

# Immunopathologic Evaluation of Estrogen Receptor, Progesterone Receptor, and Ki-67 Antibody Expressions in Canine Mammary Tumours <sup>[1]</sup>

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## Abstract

This study evaluates the expressions of Estrogen Receptor (ER), Progesterone Receptor (PR) and Ki-67 antibodies in canine mammary tumours and their prognostic values in regard to their histomorphologic subtypes. For this purpose, 60 tissue samples suspected as canine mammary tumour were examined and classified according to World Health Organisation's (WHO) criteria. Samples were labelled with anti ER, PR and Ki-67 using immunohistochemistry while ER, and PR values were showed significantly higher in benign tumours ( $P<0.001$ ,  $P<0.001$ , respectively), contraversely Ki-67 value was significantly greater in malignant tumours ( $P<0.001$ ). The statistical difference between malignant tumour types according to ER, PR and Ki-67 expressions were significant ( $P<0.001$ ,  $P<0.01$ ,  $P<0.001$ , respectively). Among malignant tumours, solid carcinomas and spindle cell carcinomas had the highest Ki-67 and lowest ER and PR expressions, complex adenocarcinomas and carsinosarcomas had the lowest Ki-67 and highest ER and PR expressions. It is concluded that presentation of immunohistochemical expression of differences between ER, PR and Ki-67 in canine mammary tumours, will provide contribution to the evaluation of tumours and determination of treatment processes in veterinary clinical pathology.

**Keywords:** Canine mammary tumour, Estrogen receptor, Progesterone receptor, Ki-67 antibody, Immunohistochemistry

## Köpek Meme Tümörlerinde Östrojen Reseptör, Progesteron Reseptör ve Ki-67 Antikor İşaretlenmelerinin İmmunopatolojik Olarak Araştırılması

### Özet

Bu çalışmada, köpek meme tümörlerinde Östrojen Reseptör (ER), Progesterone Reseptör (PR) ve Ki-67 antikorlarının işaretlenmeleri ve bunların histomorfolojik alt tiplerine göre prognostic önemi değerlendirilmiştir. Bu amaçla, köpek meme tümörü şüpheli 60 doku örneği incelenip, Dünya Sağlık Örgütü'nün (WHO) kriterlerine göre sınıflandırılmıştır. Örnekler, anti ER, PR ve Ki-67 ile immunohistokimyasal yöntem kullanılarak işaretlenmiştir. ER ve PR değerleri iyi huylu tümörlerde belirgin olarak yüksek iken, tam tersine kötü huylu tümörlerde Ki-67 değerleri daha yüksekti ( $P<0.001$ ). Kötü huylu tümör tipleri arasında da ER, PR ve Ki-67 işaretlenmeleri bakımından istatistiksel farklılıklar belirlendi (sırasıyla;  $P<0.001$ ,  $P<0.01$ ,  $P<0.001$ ). Kötü huylu meme tümörlerinden solid ve spindle hücreli karsinomalarda en yüksek Ki-67 ve en düşük ER ve PR işaretlenmeleri saptanırken, kompleks adenokarsinomalar ve karsinosarkomalar ise, en düşük Ki-67 ve en yüksek ER ve PR işaretlenmelerine sahipti. Köpek meme tümörlerinde ER, PR ve Ki-67 işaretlenmelerindeki farklılıkların immunohistokimyasal olarak gösterilmesi, veteriner klinik patolojide tümörlerin değerlendirilmesine ve tedavi süreçlerinin belirlenmesine katkılar sağlayabileceği sonucuna varılmıştır.

**Anahtar sözcükler:** Köpek meme tümörü, Östrojen reseptör, Progesteron reseptör Ki-67 antikor, İmmunohistokimya

## INTRODUCTION

Among all canine neoplasias mammary gland tissue tumours take the second place after skin cancers <sup>[1]</sup>. In dogs, the developmental risk of malignant mammary tumours throughout life time ranges between 2-20% and this value is 2-5 times greater than the benign mammary tumours <sup>[2]</sup>.

