

## PAPER DETAILS

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PAGES: 35-41

ORIGINAL PDF URL: <https://dergipark.org.tr/tr/download/article-file/2224460>

## Determination of estrogen receptor, progesterone receptor and epidermal growth factor receptor in canine vaginal tumors and vaginal fold prolapse: A preliminary study

### Research Article

Volume: 6, Issue: 1  
April 2022  
Pages: 35-41

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### ABSTRACT

The objective of this study was to determine steroid receptors and epidermal growth factor receptor in canine vaginal tumors and vaginal fold prolapse. Eight dogs with a vaginal mass were incorporated into the study. According to the vaginal examination (vaginocopy, exfoliative vaginal cytology) and histopathology, the groups were designed as vaginal fold prolapse (Group VFP; n=3) and vaginal tumor (Group VT; n=5). Vaginal tissue samples were homogenized. Tissue homogenates were analyzed with an enzyme-linked immunosorbent assay (ELISA) to determine the levels of estrogen receptor (ER), progesterone receptor (PR), and epidermal growth factor receptor (EGFR). The mean ER level was significantly higher ( $P<0.05$ ) in group VT ( $8.06\pm0.45$ ) compared to the group VFP ( $5.93\pm0.36$ ). However, PR and EGFR levels did not show significance related to the groups ( $P>0.05$ ). In conclusion, significant differences were obtained between the bitches with VT and VFP related to ER levels although both groups had pathological conditions. Further studies are needed to discuss these pathologies with healthy bitches in terms of steroid receptor and EGFR levels in both vaginal tissue and blood sera.

**Keywords:** canine, leiomyoma, leiomyosarcoma, vaginal fold prolapse, receptor

### Article History

Received: 31.01.2022  
Accepted: 29.04.2022  
Available online:  
30.04.2022

DOI: <https://doi.org/10.30704/http-www-jivs-net.1066060>

**To cite this article:** Günay Uçmak, Z., Ateş, A., Erdoğan Bamaç, Ö., Öztürk Gürgen, H., Kırşan, İ., Tek, Ç., Küçükbeğir, Ç. N., & Kurban, İ. (2022). Determination of estrogen receptor, progesterone receptor and epidermal growth factor receptor in canine vaginal tumors and vaginal fold prolapse: A preliminary study. *Journal of Istanbul Veterinary Sciences*, 6(1), 35-41.  
**Abbreviated Title:** J. Istanbul vet. sci.

## Introduction

The vaginal masses in bitches are seen frequently as vaginal fold prolapse (VFP), vaginal tumor, or urethral neoplasia protruding into the vagina (Manothaiudom and Johnston, 1991). In bitches, VFP is defined as a protrusion of edematous vaginal tissue through the opening of the vulva which appears due to the

excessive response of the vaginal mucosa to estrogens (Sontas et al., 2010). Clinically, VFP is classified into three different types based on the degree of vaginal tissue protrusion. A slight to moderate eversion of the vaginal floor to the urethral opening is defined as type I VFP. In type II, the vaginal fold is prolapsed through the

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vulvar lips thus becoming visible from outside. When the entire circumference of the vagina protrudes through the vulva, it is described as type III (Shuttle, 1967; Anya et al., 2020). Tumors affecting canine vaginal vestibule or vulva are rare (Brodey and Roszel, 1967; McEntee, 2002). hyperplasias ). Vaginal tumors usually appear in sexually intact bitches (Thacher and Bradley, 1983). The occurrence ages of vaginal tumors in bitches are reported to be ranging from 5 to 16 years of age (Cotchin, 1954). Tumors affecting the canine vaginal tissue have a mostly benign character such as leiomyomas and fibromas (Brodey and Roszel, 1967; McEntee, 2002). In dogs with benign tumors, surgical excision of the tumor combined with ovariectomy provides the treatment and prevention of the recurrence of the disease (Thacher and Bradley, 1983). However, the use of steroidal hormones receptor antagonists was reported as an alternative treatment option because the growth of these masses may be stimulated by ovarian steroids (Rollón et al., 2008; Sathya and Linn, 2014). Ferré-Dolcet et al. (2020) reported a case of progesterone-sensitive vaginal leiomyoma in which they emphasized the importance of identifying progesterone-related conditions in order to decide on effective treatment as a combined medical and surgical approach.

