostatic diseases with ageing in this rodent model.

Recent studies have led to the development of a new hypothesis on prostate-cancer development. According to De Marzo *et al.* (2007), exposure to environmental factors such as infectious agents and carcinogenic substances in the diet, along with hormonal imbalances, may lead to injury of the prostate and to the development of chronic inflammation, events that may lead to the development of proliferative inflammatory atrophy (PIA). These lesions then have the potential to become malignant over time and eventually turn into prostate cancer (De Marzo *et al.* 2007).

Indeed, especially when considered together with the report by De Marzo *et al.* (2007), our data suggest a straightforward relationship between inflammatory events and the onset of prostatic lesions, predominantly in the TG and TG + T groups. Our findings suggest that chronic inflammation status may arise due to the retention of a huge volume of inflammatory cells inside the vaginal cavity (hydrometrocolpos), which affects the prostate, which is located around the vaginal wall.

According to Schaeffer *et al.* (2008), the mechanisms involved in early prostate developmental processes are quite similar to those that control prostate-cancer development. The program of mechanisms that control prostate cancer development is determined through the regulation of genes related to the acid phosphatase pathway, Wnt, as well as others related to angiogenesis, apoptosis, migration and cell proliferation. Moreover, it has been suggested that the origin of prostate cancer may occur during the initial phases of prostate development, a process which may be potentiated by an abnormal hormonal environment, increasing the propensity for the development of prostatic lesions throughout life (Schaeffer *et al.* 2008).

Certain events, such as the aromatisation of testosterone into oestradiol by aromatase or its conversion into dihydrotestosterone by 5α-reductase, require detailed evaluation in future studies, as they may furnish straightforward answers concerning the mechanisms by which exogenous testosterone is metabolised during critical events of prostate organogenesis.

Taken together, the evidence in the literature demonstrates that exposure of female rodents to androgenic substances during the prenatal period may be very harmful to the normal processes of urogenital tract development (Wolf *et al.* 2002; Hotchkiss *et al.* 2007*a*, 2007*b*), especially for the prostate gland (Biancardi *et al.* 2012; Perez *et al.* 2012), an organ with a high potential for developing malignant lesions, such as cancer, throughout life. Therefore, several conditions, including PCOS, adrenal hyperplasia and exposure to EDCs, or even to drugs with androgenic potential, may cause irreversible interferences during the developmental process and increase the likelihood of developing prostatic lesions during adulthood and ageing. Thus, it is extremely important to elucidate the mechanisms underlying these events and to identify ways to avoid exposure to harmful exogenous androgens, as well as to come up with new ways of diagnosing and treating female patients who have experienced these kinds of hormonal interference during their prenatal development.

Although the programming window for reproductive tract masculinisation has not been established in gerbils as it has been in rats (Welsh *et al.* 2008), our research group has successfully used this rodent model in several new studies focusing on reproductive tract masculinisation and

prostate-cancer development in order to improve the methodology for reproductive approaches to prostate-cancer treatment.

In conclusion, the results of the present study, along with a growing body of scientific literature on female prostate biology, have highlighted several aspects related to the presence, function, development, physiology and diseases of the prostate in the female organism. Besides, our attempts to understand this gland more thoroughly have shown that the prostate in the female deserves more attention, considering its strong potential to respond to different treatments, as shown in the present work and others by our research group (Santos *et al.* 2006; Perez *et al.* 2011; Biancardi *et al.* 2012). Furthermore, the presence of this gland in females, as extensively demonstrated by Zaviačič (1999), alerts us to the possibility that women may be exposed to exogenous androgens by various means, mainly during the developmental period, which is a very sensitive phase for prostate establishment. The evidence suggests a potential risk for pregnant women, as they may be exposed to exogenous androgens in various ways and even have their female fetuses affected by this exposure. Moreover, some direct evidence has recently emerged from clinical cases (Reis *et al.* 2011), demonstrating the exigency of more accurate diagnoses. These recent studies have reported important improvements in medical accuracy regarding diseases with possible origins from the female prostate (Skene's gland).

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