The prognosis of canine mammary tumours, their histopathology and proliferative activity have been intensively studied <sup>[3-6]</sup>. Intracellular steroid-hormone receptor proteins, primarily Estrogen Receptor (ER) and Progesterone Receptor (PR) have been studied both as indicators of prognosis and as guides to hormone and endocrine therapy <sup>[7-9]</sup>. Estrogen and progesterone hormones are known to play an



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important role in the normal physiology of the mammary gland but these hormones have also been implicated in tumour development [10-13]. The Ki-67 antigen is a nuclear protein expressed in all active phases (G1, G2, S) of the cell cycle except G0, thus it demonstrates the proliferative activity of cells [14-16].

In the presented study about canine mammary tumours the association between histomorphological tumour types, their steroid hormone expression values and proliferation activity was aimed to be determined. For this purpose, we meant to evaluate ER, PR and Ki-67 expressions in 60 tumour suspected canine mammary tissues by immunohistochemistry (IHC). Demonstration of ER, PR and Ki-67 expression in canine mammary tumours, and the histomorphological differences between benign and malignant mammary tumours are valuable data in veterinary clinical pathology for prognosis. These findings are supportive for the prediction of the possible response of the patients to the hormonal therapy and therefore will be useful in enlightenment of the further studies to be carried out in this field.

## MATERIAL and METHODS

### *Animals and Tissue Samples*

Routine mastectomy materials from 60 female dogs admitted to Department of Obstetrics and Gynecology aging between 4-15 years were investigated. The collected tissue samples were fixed in 10% buffered formalin, processed by routine methods, embedded in paraffin wax, sectioned at 4- $\mu$ m and stained with haematoxylin and eosin (H.E.) and labelled with immunohistochemistry.

### *Histologic Examination*

The tumours were examined histologically and classified independently by two pathologists according to the criteria of the World Health Organization [17]. Tumour malignancy was graded histologically with the method of Nottingham modified by Elston and Ellis [18]. The histologic grade for each case was derived from the assessment of 1- tubule formation, 2- nuclear pleomorphism, 3- mitotic figures, and each feature being scored 1 to 3 points. The scores were then added together to obtain an overall tumour grade as follows: 3-5 points: well differentiated cells (grade 1); 6-7 points: moderately differentiated cells (grade 2); 8-9 points: poorly differentiated cells (grade 3). Tumours were classified histologically according to their most aggressive components.

### *Immunohistochemical Assay*

Tissue sections from paraffin blocks collected into poly-L-lysine-coated slides. They were put through deparaffinization, antigen retrieval was performed using citrate buffer solution (pH 6.0, 10 nM), and endogen

peroxidase and then protein blocking procedures were applied. Slides were incubated with the primary Ab ER (Zymed<sup>®</sup>, UK, Lot.No. 18-0174Z), PR (Clone 10A9; Immunotech, France, Cat.No. 1546), Ki-67(Clone MIB-1; Dako, Denmark, Code No. M 7240) overnight 4°C with dilutions of 1:50, 1:30, 1:100 respectively. Then they were treated with commercially seconder antibody kit (2<sup>nd</sup> Generation LAB-SA Detection System, Histostain<sup>®</sup>-Plus Kits Zymed<sup>®</sup>, Carlsbad, Cat. No. 85 9843) and marked with DAB chromogen. Section for Ki67 were marked with AEC chromogen. Finally, the sections were counterstained with Mayer's haematoxylin. The negative control sections were incubated with PBS instead of the primer antibody.

### **Immunohistochemical Ki-67, ER, PR Assay Scoring System**

In the study positively stained cells in neoplastic and non-neoplastic epithelial mammary gland sections were counted and scored. For each antibody, the sections were examined under light microscope (Olympus BX50) first at low power (40X) to identify 10 areas with high numbers of positive cells and a total of 2.000 cells were counted within these 10 random high-power (400X) fields and their arithmetical mean was calculated. Ki-67, ER and PR were scored on the basis of their IHC staining intensity in positive control tissues sections (canine small intestine for Ki-67 and uterus for ER and PR). Each high-power magnification scored as;- (none or less than 5% positive nuclei), + (between 5-19% positive nuclei), ++ (between 20-59% positive nuclei), and +++ (60% or more positive nuclei).

### *Statistical Analysis*

The tumours were grouped according to their histopathological features. Kruskal- Wallis and Mann-Whitney tests were used to compare the ER, PR and Ki-67 expressions of different tumour types. Besides, to assess the effects of giving birth or spaying on benign and malignant tumours Chi-Square test was performed.