The aim of the present study was to evaluate the differences in the amount of estrogen receptor (ER), progesterone receptor (PR), and epidermal growth factor receptor (EGFR) in tissue homogenates of the bitches with VFP and VT.

## Materials and Methods

**Animals and study design:** All animal procedures were carried out in accordance with the approval of the Istanbul University-Cerrahpaşa Animal Experiments Local Ethical Committee (HADYEK) (Approval number: 2021/256807). Eight dogs with a vaginal mass were incorporated into the study. All bitches were clinically and gynaecologically examined. The groups were designed according to vaginal examination (vaginotomy, exfoliative vaginal cytology) and histopathology. Vaginal smear was obtained for cytological examination of the vagina for both groups. The samples were collected with a cotton swab from all the bitches. The smears were stained with Diff Quick method according to manufacturers' instructions (ADR Group®, Mediko Kimya, Turkey). The slides were examined by using a light microscope (BAB-LAM®, BAB, Turkey) at x400 magnification. The bitches were divided into 2 groups which consist of vaginal fold prolapse (Group VFP; n=3) and vaginal tumor (Group VT; n=5). Clinical staging of VFP (Type I, Type II, Type III) was performed as Schutte (1967). The

ages of the bitches in group VFP and group VT ranged from 1 to 6 years of age and from 6 to 12 years, respectively. The breeds of the eight bitches were Cocker spaniel, Golden retriever, English Setter, Pekingese, Akita Inu, King Charles, and mixed breed. The bitches in group VT had three-view thoracic radiography and abdominal ultrasonography to determine the any metastasis to the distant organs.

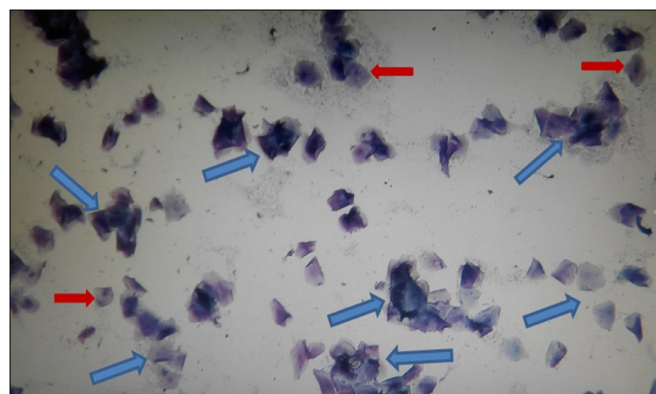
**Histopathological examination:** Vaginal tissue samples in group VFP and group VT were fixed in 10 % neutral buffered formalin, embedded in paraffin, sectioned at 4 µm, and stained with hematoxylin and eosin (HE) for histopathological examination by light microscopy. The vaginal tumors were classified according to Baba and Catoi, 2007.

**Sample preparation and enzyme-linked immunosorbent assay (ELISA):** Vaginal tissue samples were handled with PBS (PH 7.4) (1 ml PBS for 100mg tissue). They were homogenized by grinders and then centrifuged 20 min at the speed of 2000-3000 r.p.m. At the end of this procedure, supernatant was collected into a sterile eppendorf tube. Homogenized tissue samples were stored at - 20°C until ELISA were applied. Levels of ER, PR and EGFR in tissue homogenates were determined with commercially available canine-specific ELISA kits according to the manufacturer's instructions (ER Catalogue no: E0414Ca, PR Catalogue no: E0058Ca, EGFR Catalogue no: E0301Ca Bioassay Technology Laboratory, Shanghai, China).

**Statistical analysis:** Statistical analyses were performed with SPSS 13.0 (SPSS Inc, Chicago, Illinois, USA). The comparison of the groups in terms of the ER, PR and EGFR was performed by Mann Whitney-U test. The significance level was accepted as P<0.05.

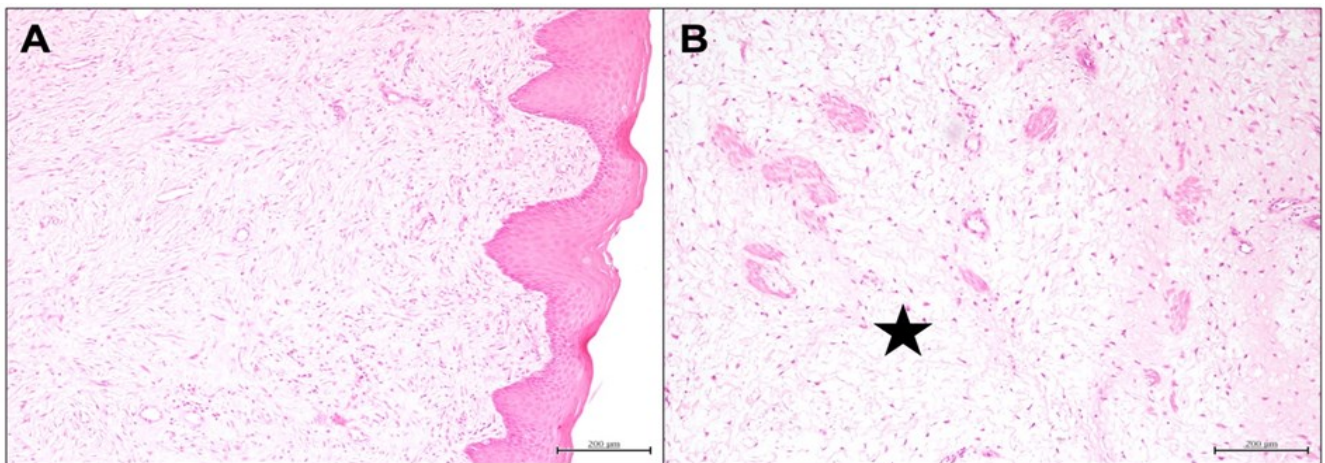
## Results

The mean ages and standard errors of the mean (SEM) in group VFP and group VT were  $2.66 \pm 1.66$  years and  $9.20 \pm 1.06$  years; respectively. In vaginal cytology



**Figure 1.** Superficial cells in the smear (red arrows). Keratinized (anuclear) superficial cells (blue arrows) in the smear.