## RESULTS

### *Clinic and Pathologic Findings*

The mean ages of the 60 female dogs at the time of tumour removal was 9.2 years. No significant difference between the ages of dogs with benign and malignant tumours was determined ( $P>0.05$ ). The results were summarised in [Table 1](#).

Of 60 dogs in the study, 24 of them been were spayed, the others were intact female dogs and among them only 27 of them gave birth previously. When the results from these dogs were compared, while there was no significant difference according to status of giving birth ( $P>0.05$ ), a difference was determined according to their status of spaying ( $P<0.05$ ) ([Table 1](#)).



Time-to-onset of metastasis and overall survival times were not included in analysis because this information was incomplete in medical records examined.

Of the 60 tumours, 13 (21.7%) were histologically benign and 47 (78.3%) were malignant. The ratio of benign to malignant tumours was 1:3.6. 10 different histologic types of mammary tumours were represented. In the benign mammary tumours group; simple adenoma (N:7), benign mixed tumour (N:3), fibroadenoma (N:2), basaloid adenoma (N:1) were determined, and in the malignant mammary tumours group; complex carcinoma (N:12), simple tubulopapillary carcinoma (N:11), simple solid carcinoma (N:8), carcinosarcomas (N:7), osteosarcomas (N:6), and spindle cell carcinomas (N:3) were determined.

From those malignant tumours (except carcinosarcomas and osteosarcomas) 24 were classified as grade I (WDC), 4 were classified as grade II (MDC), 6 were classified as grade III (PDC) by use of histologic grading.

**Immunostaining**

In neoplastic mammary tissues, ER and PR expressions was observed in the nucleus of normal and tumoural epithelial cells, but stromal cells did not have positive staining results for ER and PR. Characteristics of PR expressions in positive control tissues were similar to those for ER expression. Ki-67(MIB 1) positive reaction was expressed as a granular nuclear staining often with prominent nucleolar positivity only in neoplastic epithelial cells.

Homogeneous ++ and +++ ER and PR positive staining in ductal and alveolar epithelial cells were observed in all benign tumours and normal mammary glands. Ki-67 positive staining was not observed in any of these samples.

ER, and PR values were showed significantly higher in benign than in malignant tumours (P<0.001, P<0.001, respectively), but conversely Ki-67 value was significantly

greater in malignant tumours than in benign tumours (P<0.001). The ER, PR and Ki-67 expressions in benign and malignant mammary tumours are given in *Table 1*.

ER, PR and Ki-67 expressions of malignant mammary tumours are given in *Fig. 1*. The statistical difference between malignant tumour types according to ER, PR and Ki-67 expressions were significant (P<0.001, P<0.01, P<0.001, respectively). Among malignant mammary tumours 35 were ER (+), 12 were ER (-) and 29 were PR (+), 18 were PR (-). All of the complex adenocarcinoma and carcinosarcomas were ER (+), PR (+) and they represented the highest values (*Fig. 2* and *Fig. 3*). Simple solid and spindle cell carcinomas had low values of ER (+) and PR (+) expressions (*Table 1*).

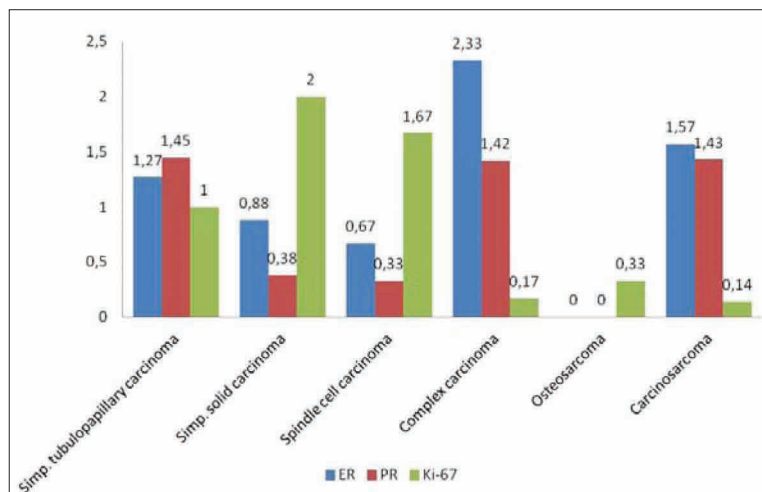
Among malignant mammary tumours 23 were Ki-67 (+), 24 were Ki-67 (-). Most of the simple solid carcinoma, spindle cell carcinoma and simple tubulopapiller carcinomas were Ki67 (+) and they had high expression values (*Fig. 4*). Carcinosarcoma, complex carcinoma and osteosarcomas had low values of Ki-67 (+) expressions.