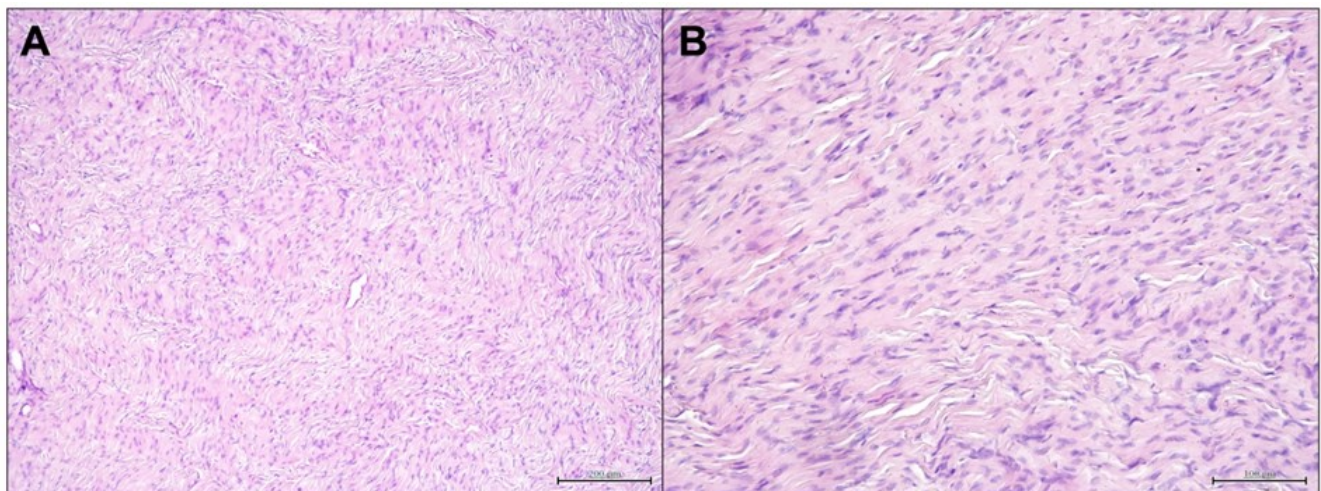




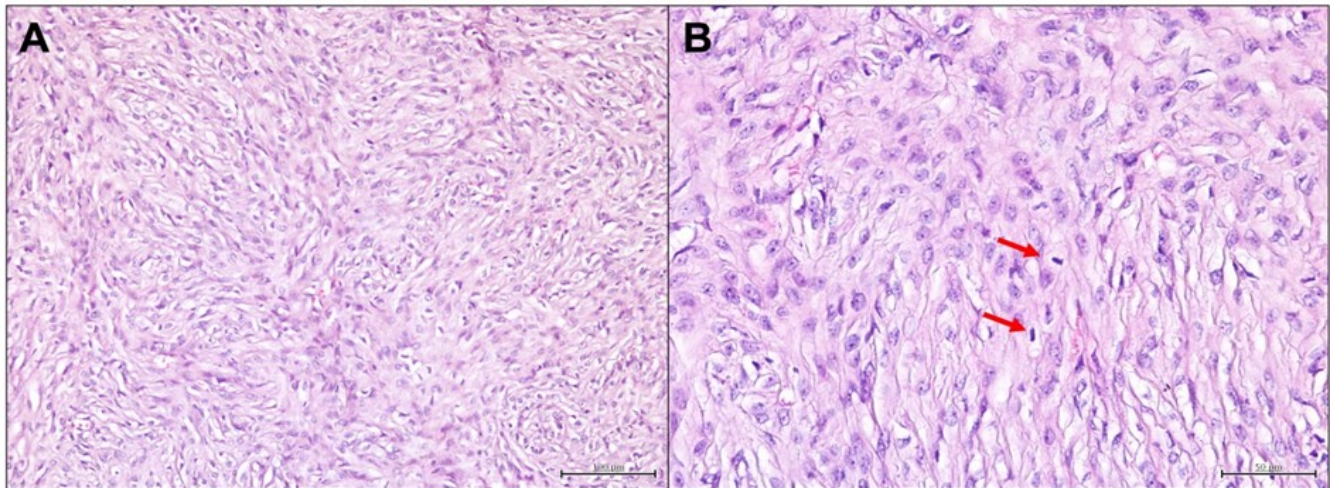
**Figure 2.** Vagina. Hyperplasia. A. Mild submucosal mononuclear inflammatory cellular infiltration, edema, and increased vascularization, H&E, Bar = 200 µm. B. Marked submucosal edema (star), H&E, Bar = 200 µm.

smears of all bitches incorporated into the study, keratinized superficial cells constituted 75% of all the cells in the slide (Figure 1) and they had their proestrus bleeding 7-14 days before they presented to the clinic. According to these data, it was determined that all bitches were in estrus in this study. In group VFP, two bitches had type 2 VFP and one bitch had type 3 VFP. Histopathological examination revealed vaginal hyperplasia characterized by inflammatory changes within the submucosa indicating marked edema, hyperemia, neovascularization, and mild infiltration of mononuclear cells (Figure 2). In group VT, three bitches had leiomyoma, which was composed of a circumscribed proliferation of monomorphic smooth muscle cells having abundant eosinophilic cytoplasm without mitotic figures (Figure 3) and two bitches had

leiomyosarcoma, which showed interwoven histological pattern composed of the pleomorphic spindle to ovoid cells with numerous atypical mitoses (Figure 4). As a result of examination with diagnostic imaging techniques (radiography and ultrasonography), any metastases were not observed to the distant organs in group VT. According to gynaecological examination, accumulation of the pus into the uterine lumen, purulent vaginal discharge, and increase in uterine diameter were determined in bitches with vaginal leiomyoma. Mean, minimum and maximum levels of ER, PR, and EGFR related to groups were given in Table 1. The mean level of ER was significantly higher in group VT compared to the group VFP ( $P < 0.05$ ). However, PR and EGFR levels were not shown a significance related to the groups ( $P > 0.05$ ).