According to ER, PR and Ki-67 expression values statistically significant differences were observed between tumoural grades of malignant mammary tumours (P<0.01, P<0.01, P<0.001, respectively).

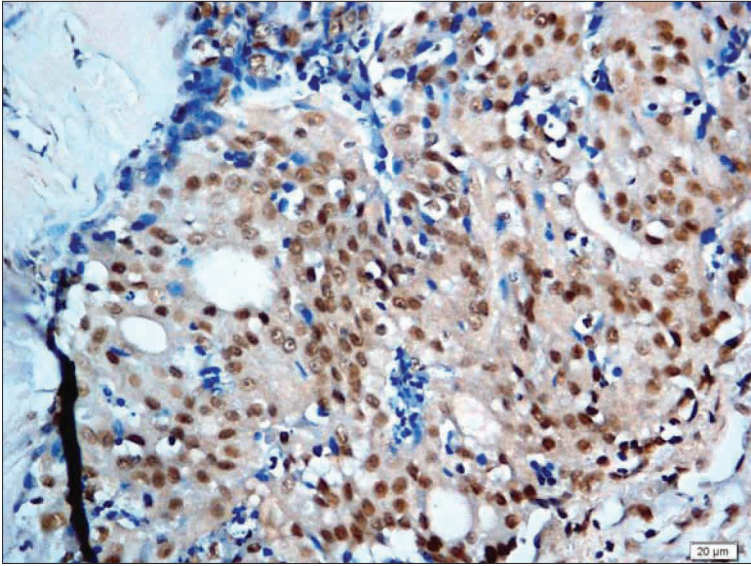
**DISCUSSION**

In the canine mammary tumour, age is clinically an important risk factor. It is a known fact that the frequency of mammary tumour development is directly proportional with age [19-21]. In the present study, average age at the time of surgery was 9.2 years (ranging from 4 to 15 years) which was similar to ages reported in previous studies [22,23]. In the presented study the benign mammary tumours were generally observed in dogs aged ≤7 years old and malignant mammary tumours were generally observed in dog aged between 10-11 years old. The difference, between benign and malignant mammary tumours was

**Fig 1.** ER, PR and Ki-67 levels of malignant mammary tumours  
**Şekil 1.** Malignn meme tümörlerinde ER, PR ve Ki-67 seviyeleri

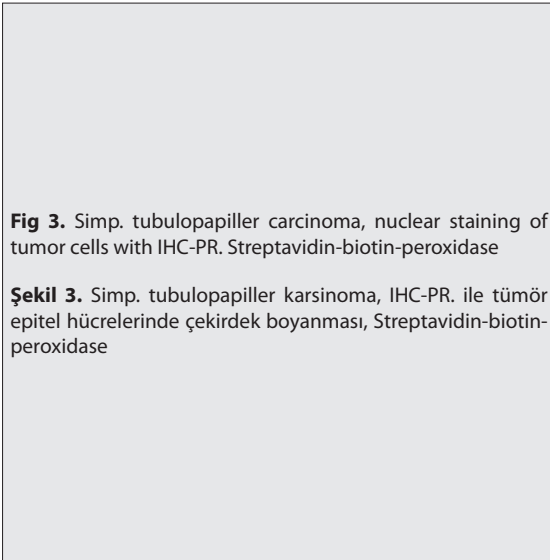






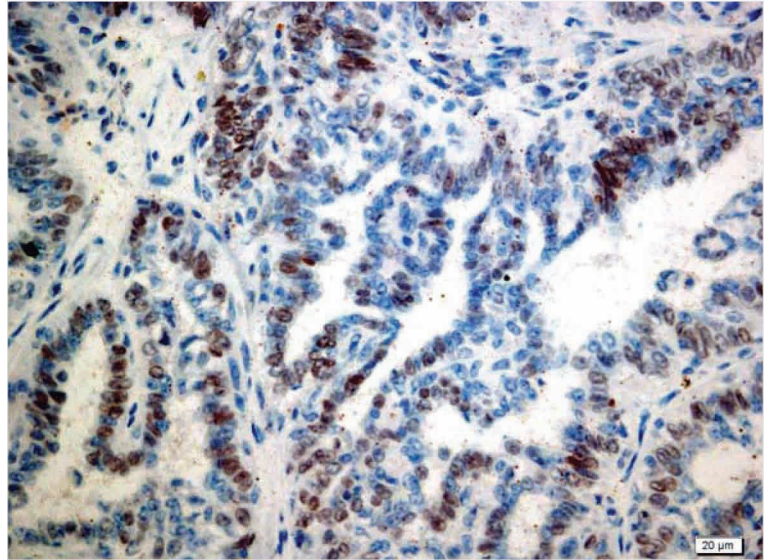
**Fig 2.** Complex carcinoma, diffuse, nuclear staining of tumor cells with IHC-ER. Streptavidin-biotin-peroxidase

**Şekil 2.** Kompleks karsinoma, IHC-ER ile tümör epitel hücrelerinde yaygın çekirdek boyanması, Streptavidin-biotin-peroxidase



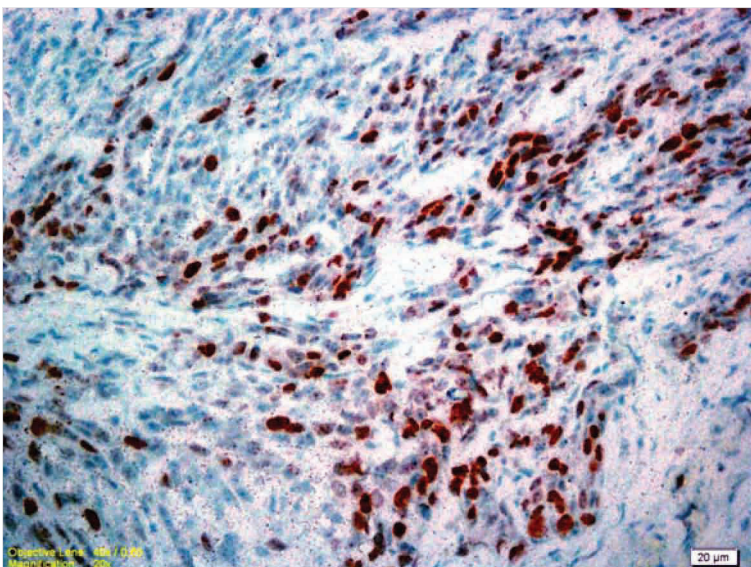
**Fig 3.** Simp. tubulopapiller carcinoma, nuclear staining of tumor cells with IHC-PR. Streptavidin-biotin-peroxidase

**Şekil 3.** Simp. tubulopapiller karsinoma, IHC-PR. ile tümör epitel hücrelerinde çekirdek boyanması, Streptavidin-biotin-peroxidase



**Fig 4.** Spindle cell carcinoma, Diffuse, nuclear staining of tumor cells with IHC-Ki-67. Streptavidin-biotin-peroxidase

**Şekil 4.** Spindle hücreli karsinoma, IHC-Ki-67 ile tümör epitel hücrelerinde yaygın çekirdek boyanması, Streptavidin-biotin-peroxidase



not significant ( $P > 0.05$ ). Also, it was observed that the dogs diagnosed as simple solid carcinoma had highest mean age values ( $\geq 11$ ) than other dogs. But conversely to our findings, Chang SC et al.<sup>[22]</sup> announced that age was not a significant prognostic factor in his research. Philibert et al.<sup>[24]</sup> reported that in dogs' age was not an important risk factor as in humans.

There was a statistical difference between benign and malignant mammary tumours according to the status of spaying ( $P < 0.05$ ), but there was no statistical difference according to the status of giving birth ( $P > 0.05$ ). Although spaying did not have statistically significant effect on ER and PR expression ( $P > 0.05$ ), it had a significant impact on the proliferative capacity (Ki-67) of the neoplastic cells ( $P < 0.01$ ). By these results we can conclude that spaying is a more preservative factor than giving birth for the formation of malignant mammary tumours. Parallel to our findings previous researchers<sup>[22,25,26]</sup> verify that in bitches ovariectomy offered a protective effect against the development of this condition and this effect is most pronounced if this operation is performed early in life, before 2.5 years of age.