**Figure 3.** Vagina. Leiomyoma of the vaginal wall. A. Interwoven fascicles of the spindle cells, H&E, Bar = 200 µm. B. Higher magnification of well-differentiated spindle cells, H&E, Bar = 100 µm.



**Figure 4.** Vagina. Leiomyosarcoma of the vaginal wall. A. Proliferation of the neoplastic spindle cells arranged as interwoven fascicles, H&E, Bar = 100 µm. B. Higher magnification of neoplastic cells showing marked pleomorphism, numerous atypical mitosis (arrows), H&E, Bar = 50 µm.

## Discussion

Vaginal fold prolapse occurs due to the elevated levels of estrogens during the proestrus and estrous stages of the sexual cycle (Sontas et al., 2010; Anila et al., 2020). The development and growth of the vaginal masses are considered to be stimulated by ovarian steroids and growth factors (Zhao et al., 2003; Ferré-Dolcet et al., 2020). The stage of the estrous cycle can be interpreted by vaginal cytology. Estrous is cytologically defined by 100% cornification with greater than 50% anuclear squames in epithelial cells (Root Kustritz, 2006). Similar to Root Kustritz (2006), in the current study, keratinized superficial cells constituted 75% of all epithelial cells in all cytology smears.

Anila et al. (2020) reported that nearly 75% of all cases of Type II and Type III vaginal prolapse were recorded in medium-sized breeds. Likewise the previous report, group VFP which had Type II and

Type III vaginal prolapse, constituted of medium-sized breeds in this study. Although Boxers are the most represented breed for vaginal leiomyoma incidence, there is no apparent breed predisposition reported for vaginal leiomyosarcoma (Cooper and Valentine, 2016). In the current study, no breed predisposition was observed in group VT as reported by Copper and Valentine (2016).

The highest occurrence age of VFP was reported to be between 1.5 to 2.7 years of age (Schutte, 1967), while Greenberg et al. (2002) indicated the occurrence of VFP in older bitches. Although the mean age of the bitches in group VFP was 2.66 years old in this study as reported by Schutte (1967), in one of the VFP cases, the affected bitch was 5 years old as indicated by Greenberg et al (2002). The smooth muscle tumors, leiomyomas and leiomyosarcomas, are observed in the vagina of the bitches, with leiomyomas being the most encountered ones

**Table 1.** Mean, minimum and maximum levels of ER, PR and EGFR related to groups.

Groups	Parameters	Minimum	Maximum	Mean ± SEM
Group VFP	ER (ng/ml)	5.236	6.467	5.93 ± 0.36 <sup>a</sup>
	PR (ng/ml)	8.741	15.217	11.01 ± 2.10
	EGFR (ng/ml)	7.839	19.381	12.49 ± 3.51
Group VT	ER (ng/ml)	6.764	8.832	8.06 ± 0.45 <sup>b</sup>
	PR (ng/ml)	6.486	14.209	10.21 ± 1.44
	EGFR (ng/ml)	8.102	17.414	11.14 ± 1.10

VFP: Vaginal fold prolapse. VT: Vaginal tumor. ER: Estrogen receptor. PR: Progesterone receptor. EGFR: Epidermal growth factor receptor. SEM: Standard error of mean. <sup>a,b</sup> Different letters in ER lines indicate the significance (P<0.05).



(Manothaiudom, & Johnston, 1991). The incidence age of the bitches with vaginal leiomyomas and leiomyosarcomas are averaging 10.8 years old (Klein, 2001). Consistent with the previous report, vaginal tumors were observed in older intact bitches in this study.

Leiomyomas are non-invasive and non-metastatic vaginal tumors (Cooper and Valentine, 2016) whereas canine vaginal leiomyosarcomas can metastasize to the spleen (Brodey and Roszel, 1967). Macroscopic features of the vaginal leiomyomas are polypoid and/or pedunculated (Brodey and Roszel, 1967). In accordance with the researchers (Brodey and Roszel, 1967; Cooper and Valentine, 2016), leiomyomas and leiomyosarcomas had the similar clinical and histological characteristics but as opposed to Brodey and Roszel (1967), metastasis was not observed in any of the leiomyosarcomas in this study. Enginler et al. (2014) reported a vaginal leiomyosarcoma following pyometra in a 10-year old Labrador Retriever bitch. In this study, pyometra was diagnosed in group VT but the bitches with pyometra had vaginal leiomyoma as opposed to Enginler et al. (2014).