In the presented study from 60 tumours 47 of them were malignant (78.3%) and 13 of them were benign (21.7%). This finding is compatible with previous reports about the prevalence of canine mammary tumours<sup>[20,27]</sup>. Tumours are recognised late because of dogs' long hair or late operative intervention. Even though those tumours are recognised in early stages, depending to the time period spent they generally expected to have malign transformation to take form because in dogs the prevalence of malignant mammary tumours are 3-4 times greater than benign tumours.

The clinical and histological characteristics of canine mammary tumours often do not provide sufficient predictive information for the clinician to know how the disease will behave in a given individual and how to implement therapy based on the predicted behaviour. Analysis of potentially important prognostic factors has been the focus of many studies<sup>[20,22,23,28,29]</sup>. Both ER and PR expression values and tumour cell proliferation (Ki-67) value are accepted to be important parameters for the evaluation of biologic behaviour and demonstrating the malignity potentials of mammary tumours<sup>[30]</sup>. Variable values detected in ER and PR levels of the mammary gland tumours are accepted to be an important evidence of the possible relation of the tumours with the hormonal factors<sup>[31]</sup>. In this study, 50 of 60 (83%) canine mammary tumours were positive for ER and/or PR, and 23 of 60 (38%) canine mammary tumours were positive for Ki-67. Statistically significant differences were detected in the expression of ER, PR and Ki-67 between benign and malignant mammary tumours. While ER and PR expression levels were high in benign mammary tumours conversely Ki-67 levels were only high in malignant tumours. This

significant differences between benign and malignant mammary tumours demonstrated that both hormone receptors and Ki-67 receptors had independent prognostic value. De Las Mulas et al.<sup>[4]</sup>, Galdes et al.<sup>[28]</sup>, Rutteman et al.<sup>[32]</sup>, Donnay et al.<sup>[33]</sup> and Nieto et al.<sup>[34]</sup> reported compatible results to present study.

In this study, it is determined that, among diagnosed malignant mammary tumours, tumours with dominant epithelial morphology such as simple solid carcinomas, spindle cell carcinomas and simple tubulopapillary carcinoma and tumours with dominant mesenchymal morphology such as carcinosarcomas, and complex carcinomas there were statistically significant differences between steroid hormone receptor expressions and tumour cell proliferation capacities just like their cellular morphologic characteristics differences. In other studies<sup>[13,30,35,36]</sup>, parallel to our findings it was reported that solid carcinomas of the malignant mammary tumours have highest Ki-67 and lowest ER expressions. De Las Mulas et al.<sup>[4]</sup> determined that complex carcinoma and carcinosarcomas have higher ER and PR expression values than simple histologic subtypes of tumours. On the contrary, Yang et al.<sup>[37]</sup> stated that simple tubulopapillary carcinomas have higher Ki-67 expression than solid carcinoma and complex carcinoma had the highest Ki-67 expression values.

In the presented study, there was no significant difference between tumours with different grades according to their steroid hormone expressions, but there was significantly important difference according to their Ki-67 expression values.

Rutteman et al.<sup>[32]</sup> reported that ER, PR scores and Ki-67 values of both benign and malignant mammary tumours showed a non-significant inverse correlation. ER and PR expression decreased significantly with increased values of Ki-67, they reported that these decreases in hormone receptors depend to the increased cellular transformation and the loss of stimulant effects of hormones on epithelial tumour cells. According to the data we established in the presented study we share this reasoning.

Different immunohistochemical investigations contribute new possibilities to the veterinary clinic pathology, which are still ill-defined to demonstrate the differences and for the evaluation and treatment of these tumours even with the golden criteria of histomorphologic structures of canine mammary tumours.

In the presented study about canine mammary tumours an association between histomorphological tumour types, their steroid hormone expression values and proliferation activity was determined. We think that the presentation of these values contribute hugely to the determination of clinical and biologic behaviour of tumours and their malignancy potentials and indirectly interests the course of disease and determination of treatment.



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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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