It was reported that VFP occurs due to a strong response of vaginal mucosa to the estrogens during pro-estrous and early estrus (Sontas et al., 2010; Alan et al., 2007). In healthy bitches, reduction of ER expression in vaginal tissue was detected from pro-estrus and late estrus (Ithurrealde et al., 2013). de Brito et al. (2006) compared ER- $\alpha$  in the vaginal epithelium of bitches with transmissible venereal tumor (TVT) or control bitches, and they indicated that there was a close relationship between neoplastic cells and cells of the vaginal stroma, which were highly positive for ER- $\alpha$ . Even though tumor types were different from the previous report (de Brito et al., 2006), the mean ER level of the vaginal tissue homogenates was higher in group VT than group VFP. A decrease in ER in bitches with VFP could be explained by the research of Uchima et al. (1987) reported that continuous exposure to estradiol resulted in a reduction in cytosolic estrogen receptors associated with nuclear accumulation of estrogen receptors.

In canine vaginal wall, both ER and PR are localized in the nuclei of the epithelial cells, stromal cells of the mucosa, and smooth muscle cells of the muscular layer. When the epithelium has a multilayered squamous stratified structure during pro-estrus and estrus, ER and PR receptors are predominantly observed in the basal and parabasal cell layers. Immunohistochemical intensity scores for PR in the canine vaginal epithelium, stroma and

muscular layer were higher in pro-estrus and estrus than in met-estrus (Vermeirsch et al., 2002). A positive correlation between estrogen concentration and PR expression in vaginal stroma and epithelium was observed in bitches and rats (Vermeirsch et al., 2002; Ohta et al., 1993). Benign tumors of the vagina that have progesterone receptors such as fibroleiomyoma can be reduced in size by using the progesterone receptor antagonist aglepristone (Rollón et al., 2008). In canine vaginal leiomyoma and leiomyosarcoma, PR expressions were determined as 82.1% and 100%; respectively (Millian et al., 2007). In this study, tissue PR levels were not different between the groups ( $P > 0.05$ ). It was hypothesized that insignificant results were obtained due to the increase of tissue PR levels in both VFP and VT.

Sağsöz et al. (2019) reported that EGFR expression in the bovine vagina, which alters according to the phase of the oestrous cycle, and a significant increase in EGFR expression had been detected in the follicular phase compared to the luteal phase. Iguchi et al. (1993) indicated that reduction of EGFR level in the vagina correlates with persistent proliferation and keratinization of the vagina in mice. Neonatally diethylstilbestrol-exposed mice had lower EGFR levels in the vagina than untreated mice (Iguchi et al., 1993). Mukku et al. (1985) emphasized that estrogen induction of physiological processes, including proliferation and differentiation, is mediated by EGF-EGF receptor interaction. In humans, EGFR is frequently expressed in invasive squamous cell carcinoma of the vagina. However, there was no statistically significant relationship between EGFR expression and clinical stage, grading, and tumor size in vaginal cancer (Brunner et al., 2011). In this study, EGFR levels did not show an alteration related to the groups ( $P > 0.05$ ). Because all bitches in the present study were in estrus and they were influenced by estrogen. As in previous reports (Mukku et al. 1985; Iguchi et al. 1993; Brunner et al. 2011; Sağsöz et al. 2019), in which the increase in EGFR in the follicular phase and in carcinogenic cases were noted, insignificant results in terms of EGFR levels were obtained between the group VFP and group VT in the present study.

## Conclusions

In conclusion, significant differences were obtained between the bitches with VT and VFP related to ER levels although both groups had pathological conditions. Further studies are needed to discuss these pathologies with healthy bitches in terms of steroid receptors and EGFR levels in both vaginal tissue and blood sera.

**Declaration of interest.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of paper.

**Acknowledgements.** The authors are also grateful to Prof. Dr. Bülent Ekiz from the Department of Animal Breeding and Husbandry for his help with the statistical analysis.

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